Hydrolysis of cassava cell walls through alkaline treatment and fermentation with alkaliphilic bacteria

By

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Submitted in the partial fulfilment of the requirements for the Degree

PhD Food Science

in the

Department of Food Science
Faculty of Natural and Agricultural Sciences
University of Pretoria
South Africa

November 2017

DECLARATION

I hereby declare that this thesis submitted at the University of Pretoria for the award of PhD degree is my work and has not been submitted by me for a degree at any other University or Institution of Higher Education.

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November 2017

ABSTRACT

Hydrolysis of cassava cell walls through alkaline treatment and fermentation with alkaliphilic bacteria

by

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Efficient processing of cassava roots by wet milling requires overcoming challenges associated with disaggregation of the starch-containing parenchyma cells. These cells entrap starch granules and hinder their release during wet milling.

Steeping of ground cassava in 0.75% (w/v) NaOH in combination with wet milling was investigated to determine whether and how dilute NaOH modifies cassava cell walls. Gas chromatography (GC) data of cell wall constituent sugar composition and Fourier transform infrared (FTIR) data showed that NaOH steeping caused solubilisation of the cell wall pectin fraction. FTIR and wide-angle x-ray scattering (WAXS) spectroscopy indicated that NaOH steeping combined with fine (500 μm opening screen size) wet milling reduced cellulose crystallinity. Dilute NaOH steeping also produced pits (micropores) through the cell wall structure as shown by scanning electron microscopy (SEM). The micropores seemed to have weakened the cell walls, as revealed by increased cellular disaggregation as viewed by light microscopy. Disaggregation of cassava root cells was associated with a reduction in large (diameter > 250 μm) residue particle size in the bagasse and consequently more starch yield. Thus, it seems that mechanistically, dilute NaOH solubilisation of alkaline-soluble pectin weakens the cell walls of starch-containing cassava root parenchyma cells.

Weakening of cassava cell walls with a combination of biological (14 day endogenous fermentation under microaerophilic conditions) and dilute alkaline pre-treatment (0.75% NaOH steeping) was investigated in an attempt to further increase starch yield by wet milling. However, the combined pre-treatment resulted in approx. 11.8% more starch yield, slightly less than the 12.3% increase obtained by using endogenous fermentation alone. The absence of an additive effect was probably because although endogenous fermentation (retting) and dilute NaOH steeping weakened cassava cell walls through different mechanisms (hydrolysis/solubilisation of pectin), the resultant loss in pectin cohesiveness was similar.

Solid state fermentation of ground cassava using various alkaliphilic *Bacillus* spp. starter cultures separately and in combination was also investigated to determine their extracellular hydrolytic enzyme induced weakening effects on parenchyma cell walls. GC and FTIR data indicated that fermentation with *Bacillus akibai* + endogenous microflora (EM), *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM caused reduction in cell wall pectin, xyloglucan and cellulose contents. Cell wall solubilisation/hydrolysis seemed to have primarily involved the amorphous constituents, as indicated by an increase in cellulose crystallinity by WAXS spectroscopy. Enzyme assay and SEM indicated that *Bacillus* spp. extracellular cellulase and polygalacturonase weakened the cell walls through formation of micropores and possible rupturing of cellulose microfibril structures. These modifications seemed to have aided disaggregation of the cassava parenchyma cells and consequently liberation of more starch granules as indicated by light microscopy. Fermentation with *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM also resulted in less large (diameter > 250 μm) residue particle size in the bagasse and consequently higher starch yield.

Thus, dilute NaOH steeping and fermentation with alkaliphilic *Bacillus* spp. starter cultures are techniques capable of improving the effectiveness of wet milling in disintegrating cassava cell walls. However, with regard to the demand for environmentally cleaner production, potential utilisation of alkaliphilic *Bacillus* spp. and more specifically *Bacillus cellulosilyticus* appears more promising.

DEDICATION

This thesis is dedicated to:

My dear wife Sheila Kamayangi Odoch,

And our lovely children Kelsey Lagum and Jonathan Rwotlonyo

For their love, patience, understanding and support

ACKNOWLEDGEMENTS

I am forever grateful and thankful to my Lead Supervisor, Prof John R. N. Taylor, for the life changing opportunity of working and learning under his dedicated and renowned supervision. I am also appreciative of the emotional and financial support he offered during my studies. I am grateful to my Co-Supervisor, Prof Elna M. Buys for her guidance in the microbiological aspects of my work.

I am very appreciative of the support by the International Centre for development oriented Research in Agriculture (ICRA) and the University of Pretoria for financing the study.

I am thankful to Mr. Thulani Sibanda and Dr. Adeoluwa Adetunji for their assistance in practical skill learning. I am also grateful to all the academic staff, administrative staff and my fellow postgraduate colleagues at the Department of Food Science, University of Pretoria, for their assistance at several occasions.

I also thank Dr. Janet Taylor, for the Taste of Africa and Santa Shoe Box activities. The activities were of a noble cause and a worthy relief from academia.

Above all, I give praise and honour to the Almighty God.

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1 INTRODUCTION

Worldwide, cassava is utilized as a source of carbohydrate in human diets and animal feeds (El-Sharkawy, 2003). In sub-Saharan Africa, cassava ranks second after maize as the most important source of calories (Nweke, Lynam and Spencer, 2002). During the period of 2003 to 2013, cassava production in Africa increased from 104 million tons to 157 million tons, approx. 51% increment (FAOSTAT, 2015). The prominence of cassava is attributed to increased utilization in industrial purposes and novel foods (Shittu, Dixon, Awonorin, Sanni and Maziya-Dixon, 2008). Additionally, African countries are increasingly substituting expensively imported wheat and barley with cassava in bread and beer production, respectively (Shittu, Raji and Sanni, 2007; Brian, 2015). Cassava's high starch content, relatively low requirement for agricultural fertilizers and drought tolerance makes it an attractive source for starch extraction (Jansson, Westerbergh, Zhang, Hu and Sun, 2009). Cassava starch is used in many industrial products, both food and non-food. The main applications include adhesives, textiles and paper (Santisopasri, Kurotjanawong, Chotineeranat, Piyachomkwan, Sriroth and Oates, 2001). Therefore, improving cassava starch extraction through novel processes is important as the starch could serve as a means of developing the economies of sub-Saharan African countries.

As reviewed by Moorthy (2002), cassava contributes 11% of the global starch production and has been exploited for several industrial purposes. However, recovery of starch granules from within root parenchyma cells during wet milling presents challenges, resulting in approx. 10% to 15% original root weight as solid waste (Sriroth, Chollakup, Chotineeranat, Piyachomkwan and Oates, 2000), containing 50% to 60% starch (dry basis) (Balagopalan, Ray, Sheriff and Rajalekshmy, 1994). This is due to the lignocellulosic cell wall materials entrapping starch granules within the cells (Sriroth et al., 2000). Cell wall material, a complex of cellulose, hemicelluloses and lignin, is recalcitrant to fragmentation by physical, chemical, enzymatic and microbial techniques (reviewed by Haghighi, Hossein, Tabatabaei, Salehi, Najafi, Gholami and Ardjmand, 2013). Separately, wet milling, ammonia steeping, enzymatic hydrolysis and microbial fermentation techniques have been developed to improve cassava starch extraction (reviewed by Moorthy, 2002). However, combined pre-treatments have recently been considered as a promising approach to overcome cell wall recalcitrance. Therefore, development of compatible pre-treatment combinations for ground cassava roots

in order to reduce the negative effect of cellular entrapment of starch granules during extraction is necessary.

Effective cassava starch extraction requires optimum disintegration of root parenchyma cell walls to release entrapped starch granules (Da, Dufour, Marouzé, Thanh and Maréchal, 2008). Some microorganisms are able to produce exo-enzymes that hydrolyse cell wall constituents, hence facilitating the release of starch granules (George, Padmaja and Moorthy, 1991). Alkaline pre-treatment is known to remove lignin, acetyl groups and different uronic acid substitutions which inhibit accessibility to cellulose by hydrolytic enzymes (Li, Kim and Nghiem, 2010). Therefore, a potential technique for improving cassava starch extraction is through combining alkali pre-treatment with high pH starter culture induced fermentation, alkaliphilic fermentation. Hence, there is need to investigate suitable alkaliphilic (microorganisms that grow optimally at pH values between 9 to 12 (Horikoshi, 1999)) bacteria that will be effective in fragmenting cassava cell walls during alkaliphilic fermentation, for application in improving starch extraction yield.

The main aim of this study was to improve commercial utilisation of cassava starch in sub-Saharan Africa through better starch extraction techniques using alkaline steeping and fermentation with alkaliphilic *Bacillus* spp. cultures, with specific interest in improving cell wall fragmentation and starch yields, which will facilitate economic development of small-holder cassava farmers.

2 LITERATURE REVIEW

2.1 INTRODUCTION

This review focuses on the science and mechanisms of different processes to improve disintegration of cassava root parenchyma cell walls. It summarizes current knowledge on cassava root physico-chemical properties, the nature of root cell wall polymers and the bio-mechanical modifications induced by physical, chemical and microbial/enzymatic treatments. Studies conducted to improve cassava cell wall disintegration in order to reduce its negative effect on starch granule release during extraction are reviewed. Lastly, valuable analytical methods to determine chemical and structural properties that underpin cell wall strength are discussed.

2.2 PHYSICO-CHEMICAL PROPERTIES OF CASSAVA ROOTS

Cassava roots are usually elongated, with depressions and crevices along their length and taper towards the distal end and are approx. 22.8 cm and 4.6 cm in length and diameter, respectively (Adetan, Adekoya and Aluko, 2003). The root starchy flesh is protected by the peel, which constitutes approx. 10.6-21.5 g/100 g (as is basis) of the total root weight. However, authors have reported wide variations in cassava root characteristics, due to the influences of genotype, environment and maturity among others (Benesi, Labuschagne, Herselman, Mahungu and Saka, 2008; Ebah-Djedji, Dje, N'zue, Zohouri and Amani, 2012). Data highlighting the wide variations in cassava root composition, i.e. starch, dry matter, fibre, protein and lipid contents are presented in Table 2.1.

Sánchez, Salcedo, Ceballos, Dufour, Mafla, Morante, Calle, Pérez, Debouck, Jaramillo and Moreno (2009) in a comprehensive study involving over 4,000 cassava genotypes reported the root starch content to vary between 65.0 and 91.0 g/100 g (dry basis). In the same study, root dry matter content averaged 33.6 g/100 g (is basis). Ceballos, Sánchez, Chávez, Iglesias, Debouck, Mafla and Tohme (2006) in a study of 149 cassava clones, reported an average root dry matter content of approx. 36.1 g/100 g (as is basis), with a range of 25.7 to 44.0 g/100 g (as is basis). These two studies highlight genotypic diversity in cassava root starch and dry matter composition. Despite the variations, a strong correlation exists between cassava root

starch and dry matter contents (Breuninger, Piyachomkwan and Sriroth, 2009). A relationship utilised by the cassava starch industry in gravimetric based root procurement.

Although rich in starch, from a human nutrition standpoint cassava roots are deficient in protein, fat, vitamins, and several minerals (Charles, Sriroth and Huang, 2005). However, recent research has found the existence of β-carotene rich non-transgenic cassava roots (Vimala, Thushara, Nambisan and Sreekumar, 2011). This discovery resulted from the significant interest in specific cassava root physio-chemical attributes to satisfy different end uses. In fact, authors have found non-transgenic cassava lines with unique native starch compositions, i.e. amylose-free and high-amylose types (Ceballos, Sánchez, Morante, Fregene, Dufour, Smith, Denyer, Pérez, Calle and Mestres, 2007; Ceballos, Sánchez, Tofiño, Rosero, Denyer, Smith, Dufour, Morante, Pérez and Fahy, 2008). Studies have also investigated the suitability of cassava genotypes for industrial starch extraction with a focus on reducing water consumption and higher starch yields (Maieves, Oliveira, Frescura and Amante, 2011).

2.3 CASSAVA ROOT PARENCHYMA CELL WALLS

The “tethered network model” for plant cell wall structure postulates a pectin matrix embedded within cellulose microfibril structures cross-linked to xyloglucans, and a web of structural proteins cross-linked together and to other wall components (Reilly, Bernal, Cortés, Gómez-Vásquez, Tohme and Beeching, 2007). The cell wall chemical composition and microstructure of cassava roots influence several physio-chemical attributes (Padonou, Mestres and Nago, 2005). Thus, detailed information concerning cassava cell wall polymers is important to improve root processing (Salvador, Suganuma, Kitahara, Tanoue and Ichiki, 2000). Salvador et al. (2000) found cellulose to constitute approx. 48% of cassava cell wall material, followed by hemicelluloses (22%) and pectin (18%). Pereira and Beléia (2004) in a study of two cassava varieties reported cellulose as 70-57% of the cell wall material, followed by pectin (11-16%) and hemicelluloses (1.2-2.7%). Despite Salvador et al. (2000) and Pereira & Beléia (2004) reporting 88.2% and 81.2% recovery of total cell wall material, respectively, the large differences are probably because cell walls are dynamic in composition and structure (Pattathil, Avci, Zhang, Cardenas and Hahn, 2015). For example, crude fibre is reported to decrease with maturity of cassava roots (Pereira and Beléia, 2004).

Table 2.1 Data on cassava root chemical composition

No. of genotypes studied	Age of roots (months)	Root dry matter (g/100 g)	Starch (g/100 g)	Sugars (g/100 g)	Crude fibre (g/100 g)			Protein (g/100 g)	Lipid (g/100 g)	Ash (g/100 g)	Reference
					Insoluble	Soluble	Total				
10	8		72.92-74.51		3.73-5.20	0.71-1.28	4.70-7.12				Maieves, Oliveira, Bernardo, Müller and Amante, 2012
10	6	27.73-40.62	74.94-82.26	2.25-3.25			0.36-1.18				Sajeev, Sreekumar, Moorthy, Suja and Shanavas, 2008
3	7	37.1-40.1						2.2-2.7	0.4-0.5	1.4-1.9	Favaro, Beléia, Da Silva Fonseca Junior and Waldron, 2008
20	13-15	12.9-39.7		1.76-8.93			2.63-4.92	1.66-4.08	0.42-0.65		Padonou et al. (2005)
3	10		86.53-91.07				3.14-3.77	2.11-4.55	0.08-0.16	0.58-0.4	Saengchan, Nopharatana, Lerdlattaporn and Songkasiri, 2015
3	12	37.82-39.64	75.36-77.70	2.10-2.64				1.95-2.27	0.58-1.04	2.29-2.67	Koko, Kouame, Anvoh, Amani and Assidjo, 2014
6			71.64-84.15		2.72-5.14	3.28-5.50	7.36-8.54	1.46-2.76	0.58-1.40	1.46-2.71	Rojas, Nair, Herbas and Bergenståhl, 2007
2	7-19	40.9-42.3	84.8-89.6				1.6-2.0	1.47-3.42	0.52-0.78	1.88-2.29	Pereira and Beléia, 2004

All proximate data are expressed on a dry weight basis.

However, dietary fibre, which is a more comprehensive measure of cell wall material, reportedly increases with age of the cassava roots (Pereira and Beléia, 2004).

Research on cassava cell walls, i.e. cell wall composition and characterization of the fractionated constituent sugars has been limited (Table 2.2). The reported differences in cell wall constituent sugar values are probably because of differences in material preparation (Adetunji, du Clou, Walford and Taylor, 2016). Cassava root parenchyma cell walls generally comprise non-starch polysaccharides formed by condensation of monosaccharide units into glucan, xylan and arabinan chains (Buschmann, Potter and Beeching, 2002). The xylem tissue and schlerenchymous fibres also contain lignin, a complex macromolecule of cross-linked phenylpropanoid monomers, which adds stiffness to the cell walls and acts as a protective barrier against enzymatic hydrolysis (Liu, Pang, Wang, Li, Lu and Niu, 2015). Lignin is covalently linked to carbohydrates through benzyl esters, benzyl ethers and phenyl glycosides (Ghaffar and Fan, 2013). Figure 2.1 shows the distribution of lignin in cassava parenchyma cell walls of a tuberous root harvested 120 days after planting (Figueiredo, Moraes-Dallaqua, Bicudo, Tanamati and Aguiar, 2015).

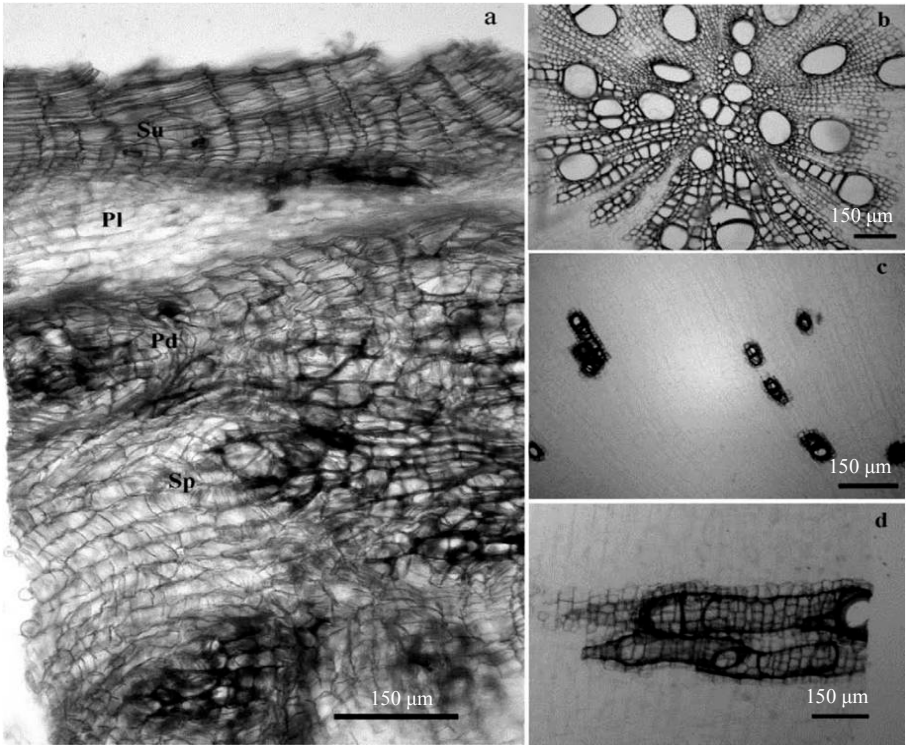

Figure 2.1 Images of a cassava tuberous root harvested 120 days after planting showing lignification of the cell walls. a= cross section of bark tissue, b= cross section of central cord showing vessel elements and fibres, c= longitudinal section showing secondary xylem with vessel elements, and d= radial section showing details of vessel elements. Su, suber; Pl, phellogen; Pd, phelloderm; Sp, secondary phloem (Figueiredo et al., 2015)

2.3.1 Cellulose

In the cassava root starchy flesh, cellulose is located mainly in the primary cell walls (Ngea, Guillon, Ngang, Bonnin, Bouchet and Saulnier, 2016). Cellulose is a linear homopolysaccharide comprised of D-glucose residues, covalently linked by (1-4)-β-glucosidic bonds. In cellulose, three reactive hydroxyl groups per anhydroglucose unit are located at positions C2, C3, and C6 (reviewed by Klemm, Heinze, Philipp and Wagenknecht, 1997). Cellulose is amphiphilic due to all three hydroxyl groups being equatorially located (Lindman, Karlström and Stigsson, 2010). Thus, the equatorial direction of a glucopyranose ring is hydrophilic, while the axial direction is hydrophobic due to hydrogen atoms of C-H bonds (Lindman et al., 2010). The hydrophobic and hydrophilic surfaces of cellulose microfibril structures are thought to bind xyloglucan through adsorption (Zhao, Crespi, Kubicki, Cosgrove and Zhong, 2014), xylan (Busse-Wicher, Gomes, Tryfona, Nikolovski, Stott, Grantham, Bolam, Skaf and Dupree, 2014), and lignin (Besombes and Mazeau, 2005).

Cellulose may be modified by chemical, enzymatic and mechanical techniques, leading to changes in its functional properties (Klemm et al., 1997; Liao, Huang, Hu, Zhang and Tan, 2011). Chemical solubilisation of cellulose is dependent upon the solvent's ability to break intramolecular hydrogen bonds that stiffen cellulose chains and intermolecular hydrogen bonds that assemble its molecules into sheet-like structures (Zhang, Ruan and Gao, 2002). NaOH solutions (7-10%) can dissolve approx. 10% Avicel (microcrystalline cellulose) below 0^{o}C (Egal, Budtova and Navard, 2007). Cai, Zhang, Liu, Liu, Xu, Chen, Chu, Guo, Xu and Cheng (2008) postulated that the hydrates formed by NaOH/water systems were able to break some inter- and intramolecular hydrogen bonds within cellulose. Cuissinat and Navard (2006) found that swelling and dissolution of cellulose in NaOH solutions proceeds by two mechanisms, dependent on solute concentrations and temperature. In Mode 3 type, cellulose fibres lose their regular diameter by swelling at distinct points ("balloons"), while Mode 4 type involves homogeneous swelling along the fibre. Importantly, the authors found that cellulose fibrils steeped in NaOH were more prone to disintegration when ground than native cellulose. Furthermore, Karuna, Zhang, Walton, Couturier, Oztop, Master, McCarthy and Jeoh (2014) found improvement in cell wall disintegration by combining extensive mechanical disruption with alkali pre-treatment. Therefore, extensive physical disruption of alkali steeped cassava roots could aid disintegration of starch containing parenchyma cell walls.

Table 2.2 Cassava cell wall extraction yield and its neutral sugar composition

No. of genotypes	Age of roots (months)	Cell wall material, (g/100 g root) (dry basis)	Cell wall sugar composition (mol %)								Reference
			Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	
3	7	3.3-3.5	2.2-2.9	0.7-0.9	4.8-6.4	9.4-4.6	1.5-2.0	18.5-22.3	42.1-50.4	13.5-21.2	Favaro et al. (2008)
		7.3*	0.9	0.7	4.0	9.9	1.6	22.1	43.2	17.0	Salvador et al. (2000)
			6.2	0.7	10.7	2.5		74.8	5.1	29.0	Salvador, Suganuma, Kitahara, Tanoue and Ichiki, 2002
		7.3	0.6	0.3	7.2	16.6	3.1	12.5	59.6	10.1	Carré and Brillouet, 1986
		4.1-5.7	1.8	0.3	2.6	6.1	1.6	10.0	35.2	6.4	Brillouet, Rouau, Hoebler, Barry, Carre and Lorta, 1988
1	12	4.4	1.0	0.8	3.2	3.8	8.0	15.4	28.4		Ngea et al. (2016)
		7.4[1]	1.4		1.8	3.4		7.5	72.7		Adetunji et al. (2016)

*Cassava starch extraction residue, and not the whole root, was used to calculate the yield of cell wall material.
[1] Cassava cell wall material was probably contaminated by trace amounts of protein, lipids and ash
All values are presented in dry weight basis

Concerning enzymic hydrolysis of cellulose, native cellulose is a semi-crystalline polymer with highly ordered crystalline domains coexisting with amorphous regions, the latter being more susceptible to enzymatic hydrolysis (Mittal, Katahira, Himmel and Johnson, 2011). Enzymic hydrolysis involves: endoglucanases which hydrolyse internal 1, 4-β-D-glucosidic linkages (preferably amorphous cellulose domains), exoglucanases that act on the existing or endoglucanase generated chain ends, and β-glucosidases, which hydrolyse the released cellobiose into glucose (Himmel, Baker, Adney and Decker, 2007). Concerning mechanisms, extracellular bacterial cellulases act as either free enzymes or cellulosomal complexes. The action of free enzymes achieves surface limited degradation, while cellulosomes accomplish pocket-type delamination (reviewed by Donohoe and Resch, 2015). Therefore, combinations of enzymes capable of both types of degradation could probably improve cell wall cellulose hydrolysis by either additive or synergistic interactions.

2.3.2 Hemicelluloses

Hemicelluloses are structurally diverse polysaccharides comprising β-1, 4-linked glycans with various sugar substituents (reviewed by Pauly, Gille, Liu, Mansoori, De Souza, Schultink and Xiong, 2013). In cassava cell walls, hemicelluloses comprise relatively very low levels of homogalacturonan and methyl-esterified homogalacturonan fractions, low levels of 1, 5-α-L-arabinan and xyloglucan fractions but much higher levels of 1, 4-β-D-galactan and 1, 5-α-L-arabinan fractions (Huang, Willats, Lange, Jin, Fang, Salmeán, Pedersen, Busk and Zhao, 2016). The authors postulated that the 1, 5-α-L-arabinan is linked to longer stretches of 1, 5-linked arabinosyl residues of arabinans.

Hemicelluloses interact with cellulose non-covalently via hydrogen bonding (reviewed by Scheller and Ulvskov 2010). The association between xyloglucan and cellulose can be very strong, enabling xyloglucan to resist dilute KOH solubilisation (Zuluaga, Putaux, Cruz, Vélez, Mondragon and Gañán, 2009). This is probably because xyloglucan binds not only to the surface of cellulose microfibril structures, but is also entrapped within the microfibril structures (Liu, Xu, Sun, Ren, Curling, Sun, Fowler and Baird, 2006). This finding appears to support the “biochemical hotspot” model that explains intricate structural arrangement of plant cell wall polymers. The model shows junctions linking irregularly arranged cellulose microfibril structures with a few xyloglucan chains (Park and Cosgrove, 2015). The intertwined junctions (hotspots) are thought to be inaccessible to chemical and enzymatic

hydrolysis. Furthermore, the “biochemical hotspot” model postulates that cellulose microfibril structures frequently make close contact with each other resulting into formation of bundles, which in turn influence cell wall bio-mechanics (Zhang, Mahgsoudy-Louyeh, Tittmann and Cosgrove, 2014). Zhao et al. (2014) postulated that the strength of cellulose–xyloglucan–cellulose junctions contribute substantially to cell wall strength. Thus, the implications of these findings are important in the design of effective cassava cell wall disintegration techniques.

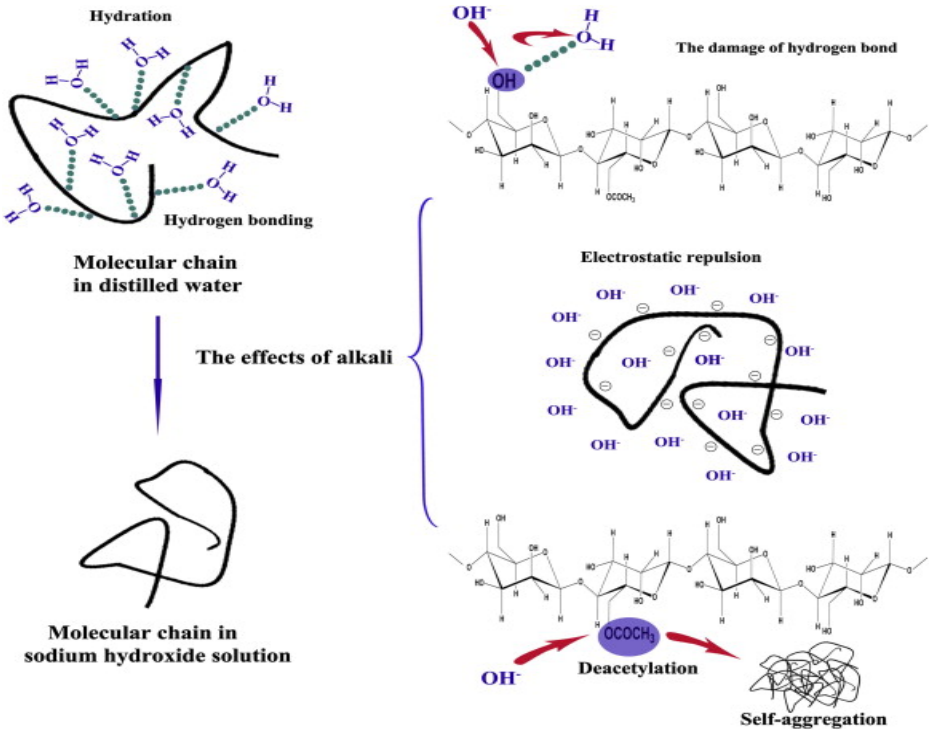

Figure 2.2 Schematic diagrams illustrating the effect of NaOH treatment on the glucomannan molecular chain (Luo, He and Lin, 2013)

The amorphous structure of hemicelluloses makes them susceptible to alkali hydrolysis (reviewed by Pauly et al., 2013). For example, Luo et al. (2013) studied NaOH solubilisation of glucomannan, a hemicellulose comprised of β-1, 4-glucosidic linked D-mannose and D-glucose monomers with a low degree of acetyl group substituents. The authors found that a higher degree of backbone substitution was positively correlated with increased polymer solubility as a result of OH^- nucleophilic attack (Figure 2.2). It is thought that the presence of backbone substituents prevents close alignment of hemicellulose chains, which improves alkali accessibility (reviewed by Pauly et al., 2013).

Concerning enzymatic hydrolysis, the great diversity in bond linkages within hemicelluloses necessitates the involvement of different enzymes (Van Dyk, Sakka, Sakka and Pletschke, 2010). Hemicellulose hydrolysing enzymes include: de-polymerizing enzymes, which cleave the polymer backbone, and de-branching/ancillary enzymes, which remove substituents posing steric hindrances to de-polymerizing enzymes (reviewed by Bhattacharya, Bhattacharya, and Pletschke, 2015). Among the many hemicellulose hydrolysing enzymes are alkaline adapted enzymes. For example, alkaline xylanases from alkaliphilic *Bacillus halodurans* S7 have been found to maintain catalytic functionality at alkaline pH due to their high percent composition of acidic amino acids (Mamo, Thunnissen, Hatti-Kaul and Mattiasson, 2009). This adaptation results in a high ratio of negatively to positively charged amino acid residues and a highly negatively charged enzyme surface (Mamo et al., 2009). Alkaline enzymes have a high percent composition of arginine and histidine, whereby arginine forms hydrogen bonds at extremely high pH, while histidine remains largely neutral, hence enabling enzyme solubility (Mamo et al., 2009). Alkaline xylanases are also reported to have relatively less asparagine, an alkali susceptible amino acid, within the polypeptide chain (Mamo et al., 2009). Therefore, application of alkaline hemicellulose hydrolysing enzymes could enable combining alkaline chemical solubilisation of cassava cell wall hemicellulose with simultaneous enzymatic hydrolysis.

2.3.3 Pectin

In models for plant cell wall structure, pectin is postulated to form a soft, gel-like viscous matrix in which cellulose–hemicellulose networks are embedded (reviewed by Cosgrove, 2005). However, it is not covalently bound to cellulose. Pectin predominantly comprises 1-4 α-D-galacturonic acid residues that are partly esterified with methanol (Palanivelu, 2006). Favaro et al. (2008) reported a composition of approx. 14% to 22% uronic acids as the main constituent of pectin in cassava root parenchyma cell walls. Hongbété, Mestres, Akissoé, Pons, Hounhouigan, Cornet and Nago (2011) estimated the pectin content of cassava cell walls as approx. 10-15% of cell wall material. Variations in pectin content in cassava roots of different ages have been found, while uronic acid content was affected by both cassava root age and cultivar type (Pereira and Beléia, 2004). Pectin is an important contributor towards plant cell wall strength, thus its content is inversely related to cassava root friability (Hongbété et al., 2011). It has been postulated that the relationship between cell wall pectin

and tissue friability is because it is the main component of the middle lamella and its cementing role in cell to cell adhesion (Parker, Parker, Smith and Waldron, 2001).

Cell wall pectin occurs as distinctive domains covalently cross-linked to each other. However, the exact nature of cross-linking is not completely understood (Cosgrove, 2014). This author's model postulates how pectin domains could covalently link to form large macromolecular networks with rhamnogalacturonan I as the anchoring backbone (Figure 2.3.a).

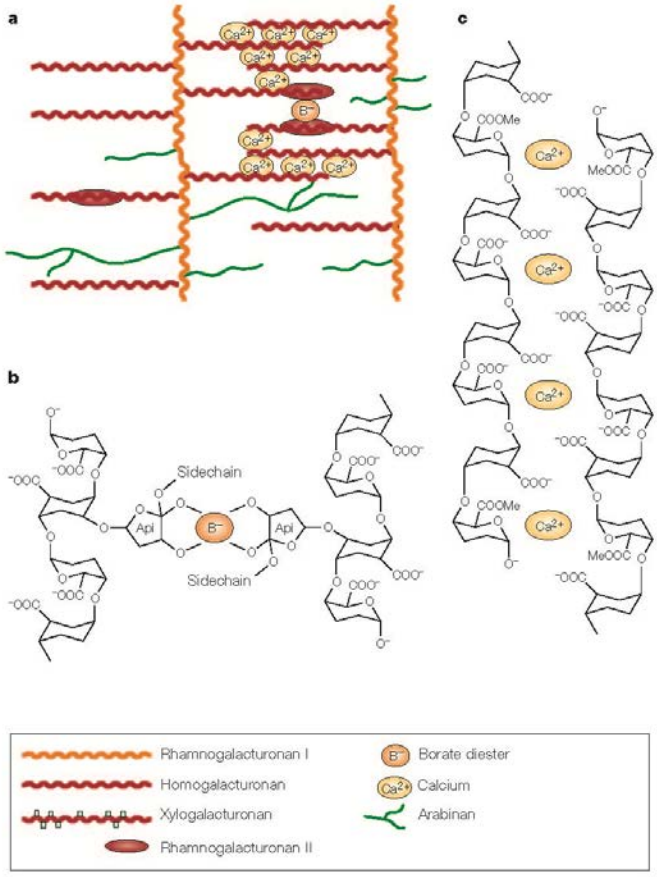

Figure 2.3 Model illustration of the formation of pectin networks within plant cell walls by covalent and ionic associations (Cosgrove, 2005)

The model shows that in cell walls, homogalacturonans are ionically cross-linked by calcium, whereas boron cross-links rhamnogalacturonan II through diester linkages (Figures 2.3b and 2.3c). Rhamnogalacturonan II is postulated to form dimers through a borate ester bond, an important cross linking in regards to cell wall strength (reviewed by Cosgrove, 2005). Approx. 75% of homogalacturonan carboxyl groups are methyl esterified, a modification that removes the carboxylate ion negative charge and blocks Ca^{2+} facilitated cross-linking.

A significant relationship between cassava root compressive strength and intercellular adhesion has been reported (Eggleston and Asiedu, 1994). Cassava root compressive strength indicates the measure of the root tissue cell walls to resist disintegration (Nanda and George, 1996). Favaro et al. (2008) found that phytate-chelation of divalent cations cross-linking pectic polysaccharides is responsible for the cassava root parenchyma cell adhesion. Research has shown that in firmer cassava (Favaro et al., 2008), pears (Murayama, Katsumata, Horiuchi and Fukushima, 2002) and tomatoes (Xin, Chen, Yang, Zhang, Deng and Yang, 2010), there are lower levels of chelate-soluble pectin, but higher levels of chelate-insoluble and sodium carbonate-soluble pectin. However, a clear distinction between water-soluble and water-insoluble pectin is difficult due to the nature of the pectin precursor, protopectin. Protopectin is water-insoluble. However, upon restricted hydrolysis by cell wall protopectinases it is converted into water-soluble pectin or pectic acid (Sakai, 1988). In spite of difficulties in defining pectin fractions by solubility, it has been demonstrated that the rate of cellular separation and loss in tissue firmness in cassava roots is linked to the availability of divalent cations that promote pectin gelation and cross-linking (Eggleston and Asiedu, 1994; Favaro et al., 2008). By contrast, addition of chelating agents such as imidazole, cyclohexane-trans-1, 2-diamine-N, N, N', N'-tetraacetate, phytic acid or sodium carbonate was found to dramatically favour cellular disruption (Favaro et al., 2008). Therefore it appears that specific pectin fractions, chelate- and sodium carbonate-soluble fractions, directly influence cassava tissue firmness (Hongbété et al., 2011).

With regard to enzymatic hydrolysis of pectin, pectinases comprise polygalacturonases, pectin esterases, pectin lyases and pectate lyases, all having different substrate specificities (Ahlawat, Dhiman, Battan, Mandhan and Sharma, 2009). Due to cellulose and hemicellulose polymers being embedded within pectin, its degradation by pectinases is believed to expose cellulose, improving the effectiveness of cellulases (Giacobbe, Pepe, Ventorino, Birolo, Vinciguerria and Faraco, 2014). Concerning alkaline pectinases which are adapted to high pH environs, the enzyme active sites are modified to contain histidine ^{223} and aspartic acid ^{201} residues, where histidine ^{223} acts as the proton donor, while aspartic acid ^{201} acts as a nucleophilic site (Palanivelu, 2006). Therefore despite alkaline influence upon histidine and aspartic acid ionisation, these molecular changes enable alkaline pectinases to maintain catalytic functionality

2.4 ALKALIPHILIC MICROORGANISMS AND CELL WALL HYDROLYSIS

Bio-conversion of plant cell wall material into valuable bio-products commonly involves enzymatic hydrolysis and/or microbial fermentation (Yoon, Ngoh and Chua, 2013). However, industrial scale hydrolysis of cassava cell walls using pectinases is considered expensive (Dzogbefia, Ofosu and Oldham, 2008). With regard to fermentation, cell wall pre-treatment may be performed to increase cellulose surface area, reduce cellulose crystallinity, partially depolymerise and remove lignin (Galbe and Zacchi, 2007). Alkali treatment is an effective and common pre-treatment technique for plant cell wall material (Yoon et al., 2013). However, improvements in cell wall digestibility by alkaline treatment may be undone when followed by acid conditioning to neutralise the material (Karuna et al., 2014). This is because the precipitation of alkali solubilised materials re-insulates the cell walls.

Alkaliphiles, i.e. microorganisms that grow optimally at pH values from 12-9 (reviewed by Horikoshi, 1999) and specifically those able to produce cell wall hydrolysing enzymes could enable bio-conversion of cell walls under alkaline conditions. The combination of alkali pre-treatment and fermentation with cultures producing alkaline stable cell wall hydrolysing enzymes should improve cassava cell wall disintegration. Alkaliphiles comprise both facultative and obligate microorganisms (Mamo and Mattiasson, 2016). Obligate alkaliphiles grow optimally at or above pH 9 but do not thrive under neutral or acidic conditions (reviewed by Horikoshi, 1999). Alkaliphiles inhabit high pH environments, but have also been isolated from non-alkaline environments (Mamo and Mattiasson, 2016). Alkaliphiles are also a source of alkaline stable enzymes (Mamo and Mattiasson, 2016). For commercial enzyme production, bacteria are usually preferred over fungi due to comparative ease of fermentation (reviewed by Prathyusha and Suneetha, 2011). Therefore among alkaliphilic microorganisms, *Bacillus* species are the most characterized (reviewed by Fujinami and Fujisawa, 2010). The authors suggested that the interest was due to *Bacillus* species producing several extracellular alkaline enzymes, their ubiquitous presence and their non-pathogenicity. Alkaline extracellular enzymes from *Bacillus* spp. include: cellulases, xylanases, pectinases, chitinases, proteases, α-amylases, pullulanases, cyclomaltodextrin glucanotransferases and lipases (reviewed by Horikoshi, 1999). Intriguingly, the alkaliphilic *Bacillus cellulosilyticus* was found to hydrolyse polysaccharides by using extracellular enzymes and unique membrane anchor proteins, unusual for Gram-positive aerobes (Mead, Drinkwater and Brumm, 2013).

The mechanism by which alkaliphilic microorganisms adapt to alkaline pH is thought to involve bacterial cell wall teichuronic acid and teichuronopeptide (a mixture of poly-γ-L-glutamate and polyglucuronate) (Aono, Ito and Horikoshi, 1992). In the facultative alkaliphile *B. halodurans* C-125, these acidic surface polymers are thought to prevent OH^- penetration into the cell protoplasm (Aono et al., 1992). Several authors have also found that alkaliphilic *Bacillus* species use protons for oxidative phosphorylation and sodium ions for adaptation to alkaline pH (reviewed by Fujinami and Fujisawa, 2010). Interestingly, some alkaliphiles are reported to produce substantial amounts of extracellular organic acids (Mamo and Mattiasson, 2016). The implication of this finding is that after alkaliphilic fermentation, alkaliphiles could potentially be used to neutralize the alkaline waste water. However, the production of sufficient amounts of organic acids to completely neutralise the alkaline effluent during a production process would likely be challenging.

2.5 DEVELOPMENTS IN CASSAVA WET MILLING STARCH EXTRACTION

2.5.1 Modification of cassava root cell walls by physical milling

Cassava starch production involves liberation of starch granules from within cells by either milling fresh or dried root pieces. However, milling of dry cassava root pieces requires more energy compared to wet milling (Abera and Rakshit, 2003). Furthermore, starch extraction by milling of dry cassava roots, results in less liberation of starch granules from the cell wall material (residue) as compared to wet milling (Abera and Rakshit, 2003). Therefore in cassava root milling processes, wet milling (Figure .2.4) is normally preferred (Saengchan et al., 2015). The process to liberate entrapped starch granules is dependent upon effective disintegration of the cell walls and separation of the resultant residues (Sriroth, Piyachomkwan, Wanlapatit and Oates, 2000). It comprises grinding the cassava roots into a slurry, followed by shredding using a saw tooth rasper to release the starch granules from within broken cells (Saengchan et al., 2015). Da, Dufour, Giraldo, Moreno, Tran, Velez, Sanchez, Le-Thanh, Marouze and Marechal (2013) reported that rasping efficiency was dependent on equipment type and specific operational details, i.e. blade height, teeth design and rotational speed.

The separation of liberated starch granules from the broken cassava root cell walls generates substantial amounts of residue (Balagopalan et al., 1994). In regard to the residue, approx.

50% to 60% (dry basis) is comprised of entrapped starch granules (Balagopalan et al., 1994). Thus, the residues are a source of financial loss (due to starch being trapped) and an environmental disposal challenge (Maieves et al., 2011). Financial losses due to unrecovered starch are estimated at approx. US$ 2 million per annum for a factory crushing 200 tonnes of fresh roots per day (Saengchan, Nopharatana and Songkasiri, 2014). Thus, improving starch extraction yield is of critical importance towards milling efficiency (Maieves et al., 2011). Therefore, physical based techniques of improving cassava starch extraction efficiency have been investigated, e.g. serration of grinding/rasping surfaces using fine wires (Da et al., 2008).

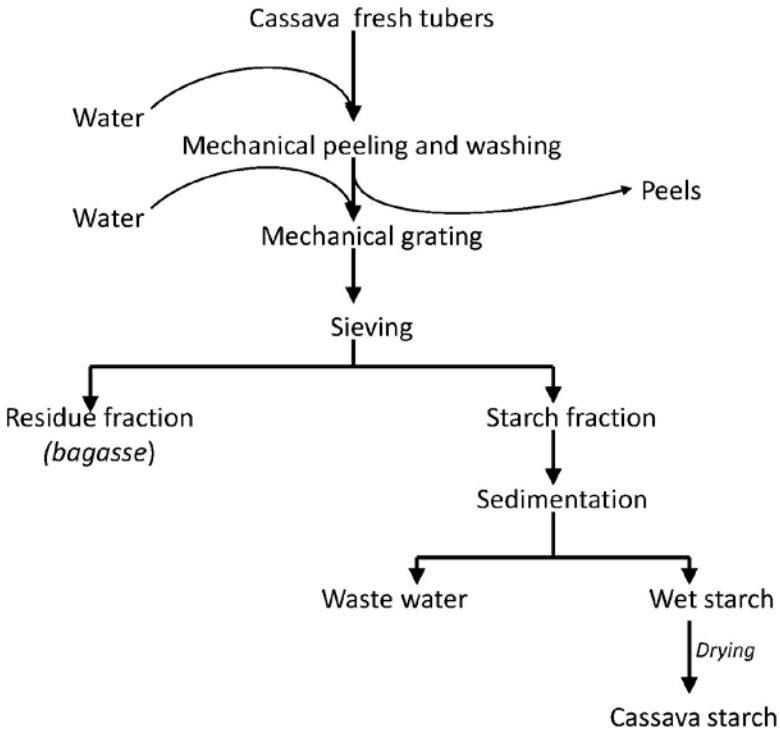

Figure 2.4 Schematic diagram of the major stages involved during starch extraction by wet milling of cassava roots (adapted from FAO, http://www.fao.org/docrep/007/y2413e/y2413e0d.htm)

The cell wall material in the cassava wet mill residue fraction is composed mainly of cellulose, hemicelluloses, lignin and trace amounts of protein (Saengchan et al., 2014). Liao et al. (2011) found that rigorous ball milling of cassava bagasse (residue cell wall-rich material) resulted in a significant reduction in cellulose crystallinity. The authors postulated that ball milling breaks inter- and intramolecular hydrogen bonds within cellulose resulting in increased lattice hydrogen bond energy. The increased lattice hydrogen bond energy is an indication that the amorphous cellulose was chemically more reactive than crystalline

cellulose. The authors suggested that cell wall modification by milling was due to the milling stress exceeding chemical bonding energy, hence bonds ruptured. This indicates that intensive wet milling of cassava roots would modify the physico-chemical attributes of the root parenchyma cell walls.

Concerning starch recovery from disintegrated cassava cell walls, recent research provides a better understanding about physical variables influencing the process. Saengchan et al. (2015) found that cassava particle size, root genotype, magnitude of the centrifugal force and the pressure drop across grinding and filtration stages affect starch recovery. Expectedly, a decrease in particle size increased starch recovery irrespective of genotype. The authors found that application of a centrifugal force during sieving improved starch recovery. However, a high pressure drop between grinding and filtration did not improve starch recovery. The authors observed that a high pressure drop caused compaction of the milled cassava and as a result, reduced the porosity. They also reported that genotype effect on starch recovery was associated with the root fibre content. Da et al. (2013) also found an inverse relationship between milled fibre size and cassava starch yield. The implication of this association is that techniques capable of fine disintegration of fibrous cassava cell walls could concomitantly improve starch granule recovery.

However, physical milling is relatively ineffective at disintegrating fibrous cassava cell walls, and starch recoveries greater than 80-85% are not achievable (Nanda and George, 1996). This is probably because unlike chemical and biotechnological treatments which weaken cell walls, milling involves physical disintegration of firm cell walls. Therefore, chemical and biotechnological approaches have been investigated in combination with milling to improve cassava cell wall disintegration.

2.5.2 Alkali hydrolysis of cassava root cell walls

Moorthy (1991) reported that 0.03 M ammonia solution steeping of cassava pieces did not improve starch yield. However, the study did not determine whether dilute ammonia steeping had any effect on the cassava cell walls. In a more recent study, Ruangudomsakul, Ruksakulpiwat and Ruksakulpiwat (2015) found that 4% NaOH steeping at 80^{o}C of the cassava residue fraction increased its cell wall crystallinity index. The authors suggested that

alkali steeping of cassava cell walls removed the amorphous cell wall constituents. Furthermore, Favaro et al. (2008) found that dilute Na_2CO_3 hydrolysed cassava cell wall polymers through a de-esterification mechanism, resulting in a cellulose-rich residual material (approx. 50% cellulose) impregnated with highly-branched galactan rich pectic polymers. In principle, this finding agrees with Chang and Holtzapple (2000) who postulated that alkali treatment of cell walls involves the hydrolysis of hemicellulose acetyl groups and lignin–carbohydrate ester linkages. Hydrolysis of lignin–carbohydrate ester linkages by alkaline conditions promotes lignin solubilisation, which in turn improves the enzymatic susceptibility of cell walls (Kim and Lee, 2005). Liu, You, Jin and Yu (2013) found that treatment of cellulose fibres with 0.2-0.5 M sodium hydroxide caused a decrease in cellulose crystallinity, which in turn increased cellulose susceptibility to enzymatic hydrolysis. Furthermore, the authors reported a strong inverse correlation between alkali concentration and cellulose crystallinity. Apiwatanapiwat, Vaithanomsat, Ushiwaka, Morimitsu, Machida, Thanapase, Murata and Kosugi (2016) also found that pre-treatment of cassava residue with ammonia and under pressure ruptured the cell walls (Figure 2.5). The authors observed that an increase in pressure resulted in an incremental increase in ruptured cell walls.

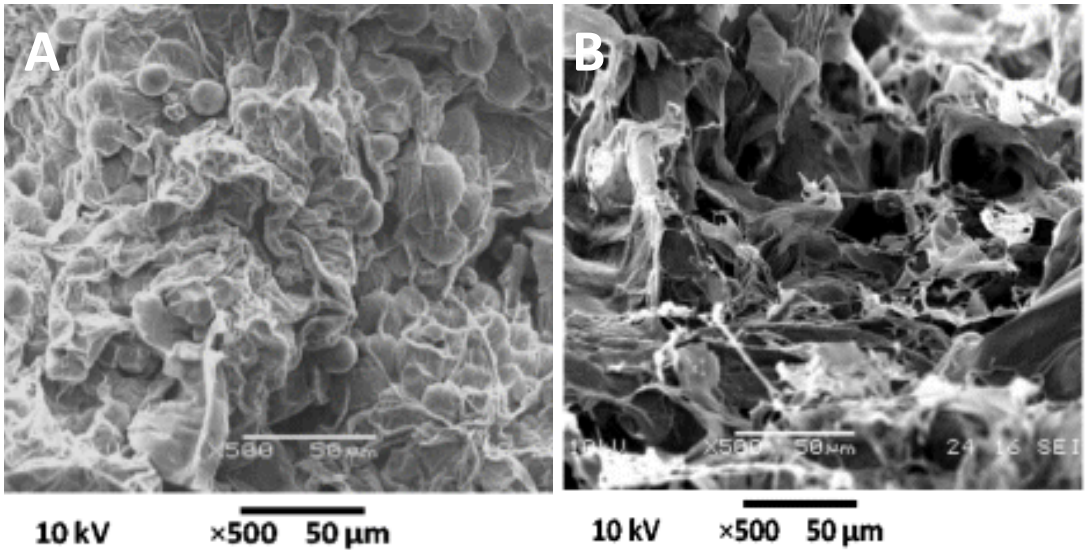

Figure 2.5 Scanning electron microscopy images of cassava residue fraction showing the effect of ammonia gas adsorption fibre expansion (AGAFE) pre-treatment (A) un-treated residue, (B) AGAFE pre-treated residue at 0.5 MPa (Apiwatanapiwat et al., 2016).

However, there is a need to better understand how cell wall chemical and ultra-structural modifications occur with alkaline pre-treatments (Da Costa Sousa, Chundawat, Balan and Dale, 2009). Such understanding of the alkali hydrolysis mechanism of cassava cell walls would be helpful in optimising existing or designing novel cell wall disintegration techniques.

2.5.3 Enzymatic/ microbial hydrolysis of cassava cell wall materials

Several enzymatic approaches to hydrolyse cassava cell wall polymers have been investigated. These include use of crude enzyme extracts, commercial enzymes (single entities and cocktails), spontaneous fermentation and starter culture led fermentation with single or co-cultures producing extracellular cell wall hydrolysing enzymes.

2.5.3.1 Hydrolysis of cassava cell walls using crude enzymes

Martos, Zubreski, Combina, Garro and Hours (2013) hydrolysed cassava root cell walls using a crude enzyme extract containing polygalacturonase from the yeast *Wickerhamomyces anomalus* (Figure 2.6). Polygalacturonase, is a depolymerizing enzyme, which hydrolyses α-(1, 4)-glycosidic bonds between galacturonic monomers in pectic substances (reviewed by Voragen, Coenen, Verhoef and Schols, 2009).

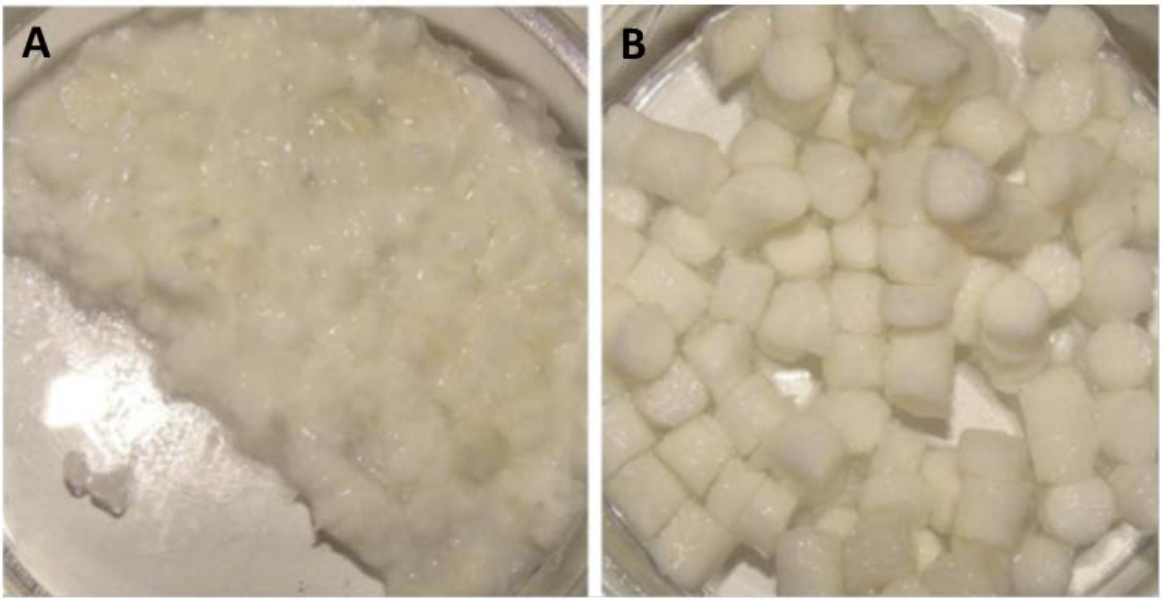

Figure 2.6 Images of raw cassava root tissue showing the loss of tissue coherence due to treatment with a polygalacturonase containing crude enzyme extract from *W. anomalus* (A) after the hydrolysis process (B) control (Martos et al., 2013).

As stated, pectin polymers in cell walls occur predominantly in the middle lamella and less in the primary cell walls (Cosgrove, 2005). Therefore the hydrolytic actions of polygalacturonases are probably more pronounced on the middle lamella tissue. Boher,

Kpemoua, Nicole, Luisetti and Geiger (1995) found that *Xanthomonas campestris pv. Manihotis* extracellular exopolysaccharide, containing pectinase and polygalacturonase, specifically degraded cassava cell wall middle lamellae. Preferential enzymatic degradation of cell walls could possibly aid the release of starch granules. This is because during milling plant tissues fracture predominantly between the adjacent cell walls (along the weaken middle lamellae) as revealed by cellular fracture analysis (Donald, Baker, Smith and Waldron, 2003).

Milling of cassava cell walls treated with crude pectinase from *Saccharomyces cerevisiae* was found to improve disintegration (Dzogbefia et al., 2008). The authors reported that cell wall disintegration was mainly influenced by the enzyme dosage and steeping time. Concerning enzymes from alkaliphilic bacteria, Sornyotha, Kyu and Ratanakhanokchai (2010) were able to disintegrate cassava parenchyma cell walls using a cellulase-free crude xylanase extract from alkaliphilic *Bacillus firmus*. However, by comparison, the authors also found that the crude enzyme of *Paenibacillus curdlanolyticus* was more effective in hydrolysing cassava cell walls. *P. curdlanolyticus* crude enzyme comprised xylanase and cellulase. Furthermore, synergistic hydrolysis of cassava cells walls was observed by using a cocktail of *B. firmus* and *P. curdlanolyticus* crude enzymes. However, the synergy was observed only at very specific enzyme ratios. This is probably because xylanases from different microorganisms not only exhibit different modes of action in hydrolysing xylan (Liab, Azadi, Collins, Tolan, Kim and Eriksson, 2000) but also appropriate proportions of xylanases in specific ratios are reported to hydrolyse xylan more effectively (Tachaapaikoon, Lee, Ratanakhanokchai, Pinitglang, Kyu, Rho and Lee, 2006).

2.5.3.2 Hydrolysis of cassava cell walls using commercial enzymes

Several authors have hydrolysed cassava cell walls using commercial cellulases, xylanases, and pectinases individually, and in combination. For example, Sriroth et al. (2000a) hydrolysed cassava cell walls using commercial cellulolytic and pectolytic enzymes. The authors found that *Trichoderma reesei* cellulase and *Aspergillus niger* pectinase were less effective individually compared to their combination in hydrolysing cassava cell walls. Rattanachomsri, Tanapongpipat, Eurwilaichitr and Champreda (2009) also found that using cellulase, pectinase and β-glucanase individually (even at high enzyme concentrations) was less effective in cassava cell wall hydrolysis compared to a combination of only cellulase and

pectinase. Interestingly, addition of β-glucanase to the cellulase and pectinase mixture did not further improve cell wall hydrolysis. The authors postulated that this was probably because of high β-glucanase side activity in the commercial cellulase.

Adetunji et al. (2016) found that treatment of milled cassava with Viscozyme, a cellulolytic enzyme cocktail, improved hydrolysis of the insoluble cell wall fraction. Furthermore, the authors observed that even an overdose of cellulolytic enzymes did not hydrolyse lignified cassava cell walls. Their data indicated that this was due to lignification of the cell walls. The intertwined and close association of cell wall lignin to cellulose micro fibrils seals off cellulase accessibility (Zhu, O'Dwyer, Chang, Granda and Holtzapple, 2008). Kajiwara and Maeda (1983) found that the liberation pattern of monosaccharides from cassava cell walls treated with *Aspergillus niger* cellulase, *Trichoderma pseudo-koningii* cellulase and a combination of *A. niger* pectinase plus *T. pseudo-koningii* cellulase were different in all treatments. Importantly, Maeda, Kajiwara and Araujo (1982) found that cassava cell walls were more disintegrated by exoglucanases in comparison to endoglucanases. These findings suggest that the action of different cellulases may differ substantially. This is probably because despite endoglucanases being non-specific nor sensitive to backbone substitution in hydrolysing β-1, 4-linked glucan chains, *in vivo* their substrate is unlikely to be native cellulose due to its predominantly crystalline configuration (Chanliaud, De Silva, Strongitharm, Jeronimidis and Gidley, 2004). Notably, the specific role of cellulases in cassava cell wall softening and subsequent disintegration is currently controversial (Ngea et al., 2016).

Intriguingly, Wehr, Menzies and Blamey (2004) reported that addition of NaOH to apple and citrus pectin solutions caused pectin de-methoxylation and dissociation of the carboxyl groups, a non-enzymic action similar to the mechanism of methyl pectin esterase. However, the specific role of pectin carboxyl group dissociation in cell wall deconstruction in not well understood. Importantly, pectin de-esterification is thought to influence several cell wall physico-chemical properties (Cosgrove, 2005). In cell walls, pectin is in contact with approx. 50% of the cellulose surface. Therefore, partial removal of pectin was found to make the cell wall material more rigid (Dick-Perez, Wang, Salazar, Zabotina and Hong, 2012). These findings suggest an association between the chemical state of the cell wall pectin to changes in cellular mechanics, a relationship of potential importance in cell wall disintegration.

Considering hemicellulose such as xyloglycans, Zhang, Chen, Jin, Xue, Guan, Wang and Zhao (2010) found that xylanase treatment of sweet potato cell wall xylans resulted in milled pulp viscosity reduction. However, with milled cassava, viscosity reduction by xylanase treatment has not been observed (Maeda et al., 1982; Huang et al., 2016). Huang et al. (2016) suggested that this was probably because commercial enzyme manufacturers produce different blends of xylanases. Furthermore, it is also probable that the cassava cell wall xyloglycans are not available for hydrolytic degradation due to being embedded.

2.5.3.3 Hydrolysis of cassava cell walls using microbial fermentation

Two ways of hydrolysing cassava cell walls using microorganisms have been reported: spontaneous and starter culture led fermentations. However, compared to use of crude or commercial microbial enzymes, the use of microorganisms in cassava cell wall hydrolysis has been less studied. George, Moorthy and Padmaja (1995) disintegrated cassava cell walls by using an inoculum containing *Lactobacillus* sp., *Streptococcus* sp., *Corynebacterium* sp. and yeasts able to produce extracellular pectinolytic and cellulolytic enzymes. The authors reported a very slight decrease in cassava root starch yield due to microbial action. They postulated that the microbial cultures utilised mainly the free cassava root sugar and less of starch. In a later study, Nanda and George (1996) spontaneously fermented cassava and reported a reduction in root compressive strength from 6.8-10.6 kg/cm^2 at 0 h to 0.5-2.1 kg/cm^2 after 48 h fermentation. These authors proposed that root softening was due to cassava cell wall degradation by microbial pectinolytic and cellulolytic enzymes. In contrast, Ampe and Brauman (1995) reported pectinolytic activities and absence of cellulolytic activity during softening of cassava root by spontaneous fermentation. This appears to suggest a principle role for cell wall pectin and not cellulose in cassava root softening. Indeed Ngea et al. (2016) found that changes to cassava cell wall polymers after spontaneous fermentation were mainly due to hydrolysis of pectic polymers namely, galactan, homogalacturonan and arabinan. With regard to the current state of knowledge concerning the application of *Bacillus* spp. Salvador et al. (2002) found that *Bacillus* M4 extracellular enzyme, exhibiting predominately arabinanase and galactanase activities with arabinoxylanase, β-glucanase and xylanase side activities but most importantly negligible cellulase activity, hydrolysed approx. 42% of cassava cell wall material. These findings indicate the need to determine the specific role of cellulolytic enzymes in cassava cell wall disintegration.

2.6 PREPARATION AND ANALYTICAL TECHNIQUES FOR STUDY OF CASSAVA CELL WALLS

2.6.1 Preparation of cassava cell wall material

In purification of cassava cell wall material, hydrolysis of the residual starch using microbial α-amylases is common. Recent studies have employed thermostable α-amylases at high temperatures > 96^{o}C (Adetunji et al., 2016; Ngea et al., 2016). Some authors have alternatively used dimethyl sulphoxide (DMSO) to solubilise and remove residual starch (Favaro et al., 2008). DMSO solubilises starch by acting as a hydrogen bond acceptor, disrupting starch-starch and water-starch hydrogen bonds (French, 1984). However, Noda, Takahata, Nagata and Shibuya (1994) found that repeated washing of sweet potato residue with 90% DMSO resulted in incomplete starch removal. This may be because DMSO is more effective at solubilising the starch amylose fraction rather than the amylopectin fraction (Jackson, 1991).

2.6.2 Techniques that can be used to analyse cassava cell walls

2.6.2.1 Wide angle X-ray scattering (WAXS)

X-rays are diffracted upon interaction with regular structures whose repeat distance is approx. equivalent to their wavelength (Khandpur, 2006). Analytical studies employ X-ray wavelengths of 0.07- 0.20 nm, similar to the inter-atomic distances between atoms (Khandpur, 2006). Given that the inter-atomic distances among chemical materials differ, the spacing of their atomic planes is not identical in all analogous directions (Khandpur, 2006). Therefore X-ray scatter gives a unique fingerprint of a material's atomic and molecular structure (Khandpur, 2006). Furthermore, X-ray diffraction techniques may be used in quantification studies because the intensities of diffraction peaks of a specific compound in a mixture are proportional to its mole fraction (Khandpur, 2006).

WAXS has an advantage over other biophysical approaches in being sensitive to a very broad range of structural changes (Makowski, 2010). Considering that cassava bagasse is structurally amorphous with a relative crystallinity of approx. 43% (Pasquini, de Morais Teixeira, da Silva Curvelo, Belgacem and Dufresne, 2010), it is probable that cassava cell wall material crystallinity can be studied using WAXS. In its simplest form, determination of

cell wall cellulose crystallinity index (CI) involves measurement of two heights in the X-ray diffractogram (Teixeira, Pasquini, Curvelo, Corradini, Belgacem and Dufresne, 2009). However, this approach significantly overestimates crystallinity (Park et al., 2010). The alternative approach of calculating relative crystallinity measures diffractograph areas (Contreras, Mendoza, Lozano and Paternina, 2016). Integrating the entire X-ray diffraction (XRD) spectra's amorphous and crystalline cellulose areas improves accuracy of the technique (Park, Baker, Himmel, Parilla and Johnson, 2010). XRD characteristic patterns have also been used to identify the different cellulose crystalline allomorphs (Park et al., 2010). With cassava, Pasquini et al. (2010) found that 6.5 M H_2SO_4 steeping of the bagasse resulted in two well-defined peaks around $2\theta = 12.5^{o}$ and $2\theta = 22.5°$. Importantly, H_2SO_4 steeping resulted in a narrowing of the XRD diffractograph peak area and an apparent increase in peak magnitude compared to un-treated bagasse. The authors attributed these differences to reduction in non-crystalline cell wall fractions (Pasquini et al., 2010).

2.6.2.2 Fourier transform infrared (FTIR) spectroscopy

FTIR analysis involves interaction of materials with infrared light energy, which excites vibration transitions at the molecular level (Wellner, 2013). The fundamental excitation (ground state to first excited state), involves energies correspondent to the mid infrared (IR) spectrum (4000–400 cm^{-1}, 2.5–25 μm λ) (Wellner, 2013). However, absorbance at each wavenumber is influenced by material molecular geometry, atomic masses, relative positions of atoms, and their inter-atomic bond strengths. Therefore specific compounds generate a unique and characteristic IR spectrum. This principle enables FTIR techniques to not only identify, but also quantify a material's constituents.

FTIR spectra of plant cell walls are influenced by the contributions of the main polymers: cellulose, hemicelluloses, pectin and lignin (Adel, El–Wahab, Ibrahim and Al–Shemy, 2010). These are pronounced within the carbohydrate fingerprint region (1200-800 cm^{-1}) due to vibrations of C–O bonds, of which vibration intensities are influenced by bond linkages and sugar types (Miller, 2001). In FTIR cassava tissue studies, both potassium bromide disc (Liao et al., 2011) and attenuated total reflectance (ATR) (Uarrota, Moresco, Coelho, da Costa Nunes, Peruch, de Oliveira Neubert, Rocha and Maraschin, 2014) procedures have been used. The latter technique is reported to improve repeatability of IR spectra (Leitermann, Syldatk and Hausmann, 2008). Relevant band regions are hemicelluloses (1732, 1240 cm^{-1}), cellulose

(1170-1150, 1050, 1030 cm^{-1}), lignin (1590, 1510 cm^{-1}), and pectin (1680-1600, 1260, 955 cm^{-1}) (Michell and Schimleck, 1996; Mascarenhas, Dighton and Arbuckle, 2000). FTIR enables identification of specific functional groups because fingerprint region absorptions are primarily caused by sensitive bending and skeletal vibrations (Stuart, 2004). Post-processing of IR spectra using e.g. Fourier self-deconvolution or calculation of 2^{nd} derivatives, improves peak resolution by sharpening spectral features (Charles, Huang and Chang, 2008; Sompong et al., 2015).

Liao et al. (2011) used FTIR to determine changes in hydrogen bond energy within cassava stillage (a cassava bioethanol production residue) as a result of impact ball milling. FTIR spectral differences have also been used by Thumanu, Sompong, Phansak, Nontapot and Buensanteai (2015) to study cassava tissue inoculated with *Bacillus subtilis*. The authors were able to elucidate complex biochemical changes, together with structural and chemical modifications. In fact, in studying cassava root postharvest physiological deterioration, a FTIR-based model outperformed biochemical assays in defect diagnosis (Uarrota et al., 2014). This is probably because FTIR enables *in situ* examination, hence avoiding loss of tissue physio-chemical information as a result of fractionation (Wellner, 2013).

2.6.2.3 Liquid and gas chromatography

For the determination of monosaccharides, the most common analytical techniques are gas chromatography (GC) and high performance liquid chromatography (HPLC) (Cheng, Tsai and Chang, 2006). In GC, flame ionization detection or mass spectrometry (MS) may be used to quantify sugars (Medeiros and Simoneit, 2007). Concerning HPLC refractive index detection, pulsed amperometric detection, evaporative light-scattering detection and MS are common (Medeiros and Simoneit, 2007). Considering cassava cell wall hydrolysates, determination of constituent neutral sugars has been specifically by GC (Favaro et al., 2008; Adetunji et al., 2016; Ngea et al., 2016) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Salvador et al., 2000; Salvador et al., 2002; Ngea et al., 2016).

A pioneering study into cassava cell walls was conducted by Salvador et al. (2000) using HPAEC-PAD. The technique is reputed to provide superior separation and detection of neutral monosaccharides (Garleb, Bourquin and Fahey, 1989). It separates monosaccharides

by using strongly alkaline solutions, in which the weak acidic properties of sugar molecules are exploited (Cataldi, Campa and Benedetto, 2000). HPAEC-PAD reportedly has an advantage over GC based methods in separation of multi-component mixtures containing monosaccharides, disaccharides and amino acids (Cataldi et al., 2000). However, the hydrolytes of purified residual cassava cell walls are most likely not complicated by the presence of disaccharides and amino acids.

In GC, separation of constituents depends on their relative vapour pressures and affinities for the stationary phase (Piantanida and Barron, 2014). Due to the use of helium or argon as the mobile phase, prior to analysis of non-volatile constituents e.g. monosaccharides, chemical derivatization is necessary (Piantanida and Barron, 2014). Derivatization techniques comprise substitution of carbohydrates polar groups to impart volatility (reviewed by Ruiz-Matute, Hernandez-Hernandez, Rodríguez-Sánchez, Sanz and Martínez-Castro, 2011). Generally, alditol acetate derivatives are preferred in analysis of monosaccharides (Blakeney, Harris, Henry and Stone, 1982). This is because use of alditol acetates simplifies chromatograms by producing a single peak for each derivatized sugar (Fox, Morgan and Gilbart, 1988).

2.7 CONCLUSIONS

The chemical composition and structure of cassava cell walls is very complex. Although some research has been conducted, the details of the cassava parenchyma cell walls are still incompletely understood. However, it appears that the pectin component plays a key role in the structural integrity of the cassava root parenchyma cell walls. As pectin in readily solubilised under alkaline conduction, alkaline steeping of cassava roots would seem to be a promising avenue to explore to improve cell wall disintegration. Additionally, application of alkaline fermentation using alkaliphilic bacteria would appear to be advantageous as their cell wall hydrolysing enzymes could complement the alkaline conditions to lead to greater cell wall disintegration.

3 HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

i) Steeping of ground cassava root pieces in dilute sodium hydroxide solution will weaken the microstructure of the parenchyma cell walls. Alkali treatment of plant cell walls solubilises hemicelluloses and lignin, hydrolyses uronic and acetic esters, and opens up cellulose network (Jackson, 1977). Furthermore, alkali induced hydrolysis cleaves the α-ether linkages between lignin and hemicelluloses, as well as ester bonds between lignin and/or hemicelluloses and hydroxycinnamic acids (Spencer and Akin, 1980). In cell walls, complex pectic cross-linkages aid in strengthening the structure cohesion (Caffal and Mohnen, 2009). Thus, opening up of the cell wall by dilute sodium hydroxide steeping will probably disrupt the various cross-linkages and result in cell wall weakening. With 0.03 M ammonia solution steeping of cassava roots followed by milling, disintegration of the cell walls was increased (Moorthy, 1991). NaOH steeping of ground cassava pieces will result in modification in the structure and composition of the cell walls, probably decreasing the root compressive strength which in turn increases tissue deformation (Nanda and George, 1996). Increased cellular rupture improves the effectiveness of grinding (Nanda and George, 1996) and fine size reduction of cassava during milling results in increased starch yield (Da et al., 2013).

ii) Endogenous fermentation of ground cassava pieces combined with dilute sodium hydroxide steeping will further weaken the cell walls. In endogenous cassava fermentation, bacteria, yeasts and moulds hydrolyse the root cell walls using extracellular cellulolytic and hemicellulolytic enzymes (Brauman, Keleke, Malonga, Miambi and Ampe, 1996). The complex microbiota efficiently hydrolyses the root cell walls due to decreased metabolite repression and feedback regulation (Soundar and Chandra, 1987; Haruta, Cui, Huang, Li, Ishii and Igarashi, 2002). Furthermore, cooperation and symbiosis within the microbiota enhances hydrolysis of cell wall cellulose (Zhang, He, Tian, Maoa, Tang, Zhang and Zhang, 2011). However, while cellulose is slowly hydrolysed during cassava fermentation, the lignin fraction is not (Okudoh, Trois, Workneh and Schmidt, 2014). Lignin shields the cell wall cellulose and hemicellulosic fractions from microbial enzymatic action (Liu et al., 2015). In cell walls, the hemicellulosic constituents bind lignin to cellulose and also provide more rigidity to the cellulose-hemicellulose-lignin network (Zhang, Xie, Yin, Khanal and Zhou, 2016). Thus, lignin most likely retards the weakening of cassava cell walls by fermentation.

Given that dilute NaOH solubilises cell wall lignin and subsequently exposes the embedded cellulose network (Li et al., 2010), the combined treatment will be more effective than either treatment alone in weakening the cell walls (Sindhu, Binod and Pandey, 2016).

iii) Fermentation of dilute sodium hydroxide treated cassava pieces using alkaliphilic *Bacillus akibai, B. cellulosilyticus* and *B. hemicellulosilyticus* cultures will weaken the root parenchyma cell walls. *B. akibai, B. cellulosilyticus* and *B. hemicellulosilyticus* cultures are reported to produce extracellular alkaline-stable cellulase (Nogi, Takami and Horikoshi, 2005). A fermentation technique using acid-tolerant cultures has been used to improve cassava root parenchyma cell wall disaggregation (George et al., 1991). Hydrolysis of the cassava cell wall constituents, the rupture/fragmentation and release of the trapped starch granules within the root parenchyma cells was primarily due to the action of microbial pectinolytic, cellulolytic and proteolytic enzymes (Okafor, Ijioma and Oyolu, 1984; George et al., 1991). Enzymic hydrolysis of the hemicelluloses, lignin and cellulose fractions alter the primary cell wall microstructure, subsequently reducing cell wall strength (Silveira, Stoyanov, Gusarov, Skaf and Kovalenko, 2013). This is primarily because pectic substances cement the cellular matrices and once hydrolysed tissue cohesion weakens (Nanda and George, 1996). Furthermore, cross-linkages between cellulose and xyloglucan polymers may also get disrupted by enzymic action, further weakening the cell walls (Whitney, Gothard, Mitchell and Gidley, 1999).

3.2 OBJECTIVES

i. To determine the effect of dilute NaOH steeping of ground cassava pieces on the root cell walls with the aim of understanding the possible weakening mechanism.

ii. To determine the effects of endogenous fermentation in combination with dilute NaOH steeping on cassava cell walls with the aim of improving starch extraction.

iii. To determine the effects of fermenting dilute NaOH wetted cassava pieces using alkaliphilic cultures of *Bacillus akibai, B. cellulosilyticus* and *B. hemicellulosilyticus* alone, and in combination on cassava cell walls with the aim of improving starch extraction.

4 RESEARCH

4.1 MECHANISM OF CASSAVA TUBER CELL WALL WEAKENING BY DILUTE SODIUM HYDROXIDE STEEPING[1]

[1] Research Chapter published as; Odoch, M., Buys, E.M. and Taylor, J.R.N., 2017. Mechanism of cassava tuber cell wall weakening by dilute sodium hydroxide steeping. *Food Chemistry*, 228, 338-347.

4.1.1 Abstract

Steeping of cassava root pieces in 0.75% (w/v) NaOH in combination with wet milling was investigated to determine whether and how dilute NaOH modifies cassava cell walls. Gas chromatography data of cell wall constituent sugar composition and Fourier transform infrared (FTIR) data showed that NaOH steeping reduced the level of pectin in cassava cell walls. FTIR and wide-angle x-ray scattering spectroscopy also indicated that NaOH steeping combined with fine milling slightly reduced cellulose crystallinity. Scanning electron microscopy showed that NaOH steeping produced micropores in the cell walls and light microscopy revealed that NaOH steeping increased disaggregation of parenchyma cells. Steeping of ground cassava in NaOH resulted in a 12% decrease in large residue particles and approx. 4% greater starch yield with wet milling. Therefore dilute NaOH steeping can improve the effectiveness of wet milling in disintegrating cell walls through solubilisation of pectin, thereby reduced cell wall strength.

4.1.2 Introduction

The cassava (*Manihot esculenta* Crantz L.) plant is mainly cultivated for its tuberous, starch-containing roots (Alves, 2002). It is widely grown in tropical regions of Africa, Asia, and Latin America, and is the third most important source of calories worldwide after rice and maize (FAOSTAT, 2015). Harvested cassava roots have the following approximate composition: moisture 60 g/100 g, total carbohydrate 38 g/100 g, dietary fibre 1.8 g/100 g, protein 1.4 g/100 g, potassium 271 mg/100 g, magnesium 21 mg/100 g, calcium 16 mg/100 g, sodium 14 mg/100 g, and ascorbic acid 21 mg/100 g (as is basis) (USDA, 2016). The high starch content of cassava roots, approx. 26-52 g/100 g (as is basis), and the plant's relatively low fertiliser requirement and drought tolerance also makes it attractive for starch production for industrial use (Jansson, Westerbergh, Zhang, Hu, & Sun, 2009).

The cassava root is generally processed fresh by mechanical attrition (wet milling). However, a major impediment in the root for food and for starch extraction is disintegration of the recalcitrant cell walls (Sriroth, Piyachomkwan, Wanlapatit, & Oates, 2000). Disintegration of cassava cell walls is also important in detoxification of processed starch (Sornyotha, Kyu, & Ratanakhanokchai, 2010), thermal softening of cooked roots (Favaro, Beléia, Junior, & Waldron, 2008) and reduction in viscosity of milled cassava (Huang et al., 2016).

Disintegration of cassava root cell walls is a multifactorial problem as plant cell wall strength depends on the properties of its components, their composition, intrastructural interactions and the molecular mechanism of cell adhesion (Favaro et al., 2008). Specifically, in cassava cell wall, pectin content has been found to be inversely related to root friability (Hongbété et al., 2011). It is recognised that, generally, pectin interactions within the middle lamella, are the principal factor responsible for cell adhesion (Parker, Parker, Smith, & Waldron, 2001). Research indicates that cassava root cell wall adhesive strength is influenced by the proportions of alkali-soluble and chelator-insoluble pectins plus the presence of divalent cations, which are thought to strengthen pectin cross-linking (Favaro et al., 2008). Cassava cell walls also contain soluble hemicellulosic mucopolysaccharides, referred to as cassava tuber mucilage (Charles, Huang, & Chang, 2008), which probably act as an adhesive component within the intercellular spaces.

Weakening of cassava cell walls by various treatments such as ammonia (Moorthy, 1991), enzymatic (Adetunji, du Clou, Walford, & Taylor, 2016; Eyini & Rajapandy, 2007) and microbial (Nanda & George, 1996) have been investigated to improve cellular disintegration by wet milling.

Recently, the mechanisms of cassava cell wall weakening by enzymatic (Adetunji et al., 2016; Huang et al., 2016) and retting (spontaneous fermentation) involving endogenous and exogenous enzymes (Ngea, Guillon, Ngang, Bonnin, Bouchet, & Saulnier, 2016) have been described. Adetunji et al. (2016) and Ngea et al. (2016) found that cassava root tissue weakening was related to opening up of the cell wall structure, partly through pectin degradation and associated the degradation with specifically modifications to galactan, homogalacturonan and arabinan pectic polymers. Weakening of cassava cell walls during retting is thought to primarily involve activities of pectin methylesterase and polygalacturonate lyase (Brauman et al., 1996).

It has been found that pectins and xyloglucans in cassava root cell walls can be solubilised by sequential extraction using the chelating agents imidazole and cyclohexane-trans-1, 2-diamine-N, N, N', N'-tetraacetate and then under alkaline conditions with Na_2CO_3 followed by KOH (Favaro et al., 2008). Furthermore, sodium hydroxide (NaOH) treatment has been shown to weaken grass/cereal cell walls by saponification of the intermolecular ester bonds cross-linking xylan hemicellulose and lignin, depolymerisation of lignin by cleavage of inner-molecular α- and β-aryl ether linkages, and removal of hemicellulose acetyl groups and uronic acid substitutions (reviewed by Zhao, Zhang & Liu, 2012). NaOH treatment also causes cellulose from plant primary cell walls to swell, with a concomitant decrease in chain crystallinity (Dinand, Vignon, Chanzy, & Heux, 2002).

However, the effect of alkaline steeping on cassava cell wall structure and disintegration has not been investigated. Hence, on the basis of the above research, it was hypothesised that steeping of cassava roots under alkaline conditions with dilute NaOH could be an effective way of chemically modifying and weakening the parenchyma cell walls to aid their disintegration during wet milling. The aim of this was to test the hypothesis that dilute alkali steeping would chemically modify cassava parenchyma cell structure and as a consequence result in greater disintegration of the cells during wet milling. The specific objective of the

study was therefore to characterize dilute NaOH induced weakening of cassava root cell walls and to identify any existent effects in relationship to starch extraction.

4.1.3 Materials and methods

4.1.3.1 Materials

Cassava roots (sweet variety '*South Africa'*) were harvested from eleven month old plants grown in a single field at Tonga, Mpumalanga Province, South Africa. Eleven month old plants were chosen on the basis that at 10 to 12 months, the roots are considered matured (Santisopasri et al., 2001).

4.1.3.2 Transport and storage

Directly after harvesting, the roots were placed into polystyrene insulated boxes with closed lids and transported in a covered vehicle with an internal temperature of 20-23^{o}C to the University of Pretoria. Directly on arrival, the roots were washed, peeled to remove the periderm and phelloderm layers, and then chopped into chips (approx. 9 cm^{3}). The chips were placed in high density polypropylene bins with closed fitting lids and stored at -20^{o}C for up to 4 months until utilized. The entire process from harvest to storage was completed within 18 h. The pH and titratable acidity of ground freshly harvested cassava roots and cassava chips after storage at -20^{o}C were determined. They both had a pH of 6.3±0.1 and a titratable acidity of 0.1% (lactic acid equivalents). This showed that there was no spontaneous fermentation during storage.

4.1.3.3 Cassava chip size reduction

Prior to utilization, the frozen chips were thawed at 4^{o}C for 24 h, then ground into smaller pieces using an electric rotary meat mincer fitted with an 8 mm opening plate.

4.1.3.4 NaOH steeping and wet milling

The starch extraction method by Moorthy (1991) was used with modifications. Thus, preliminary extraction trials were done to confirm the validity of the method. Cassava pieces

(400 g) were mixed with 400 ml 0.75% (w/v) NaOH solution, final pH 11.5. Control samples were mixed with distilled water. All samples were steeped for 2 h at 25^{o}C. The cassava pieces were pulverised in a Waring blender for 1 min using low and high speed for 30 s each. The pulp was suspended in 5x its volume distilled water and wet milled at 12,000 rpm using a Retsch EZ200 wet mill (Haan, Germany). Three screen opening sizes were investigated: 500 µm, 1000 µm and 2000 µm.

4.1.3.4.1 Preparation of cassava starch and residue fractions

The wet milled pulp was separated into residue (remaining on the sieve) and filtrate (passing through the sieve) using a 106 µm opening sieve. The residue was re-suspended in 200 ml distilled water and the sieve separation process was repeated five times in total. The collected filtrate was allowed to stand for 12 h at 25^{o}C to facilitate starch sedimentation and the clear supernatant was discarded. The wet cassava starch (the sediment) and the sieve-retained cassava residue, were dried in a forced draught oven at 50°C for 72 h. The dried starch and residue fractions were milled using a Janke and Kunkel micro hammer mill (Staufen, Germany) fitted with a 500 µm opening screen. The milled samples were stored in sealed polyethylene bags at 4^{o}C until assayed.

4.1.3.4.2 Starch removal from the cassava residue fraction

The starch in the residue fraction was removed by enzymic hydrolysis to enable characterization of the insoluble cell wall material. Residue fraction, 6 g, was suspended in 100 ml distilled water and the slurry pH adjusted to pH 5.0 using 1 M HCl. The slurry was centrifuged at 3100 g for 15 min, 4^{o}C and the clear supernatant carefully decanted. Pure thermostable α-amylase from *Bacillus licheniformis* (Megazyme International, Bray, Ireland) was used to hydrolyse the starch under conditions in accordance with the supplier's Total Starch Assay. The enzyme was diluted (1:30) using sodium acetate buffer (0.1 M, pH 5.0) containing calcium chloride (0.05 M). The diluted enzyme, 185 ml, was added and the slurry incubated at 100^{o}C for 20 minutes in a shaking water bath at 80 rpm. The starch enzymic hydrolysis step was repeated, until starch was absent in the residue fraction as assessed by the iodine test. The residue was re-suspended in 30 ml distilled water, and washed twice. The residue material after starch enzymic hydrolysis, referred to as cell wall material (CWM), was freeze dried, milled using an air-cooled, knife-type laboratory mill (IKA, Staufen,

Germany) and stored at 4°C in zip-lock type polyethylene bags until required. Figure 4.1.1 summarises the stages followed during cassava starch extraction and isolation of the cassava cell wall material.

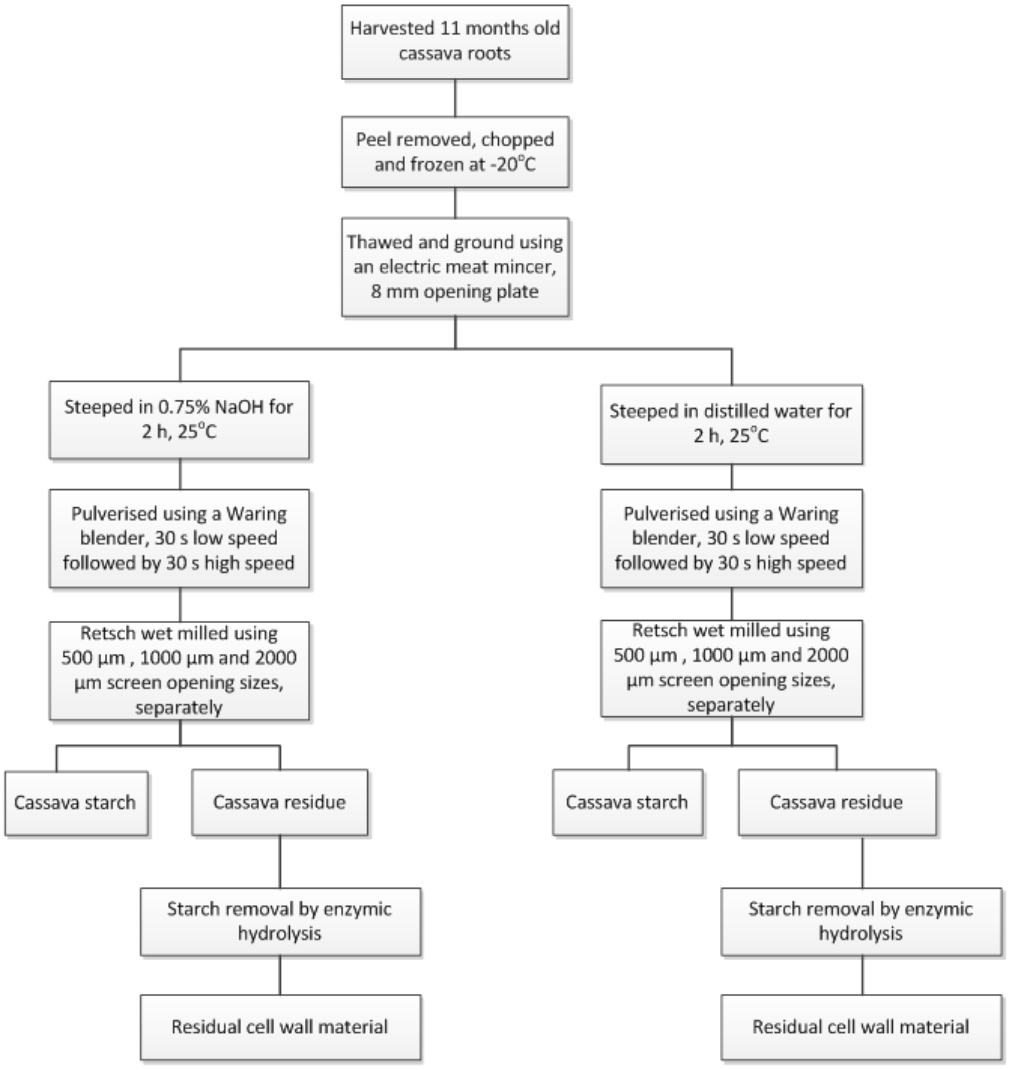

Figure 4.1.1 Flow diagram of the stages followed during cassava starch extraction and isolation of cell wall material from the cassava root residue fraction

4.1.3.5 Analyses

4.1.3.5.1 Residue fraction particle size

Residue fraction particle size, prior to starch enzymic removal, was determined by sieving 10 g wet residue through a 250 µm opening sieve followed by 25 µm opening sieve using 1 L distilled water. Retained particles on both sieves were dried separately by AACC Method 44-15A (AACC International, 2000).

4.1.3.5.2 Starch content

Starch content of the starch and residue fractions (prior to starch removal) was determined using the Megazyme Total Starch Assay procedure (Amyloglucosidase / α-Amylase Method) (Megazyme International, 2016).

4.1.3.5.3 Microscopy

Cassava residue (prior to starch removal) was suspended in distilled water and viewed using a light transmission microscope equipped with a mounted camera. For scanning electron microscopy (SEM), cassava residue was neutralised by suspending in 300x its volume of distilled water, collected over a 106 µm sieve opening size and lyophilized. Dried residue was mounted on an aluminium stub, carbon coated (<0.5 nm thickness) and viewed using a JEOL JEM-8700 SEM (Tokyo, Japan). For each replicate sample, 5 specimens were randomly collected and 8 locations per specimen viewed.

4.1.3.5.4 Attenuated total reflectance Fourier transform infrared (ATR-FTIR) and wide-angle x-ray scattering (WAXS) spectroscopy

For ATR-FTIR and WAXS measurements, the milled CWM preparations in their polyethylene bags were equilibrated for 7 days at approx. 25°C in a desiccator containing silica gel. ATR-FTIR spectroscopy was used to probe the cell wall structural and chemical features. Background spectrum of the clean ATR diamond crystal was acquired. Milled cassava CWM, approx. 30 mg, was carefully dispersed onto the ATR diamond crystal and the pressure arm locked into position. IR spectra were directly acquired using a Bruker V70x spectrometer (Ettlingen, Germany) equipped with a helium-neon laser (λ = 633 nm, power output 1 mW) and DLaTGS detector. Background corrected spectra were recorded from 4000 to 400 cm^{-1}, resolution 4 cm^{-1}. Five replicate spectra (32 co-added scans) were collected per sample. All spectra were processed using Bruker OPUS 7 software. Atmospheric contributions were subtracted and the spectra vector normalized. Automatic baseline correction (64 baseline points) was done with the rubber band function and the spectra normalised. For each sample, average spectra were generated using the software averaging function. The CWM crystalline: amorphous ratios were calculated (Colom, Carrillo, Nogués & Garriga, 2003).

For WAXS, measurements were determined using a PANalytical X'Pert Pro Powder Diffractometer (Eindhoven, Netherlands) in theta-theta configuration, fitted with an X'Celerator detector and variable divergence- and fixed receiving slits. CWM was prepared according to the PANalytical backloading technique, ensuring nearly random particle distribution. CWM analysis was performed at 25°C using Fe filtered Co-K_a radiation (1.54 A) at 35 kV and 50 mA. The recorded range was 5–90°, step size 0.0262°, scan step time 104.4 s and tube focus dimension, 12 mm x 0.4 mm. Relative diffraction intensities were plotted against 2 θ using Origin v8.0 software (OriginLab, Northampton, MA). Relative crystallinity was estimated as the ratio of the integrated area of crystalline peaks to the total integrated area above a straight baseline (Wokadala, Ray & Emmambux, 2012).

4.1.3.5.5 Gas chromatography characterization of residual cassava cell wall material

Gas chromatography (GC) analysis of the neutral sugar composition of cassava CWM and spectrophotometric analysis of uronic acid content were performed by Englyst Carbohydrates Ltd (Southampton, UK). Sugar composition was determined after sulphuric acid hydrolysis by GC of the alditol acetate derivatives (Englyst, Quigley and Hudson, 1994). CWM was pre-hydrolysed using 12 M H_2SO_4 for 1 h at 35^{o}C and then hydrolysed into constituent monomers using 2 M H_2SO_4 for 1 h at 100^{o}C. An internal standard deoxygalactose was used. Alditol acetate aliquots, 0.5 ml, were injected under the following conditions: injector temperature, 275°C; column temperature, 220°C; detector temperature, 275°C; carrier gas, helium; and flow rate, 8 ml/min. A Shimadzu 2010 gas chromatograph (Kyoto, Japan) fitted with a flame-ionization detector, auto-injector and computing integrator using an Agilent Factor Four wide bore capillary column (Stockport, UK) at 210°C was used. External sugar standards comprised rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose (dried to constant mass under reduced pressure with phosphorus pentoxide) diluted into 50% saturated benzoic acid. Uronic acid content was determined separately by a spectrophotometric procedure, as described by Englyst et al. (1994) with galacturonic acid as the standard. CWM total non-starch polysaccharide content was calculated as the sum of neutral sugars and uronic acids.

4.1.3.5.6 Statistical analysis

All experiments were performed at least twice. Data were analysed using one-way and two-

way analysis of variance (ANOVA). Fisher's Least Significant Difference (LSD) test was used to determine significant differences between means.

4.1.4 Results and discussion

4.1.4.1 Effects of dilute NaOH steeping and wet mill screen size on cassava cell walls

Steeping of ground cassava pieces in dilute NaOH resulted in 3.4 g/100 g and 4.3 g/100 g CWM (dry basis) from pulp wet milled using 500 μm and 2000 μm screen opening sizes, respectively. With water steeping, the CWM yield was higher, 4.6 g/100 g and 4.8 g/100 g, respectively. This indicates that a fraction of the CWM had been solubilised by the NaOH steeping. The yield of cassava CWM is similar to that obtained by other workers (3.3-4.5 g/100 g) (Favaro et al., 2008; Kajiwara & Maeda, 1983).

The neutral sugar constituent monomers of the hydrolysed cassava CWM comprised glucose, galactose, xylose, arabinose, mannose, rhamnose and fucose in decreasing order (Table 4.1.1). The sugar plus uronic acid recovery from both the NaOH and water steeped CWM was similar (approx. 50%); hence their sugar composition can be reliably compared. The low percentage sugars relative to total CWM is probably due to the fact that isolated cassava CWM contains lignin (Adetunji et al., 2016). However, such lignin content was derived primarily from the root xylem and central vascular fibre tissue cell walls (Figueiredo, Moraes-Dallaqua, Bicudo, Tanamati, & Aguiar, 2015). Overall, glucose, galactose and xylose were the major sugars in the water (control) steeped cassava CWM, 26%, 9% and 5% respectively. With the exception of galactose, similar data were reported by Ngea et al. (2016) for fresh cassava CWM, approx. 28% glucose, 15% galactose and 4% xylose. This indicates that cellulose and probably xyloglucans were the major NSPs in the residual cassava CWM.

Overall, steeping of ground cassava in 0.75% NaOH resulted in a slight not statistically significant ($p \geq 0.05$) increase in glucose (2.2%) and a larger significant increase ($p < 0.05$) in xylose (6.8%). Overall, there was a concomitant substantial reduction in galacturonic acid (33.0%).

Table 4.1.1 Constituent sugar composition of remaining purified cassava root cell wall material after steeping cassava pieces in 0.75% NaOH or distilled water and then wet milling (g/100 g cell wall material, dry matter basis)

Wet mill screen opening size (µm)	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size
	Rhamnose			**Fucose**			**Arabinose**		
2000	0.66±0.01[1]	0.65±0.01	0.66[a]±0.01	0.50±0.03	0.48±0.01	0.49[a]±0.02	2.69±0.05	2.44±0.07	2.57[a]±0.15
500	0.76±0.01	0.75±0.06	0.76[b]±0.03 (+15.2%) [3]	0.50±0.01	0.55±0.04	0.52[a]±0.03 (+6.1%)	2.84±0.02	2.89±0.06	2.86[b]±0.05 (+11.3%)
Overall effect of NaOH steeping	0.71[a]±0.06	0.70[a]±0.07 (-1.4%) [2]		0.50[a]±0.02	0.51[a]±0.04 (+2.0%)		2.77[a]±0.09	2.67[a]±0.27 (-3.6%)	

Wet mill screen opening size (µm)	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size
	Xylose			**Mannose**			**Galactose**		
2000	5.29±0.09	5.42±0.09	5.35[b]±0.11	1.38±0.04	1.27±0.06	1.32[a]±0.08	8.89±0.05	8.70±0.18	8.79[a]±0.15
500	4.79±0.04	5.33±0.18	5.06[a]±0.32 (-5.4%)	1.27±0.03	1.52±0.32	1.39[a]±0.24 (+5.3%)	9.14±0.18	9.41±0.33	9.27[b]±0.27 (+5.5%)
Overall effect of NaOH steeping	5.04[a]±0.29	5.38[b]±0.13 (+6.8%)		1.32[a]±0.07	1.39[a]±0.24 (+5.3%)		9.01[a]±0.18	9.05[a]±0.47 (+0.4%)	

Wet mill screen opening size (µm)	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size
	Glucose			**Galacturonic acid**			**Total NSP sugars in CWM**		
2000	26.90±0.34	26.39±0.66	26.65[a]±0.52	6.56±0.09	4.32±0.02	5.44[a]±1.30	52.86±0.60	49.66±1.08	51.26[a]±1.98
500	26.49±0.41	28.17±1.03	27.33[a]±1.16 (+2.6%)	7.05±0.14	4.79±0.17	5.92[b]±1.31 (+8.8%)	52.84±0.75	53.41±1.20	53.13[b]±0.88 (+3.6%)
Overall effect of NaOH steeping	26.70[a]±0.39	27.28[a]±1.24 (+2.2%)		6.81[b]±0.30	4.56[a]±0.29 (-33.0%)		52.85[a]±0.55	51.54[b]±2.36 (-2.5%)	

[1]Means± standard deviations of two independent experiments. [2]Row values in parentheses are the percentage differences between means of steeping solvents.
[3]Column values in parentheses are the percentage differences between means of wet mill screen opening sizes.
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

In cassava cell walls, the pectin is composed mainly of galacturonic acid and galactose residues, while the hemicellulosic fraction comprises mainly xylose, galactose and glucose (Salvador, Suganuma, Kitahara, Tanoue & Ichiki, 2000). Importantly, fucose is only located in the hemicellulosic fraction of cassava cell walls (Salvador et al., 2000). Overall, none of these sugars decreased with the NaOH steeping treatment compared to water steeping. Thus, the dilute NaOH steeping of ground cassava appears to have solubilised mainly the CWM pectin fraction but not the cellulose and hemicellulosic fractions.

Considering the FTIR spectroscopy data (Figure 4.1.2), IR spectra of CWM from ground cassava pieces steeped in NaOH and water steeped revealed two main absorption regions, 1800-700 cm^{-1} and 3500-2700 cm^{-1}. Compared to water steeped (Figure 4.1.2c.d), steeping of ground cassava in 0.75% NaOH resulted in a small decrease in vibration intensities at 1734 cm^{-1} and a larger decrease at 1234 cm^{-1} and at 1237 cm^{-1} wavenumbers (denoted by black arrows on the spectra) (Figure 4.1.2a,b). The around 1737 cm^{-1} vibration has been ascribed to C=O stretching of methyl esterified uronic carboxyl groups (Oliveira et al., 2016) and the 1234 cm^{-1} vibration to the stretching vibration of C-O methyl ester groups (Lim et al., 2014). Less prominent IR spectra differences centered at 2920 cm^{-1}, 2917 cm^{-1}, 1416 cm^{-1} and 1373 cm^{-1} could not be unambiguously identified. Furthermore, IR spectra of CWM steeped in NaOH and water revealed no spectral differences at 1770 cm^{-1}, 1740 cm^{-1}, 1595 cm^{-1} and 1510 cm^{-1} wavenumbers, which are attributed to cell wall lignin vibrations (Popescu, Popescu, Singurel, Vasile, Argyropoulos, & Willfor, 2007). Thus, the FTIR data support the chemical analysis data that NaOH steeping brought about a reduction in the level of pectin.

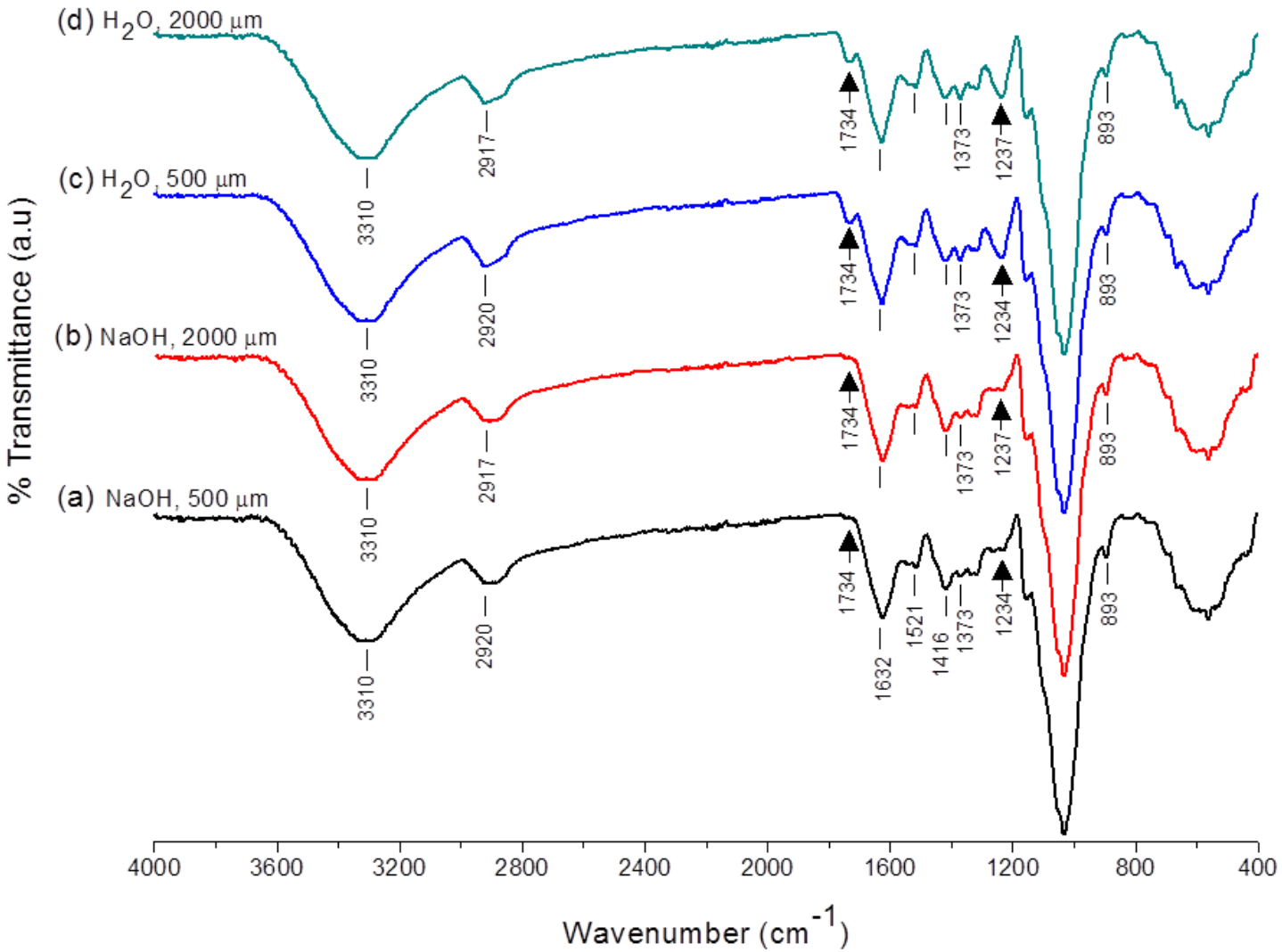

Figure 4.1.2 FTIR spectra showing the effects of steeping cassava in 0.75% NaOH or water and wet mill screen opening size on the chemical constituents of the remaining purified cell wall material. (a) NaOH steeped and 500 µm screen, (b) NaOH steeped and 2000 µm screen, (c) H_2O steeped and 500 µm screen, and (d) H_2O steeped and 500 µm screen

Dilute NaOH treatment promotes demethoxylation of homogalacturonans (Wehr et al., 2004), a non enzymatic modification similar to the action of pectin methyl esterase. However, the specific role of pectin demethoxylation in cell wall deconstruction is still unresolved. Fischer, Arrigoni and Amadò (1994) postulated that non-esterified carboxylic groups of pectin galacturonic acid residues are ester linked to hydroxyl groups of hemicelluloses. Hence, NaOH solubilisation of the pectic polymers was at least in part due to saponification. Additionally, alkali soluble pectins have been reported in plant tissues (Murayama, Katsumata, Horiuchi & Fukushima, 2002). Hence, cassava CWM pectic polymers could also have been simply solubilised by steeping in dilute NaOH. Furthermore, in cassava root parenchyma cell walls, the pectin comprises methyl-esterified homogalacturonans (Ngea et al., 2016). Methyl-esterified homogalacturonans are susceptible to β-elimination reaction under alkaline conditions (Kravtchenko, Arnould, Voragen, & Pilnik, 1992). Thus, it seems likely that the cell wall pectin reduction action of NaOH steeping was as a result of pectin degradation through β-elimination induced depolymerisation.

With regard to the overall effect of increased milling attrition brought about by a decrease in wet mill screen opening size from 2000 µm to 500 µm, there were significantly ($p<0.05$) increased proportions of galactose, galacturonic acid, arabinose and rhamnose in cassava CWM (Table 4.1.1). In contrast, the decrease in screen opening size significantly ($p<0.05$) decreased the xylose constituent. In sugar beet hemicellulose extraction using 10% NaOH, Wen, Chang, Brown and Gallaher (1988) found that a decrease in particle size increased hemicellulose solubilisation, which the authors attributed to increased particle surface area. Hence, it seems likely that fine milling enabled preferential solubilisation of the xyloglucan constituent of cassava cell walls.

Cell wall cellulose consists of crystalline and amorphous domains held together mainly by hydrogen bonding (Liao, Huang, Hu, Zhang & Tan, 2011). The amorphous domains are relatively more susceptible to hydrolytic degradation than the crystalline domains. In FTIR spectroscopy, total crystallinity index (TCI) a_{1377}/a_{2922} is proportional to cellulose crystallinity, while lateral order index (LOI) a_{1426}/a_{898}, which is a measure of cellulose structural disorder, increases with a decrease in cellulose crystallinity (Colom et al., 2003; Corgié, Smith & Walker, 2011). The TCI of NaOH steeped CWM from cassava milled at 500 µm decreased slightly (0.62) compared to the water steeping (0.65) (Figures 4.1.2a,c). In agreement, the LOI a_{1426}/a_{898} of CWM from cassava steeped in NaOH (4.27) increased

compared to the water steeped (3.56). Thus, together TCI and LOI of CWM from cassava milled at 500 µm indicated that NaOH steeping slightly reduced the proportion of crystalline cellulose compared to water steeping. The observed reduction in cellulose relative crystallinity index is similar to that found by Liao et al. (2011) using intense dry milling of cassava residue, who likewise applied FTIR to assess changes in crystallinity. However, for cassava milled using a 2000 µm screen opening size, the TCI of NaOH steeped CWM increased slightly (0.74) compared to the water steeping (0.69) (Figures 4.1.2b,d). Expectedly, the LOI a_{1426}/a_{898} of CWM from cassava steeped in NaOH (3.43) decreased compared to the water steeping (4.80). Therefore, TCI and LOI of CWM from cassava milled at 2000 µm indicated that NaOH steeping increased the proportion of crystalline cellulose compared to water steeping. As FTIR estimation of cellulose crystallinity indices involves determination of relative peak heights at different wavenumbers (Park, Baker, Himmel, Parilla & Johnson, 2010), a more detailed investigation of the effects of NaOH steeping and milling attrition on cassava cell wall cellulose crystallinity was conducted using WAXS.

All WAXS diffraction patterns of residual cassava CWM revealed three planes (denoted by small arrows) at 2θ approx. 16.0°, 22.5° and 34.5° (Figure 4.1.3). The peak at 2θ approx. 22.5° is assigned to the (0 0 2) plane of cellulose I, the broad peak at 2θ approx. 15°–17° is attributed to the overlap between (101) and (10-1) planes of cellulose I, and the peak at 34.5° is also associated with cellulose I (Leite, Zanon, and Menegalli, 2017; Liao et al., 2011). With CWM from cassava milled with a 2000 µm screen opening size it appears that NaOH steeping caused an increase in peak intensity (002) and its narrowing compared to water steeping (Figures 4.1.3D,C). These changes indicate an increase in crystalline phase (Liao et al., 2011). However, with CWM from cassava milled to 500 µm it appears that NaOH steeping did not result in increased peak intensity (002) but rather slightly flattened the diffraction peak at 2θ approx. 16.0° (Figure 4.1.3B, arrow marked a) when compared to water steeping (Figure 4.1.3A). The peak flattening suggests a slight decrease in crystalline phase. These indicated changes in CWM crystallinity are in agreement with the changes suggested by the FTIR ratios (Figure 4.1.2).

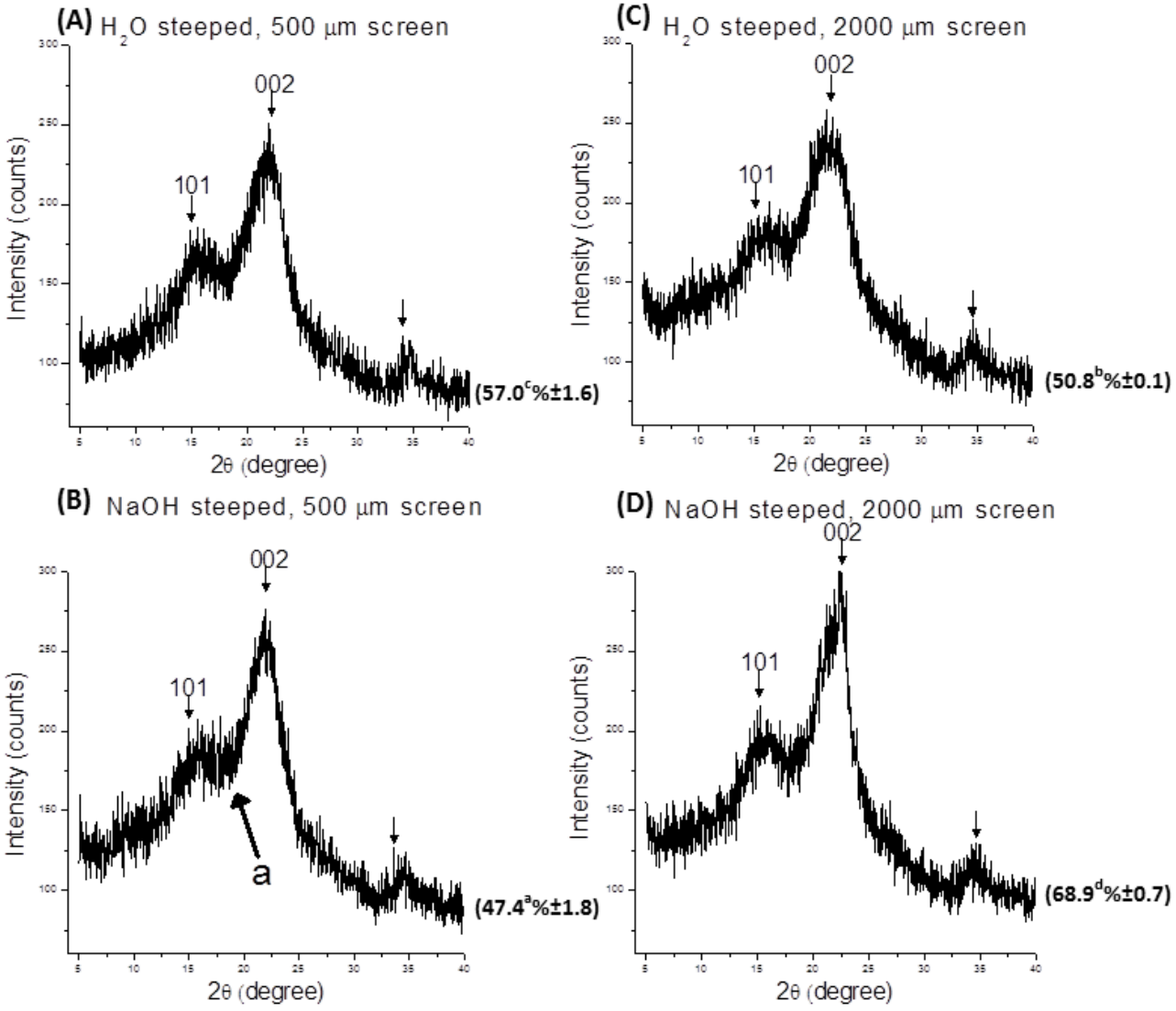

Figure 4.1.3 X-ray diffraction spectra of the remaining purified cell wall material after steeping cassava pieces in 0.75% NaOH or distilled water and then wet milling using 500 µm screen (A,B) or 2000 µm screen (C,D). *Values in parentheses are mean relative crystallinity (%) ± SD, n = 2

In fact, the WAXS data showed that fine wet milling (500 µm screen opening size) of NaOH steeped cassava resulted in a slight decrease in cellulose relative crystallinity (47.4%) compared to water steeping (57.0%), with the opposite effect for 2000 µm milled cassava, (68.9%) and (50.8%), respectively. This is probably because the more drastic condition of NaOH steeping followed by wet milling to 500 µm, may have modified not only the amorphous cell wall domains, but also some of the crystalline domains. This result is consistent with the study of Leite et al. (2017) in which the cellulose crystallinity of cassava nanofibers reduced under more intense acid treatment.

Steeping of ground cassava pieces in dilute NaOH appears not to alter the CWM cellulose crystallite structure considering that no new peaks appeared at 2θ = 12.1°, 19.8° and 22.0° (Figures 4.1.3B,D), the characteristic peaks of cellulose II (Liao et al., 2011). Thus, it appears that the mechanism by which NaOH steeping influences cassava cell wall cellulose relative crystallinity is particle size dependent. For cassava ground to ≤ 2000 µm, NaOH steeping appears to primarily reduce the cell wall amorphous pectin fraction alone. However, for cassava ground to ≤ 500 µm (fine milling), the solubilisation of pectin and reduction of probably xyloglucan facilitated opening up the cellulose microfibril structures.

Significantly, SEM (Figures 4.1.4B, C) revealed that steeping of ground cassava in NaOH resulted in micropores (dashed arrows) (1-2 µm across) in the cassava parenchyma cell wall surface, a feature not observed in the water treatment (Figure 4.1.4A). Such cell wall micropores have been reported in microwave-heated, acid pre-treated cassava cell walls (Cheng et al., 2015). These authors attributed the formation of such micropores to weakened cell walls due to hydrolysis of hemicellulose and lignin constituents within the cellulose-hemicellulose-lignin polymer matrix.

Further, light microscopy (Figures 4.1.5A,C,E) revealed that the clumps of parenchyma cells in cassava residue from the ground roots that had been steeped in NaOH had less closely aggregated starch-containing cells compared to the water steeping treatment (Figures 4.1.5B,D,F). Also in the residues from the NaOH steeping that had been milled to ≤ 500 µm and ≤1000 µm the cells contained far fewer starch granules than their water steeped controls. Furthermore, overall steeping of ground cassava in NaOH resulted in approx. 12% decrease in large residue particles, diameters ≥ 250 µm, compared to the water steeped (Table 4.1.2A).

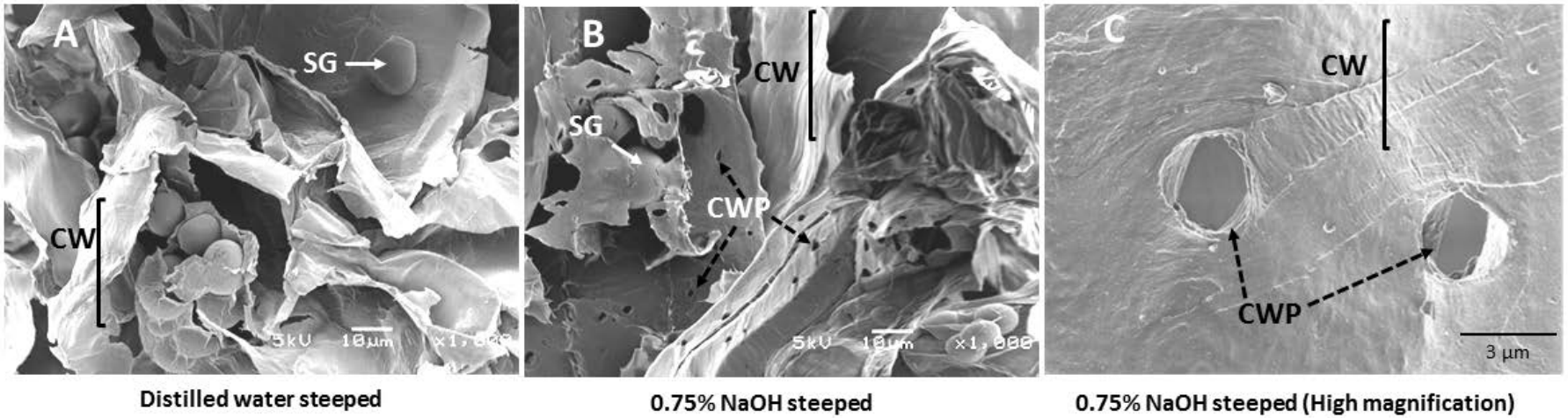

Figure 4.1.4 High resolution scanning electron microscopy showing the effects of steeping ground cassava pieces in distilled water and 0.75% NaOH, followed by wet milling using a 500 µm sieve opening size on the appearance of cassava residue fraction cell walls (A) distilled H_2O, (B, C) dilute NaOH.
CW: cell wall, **CWP**: Cell wall micropores, **SG**: starch granules

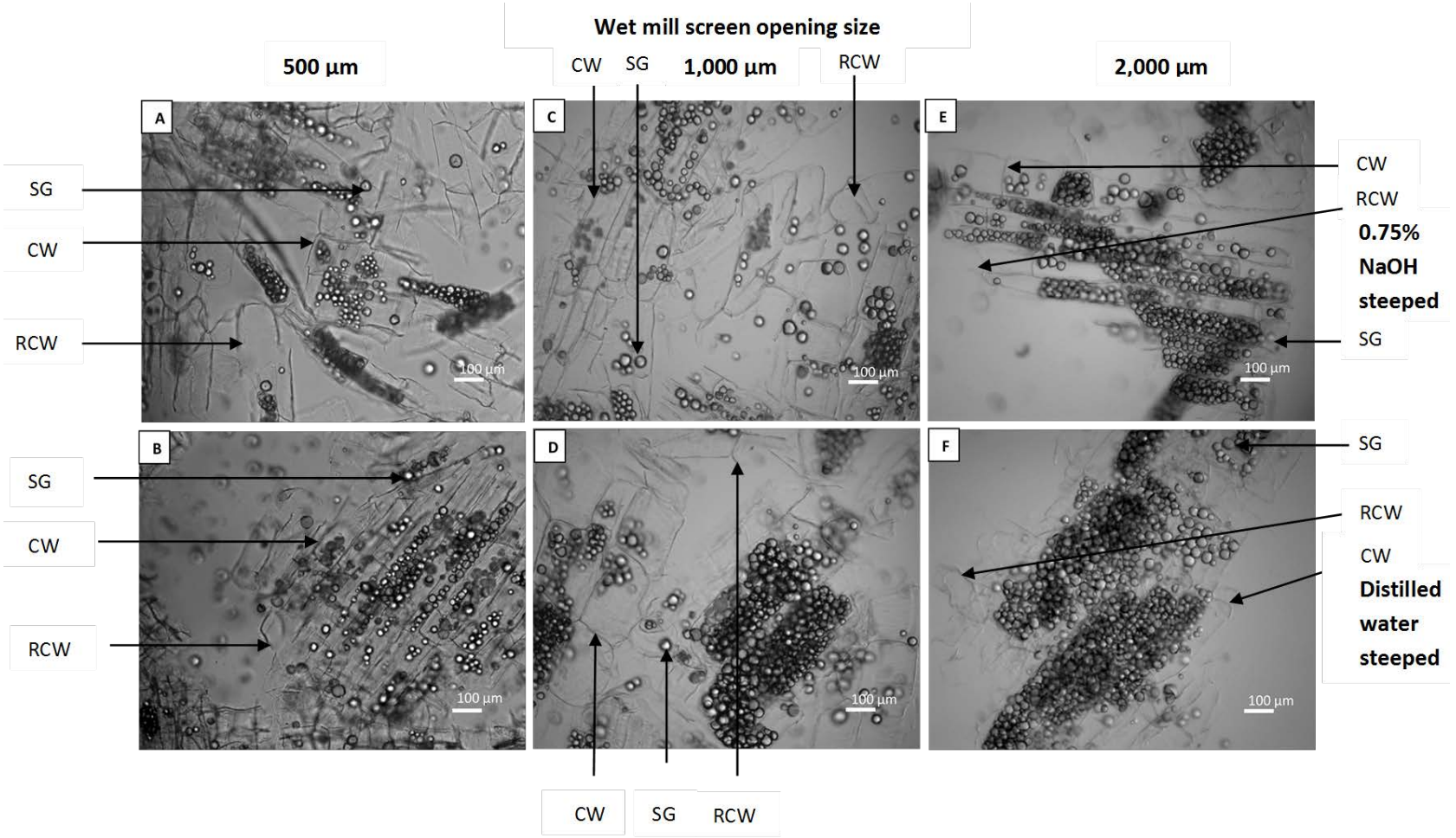

Figure 4.1.5 Light microscopy showing the effects of steeping ground cassava pieces in distilled water and 0.75% NaOH and subsequent wet milling on disaggregation of cassava root parenchyma cells and subsequent release of starch granules; (**A)** 0.75% NaOH and 500 µm, (**B)** Distilled H_2O and 500 µm, (**C**) 0.75% NaOH and 1000 µm, (**D)** Distilled H_2O and 1000 µm, (**E**) 0.75% NaOH and 2000 µm, and (**F**) Distilled H_2O and 2000 µm.
CW: cell wall, **RCW**: Rupture in cell wall, **SG**: starch granules

Table 4.1.2 Effects of sodium hydroxide steeping and wet mill screen opening size on A) percentage wet milled cassava large particles (diameters ≥250 µm), and B) starch extraction from cassava roots by wet milling (yield per 1 kg cassava roots)

A)

Wet mill screen opening size (µm)	Distilled H_2O	0.75% NaOH	Overall effect of screen size
2000	77.3±0.2[1]	65.7±1.4	71.5^{c}±6.8
1000	75.2±0.3	62.2±0.0	68.7^{b}±7.5
500	71.4±0.0	59.5±0.0	65.5^{a}±6.9
Overall effect of NaOH steeping	74.7^{b}±2.7	62.5^{a}±2.8	

B)

Wet mill screen opening size (µm)	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size
	Starch fraction wt. (g d.b[2].)			**Starch fraction – starch purity (%)**			**Starch fraction - starch wt. (g d.b.)**			**Starch yield (%)**		
2000	213.4±1.7[1]	226.3±1.2	219.8^{a}±7.5	88.8±0.6	88.8±0.4	88.8^{a}±0.4	189.4±3.0	200.8±0.1	195.1^{a}±6.8	75.7±0.5	79.7±0.1	77.7^{a}±2.3
1000	232.1±1.4	246.4±0.3	239.3^{b}±8.3	90.1±0.8	89.8±0.3	89.9^{ab}±0.5	209.0±3.2	217.6±4.2	213.3^{b}±5.9	81.3±0.5	85.4±0.4	83.3^{b}±2.4
500	236.9±2.9	248.2±6.7	242.5^{b}±7.8	91.3±0.3	89.6±2.2	90.4^{b}±1.6	216.3±1.8	222.2±0.6	219.2^{c}±3.6	84.0±0.0	87.2±0.4	85.6^{c}±1.8
Overall effect of NaOH steeping	227.5^{a}±11.2	240.3^{b}±11.3		90.0^{a}±1.2	89.4^{a}±1.1		204.9^{a}±12.6	213.5^{b}±10.2		80.3^{a}±3.8	84.1^{b}±3.5	

Wet mill screen opening size (µm)	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size	Distilled H_2O	0.75% NaOH	Overall effect of screen size
	Residue fraction wt. (g d.b.)			**Residue fraction - starch content (%)**			**Residue fraction - starch wt. (g d.b.)**			**Total starch in both fractions (g d.b.)**		
2000	84.1±0.4	71.9±0.8	78.0^{c}±7.1	72.4±0.6	71.5±0.6	71.9^{b}±0.7	60.9±0.7	51.3±0.0	56.1^{c}±5.6	250.3±2.3	252.1±0.1	251.2^{a}±1.7
1000	69.7±0.9	53.7±0.3	61.7^{b}±9.2	69.4±0.3	69.3±0.6	69.4^{a}±0.4	48.3±0.8	37.2±0.6	42.8^{b}±6.4	257.3±2.3	254.8±3.7	256.0^{b}±2.9
500	59.0±1.1	46.0±0.9	52.5^{a}±7.6	69.6±0.7	71.3±1.0	70.5^{a}±1.2	41.1±0.4	32.8±1.1	36.9^{a}±4.8	257.4±2.2	254.9±1.7	256.1^{b}±2.1
Overall effect of NaOH Steeping	70.9^{b}±11.3	57.2^{a}±11.9		70.5^{a}±1.6	70.7^{a}±1.2		50.1^{b}±9.0	40.4^{a}±8.7		255.0^{a}±4.0	253.9^{a}±2.3	

[1]Means± standard deviations of two independent experiments
[2]d.b. = dry weight basis
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

4.1.4.2 Influence of NaOH induced cell wall modifications on starch extraction

Overall, steeping of ground cassava in 0.75% NaOH resulted in an approx. 4% higher starch yield compared to water steeping (Table 4.1.2B). The starch yield was the amount of starch liberated from the cell walls expressed as a percentage of the total (liberated and residue entrapped) starch available (George et al., 1995). As expected, overall a decrease in screen opening size from 2000 µm to 500 µm also resulted in more starch being extracted. Fine wet milling of ground cassava, i.e. to a particle size ≤ 500 µm, resulted in a 2.3% and 7.9% increase in starch yield compared with the 1000 µm and 2000 µm screen opening sizes, respectively. The combination of NaOH steeping and fine milling resulted in the highest cassava starch yield of 87%, approx. 3% more than of water steeping followed by fine milling. Together, the SEM and light microscopy observations and the starch yield data suggest that steeping in dilute NaOH weakened the parenchyma cell walls, which increased the level of cell disaggregation and resulted in greater release of starch granules.

4.1.5 Conclusions

Steeping of ground cassava in dilute NaOH solubilises the parenchyma cell wall pectin, and only when in combination with fine milling affects the xyloglucan polymers. The concomitant loss in cell wall structural integrity, especially with fine milling, enables NaOH to access the embedded cellulose microfibril structures and causes a slight reduction in cellulose crystallinity. Together with solubilisation of pectin and xyloglucan polymers, the loss in cellulose crystallinity results in localised structural collapse, manifested as formation of micropores in the cassava cell walls. These cell wall micropores in turn weaken the cell walls, enabling increased disaggregation of the starch-containing parenchyma cells with wet milling. Therefore, the effect of NaOH steeping on milled cassava root pieces is in principle similar to that observed using exogenous cellulolytic enzymes and spontaneous fermentation as it results in cell wall pectin degradation. However, the exact mechanism is probably different as it is likely that NaOH steeping brings about β-elimination depolymerisation reactions. This research work clearly supports the contention that structurally, cross-linking of cell wall pectic polymers aids cellular cohesion in cassava parenchyma cells. Thus, the understanding that solubilisation of pectin by dilute NaOH steeping can facilitate greater disintegration of cassava parenchyma cell walls should help in the development of more efficient wet-based processing technologies for cassava roots.

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4.2 COMBINED EFFECTS OF ENDOGENOUS FERMENTATION FOLLOWED BY DILUTE SODIUM HYDROXIDE STEEPING ON CASSAVA ROOT STARCH EXTRACTION

4.2.1 Abstract

To further improve starch extraction from cassava roots by wet milling, the combined effects of endogenous fermentation followed by 0.75% (w/v) NaOH steeping on cell wall disaggregation was investigated. Total titratable acidity, pH and microbiological analyses indicated that cell wall disaggregation by endogenous fermentation of cassava pieces was dependent on temperature and the presence of yeasts/moulds. Light microscopy revealed that separately, endogenous fermentation and NaOH steeping increased rupture of parenchyma cell walls. Particle size analysis of the residue fractions in the bagasse showed that improved cellular rupture was associated with fewer large particles, diameter ≥ 250 μm. Total starch assay also indicated that separately, endogenous fermentation and NaOH steeping improved starch yield. In both treatments, greater starch yield appears linked to weakening of the cell walls, which in turn probably increased cellular disaggregation during wet milling. However, the combination of endogenous fermentation and NaOH steeping did not further improve starch yield. Fermentation followed by NaOH steeping resulted in approx. 89% starch yield, less than fermentation followed by water steeping, approx. 93%. The inability of endogenous fermentation combined with dilute NaOH steeping to improve the starch yield was probably due to their similar actions in weakening of cell walls. Thus, NaOH steeping has no additive effect to endogenous fermentation in improving starch extraction.

4.2.2 Introduction

Cassava, (*Manihot esculenta* Crantz L.) is one of the major commercial sources of starch, contributing approx. 12% of the global starch trade (Henry and Westby, 2000). In 2012, the global export trade was valued at over US$ 1.04 bn. (FAOSTAT, 2012). Recently, diversification in the utilisation of cassava starch i.e. biofuels and bioplastics has spurred strong market demand (Papong, Malakul, Trungkavashirakun, Wenunun, Chom-in, Nithitanakul and Sarobol, 2014).

Cassava starch production can be by dry or wet milling but wet milling is simpler and more energy efficient (Meuser, Smolnik, Rajani and Giesemann, 1978). Cassava starch extraction by wet milling is only viable when the process achieves a yield of greater than 20 g starch/100 g of root (as is basis) (Sriroth et al., 2000). During the extraction process, a significant proportion of starch is lost in the residue fraction (Sriroth et al., 2000). This is because the starch granules are entrapped within the root parenchyma cells making it difficult to liberate. In cassava, the application of combined treatments to efficiently rupture the cell walls has been attempted by several authors using combinations of different enzymes like pectinase, xylanase and cellulase (Kallabinski and Balagopalan, 1991; Sornyotha, Kyu and Ratanakhanokchai, 2010); heating and 0.3 M sulphuric acid (Agu, Amadife, Ude, Onyia, Ogu, Okafor and Ezejiofor, 1997); ultrasound and heat (Nitayavardhana, Shrestha, Rasmussen, Lamsal, van Leeuwen and Khanal, 2010); hydrothermal treatment and cellulolytic enzymes (Nair, Padmaja and Moorthy, 2011) or 250 ppm viscozyme/ultraflow and lactic acid bacteria fermentation (Adetunji et al., 2016). Applications of combined treatments to weaken cell walls are generally more effective than each treatment alone (Sindhu et al., 2016). This is because in most instances the combined treatments further increase hydrolysis of the cell wall constituents (Sun, Sun, Cao and Sun, 2016).

Biological treatments such as fermentation utilise the action of microbial extracellular enzymes to weaken and subsequently aid cell wall rupture (Sindhu et al., 2016). Fermentations are generally performed at milder pH and temperature conditions compared to chemical treatments and thus, are less environmentally harmful in terms of effluent production (Rabemanolontsoa and Saka, 2016). However, because of its typically long duration, fermentation is less amenable to some industrial applications (Plácido and Capareda, 2015). In Chapter 4.1, application of 0.75% (w/v) NaOH steeping to ground

cassava for a short duration of 2 h was sufficient to aid rupturing of the root parenchyma cell walls. This dilute alkali steeping seemed to weaken the cell wall structure through formation of perforations i.e. micropores. Consequently, dilute NaOH weakening of the cassava cell walls resulted in increased cellular disaggregation during wet milling and subsequently a higher (approx. 4%) starch yield.

The combined effects of biological treatment (endogenous fermentation) and dilute NaOH steeping on weakening of cassava cell walls and possibly a further improvement on starch yield has not been investigated. Therefore, in preparation for the non-sterile fermentation of cassava roots using alkaliphilic *Bacillus* spp. (Chapter 4.3), the combined effects of endogenous cultures and dilute NaOH steeping was investigated. Here it is hypothesized that combining endogenous fermentation and dilute NaOH steeping would have an additive weakening effect on the cassava root parenchyma cell walls. As a result, the combined treatment could further improve cell wall disaggregation during wet milling and subsequently result in greater starch liberation. Therefore the objective of the work was to test the hypothesis that combining endogenous fermentation and dilute NaOH steeping could further improve starch yield by wet milling.

4.2.3 Materials and methods

4.2.3.1 Materials

Cassava roots (sweet variety '*South Africa'*) were obtained as described in Chapter 4.1. In addition, fresh cassava roots (sweet variety '*Maputo*') grown in Mozambique were obtained from a retail outlet in Pretoria, South Africa.

4.2.3.2 Methods

4.2.3.2.1 Cassava root transport and storage

Directly after harvesting, the cassava variety '*South Africa*' roots were transported, processed and stored as frozen chips at the University of Pretoria as described in Chapter 4.1. With regard to the study on fresh cassava root fermentation, the non-frozen roots from Mozambique were utilised.

4.2.3.2.2 Effects of storage temperature and dilute NaOH steeping on starch yield

As described in Chapter 4.1, frozen cassava chips (approx. 9 cm^3) were thawed at 4^oC for 24 h prior to utilisation. Cassava chips (2.4 kg) were vacuum packed into multi-layered vacuum bags (polyethylene 55 µm, ionomer resin 10 µm and polyamide 15 µm) (Plastikon Pretoria, South Africa) and stored for 14 days. Vacuum packaging was done to inhibit growth of aerobic spoilage microorganisms. Three storage temperatures, 24-25^oC, 4^oC and -20^oC were investigated. Selection of storage temperatures was based on conditions favourable for the growth of moulds and yeasts capable of producing cell wall degrading extracellular enzymes (Amoa-Awua, Frisvad, Sefa-Dedeh and Jakobsen, 1997). After the 14 days storage, the cassava chips were ground into smaller pieces using an electric rotary meat mincer fitted with an 8 mm opening plate. Ground cassava pieces (400 g) were mixed with 400 ml 0.75% (w/v) NaOH solution, final pH 11.5. Control samples were mixed with only distilled water. All treatments were steeped for 2 h at 25^oC. The steeped cassava pieces were pulverised in a Waring blender for 1 min. using low and the high speed each for 30 s. The pulp was suspended in 5x its volume distilled water and wet milled using Retsch EZ200 wet mill (Haan, Germany) with a 500 µm screen opening size. Figure 4.2.1 summarises the stages followed during cassava starch extraction.

4.2.3.2.3 Effects of endogenous fermentation followed by dilute NaOH steeping

Frozen (-20^oC for 11 months) cassava chips were thawed at 4^oC for 24 h prior to utilisation. The cassava chips (3.2 kg) were vacuum packed into multi-layered vacuum bags and treated as follows; non-fermented and fermented for 14 days at 24-25^oC (final pH 3.8). Non-fermented samples were immediately ground and pulped as described under 4.1.3.4.1 but using a 2,000 µm wet mill screen opening size. For the endogenous fermented cassava followed by NaOH steeping, additional 1.0 M NaOH soln. was added to obtain a pH of 11.5 to enable direct comparison between the treatments. In a related study, non-frozen (fresh) cassava root pieces (3.2 kg) were vacuum packed into multi-layered vacuum bags and immediately ground (non-fermented) or fermented for 14 days at 24-25^oC (final pH 3.9) as described above (Section 4.2.3.2.3). Figure 4.2.2 summarises the stages followed during cassava starch extraction in the related experiments.

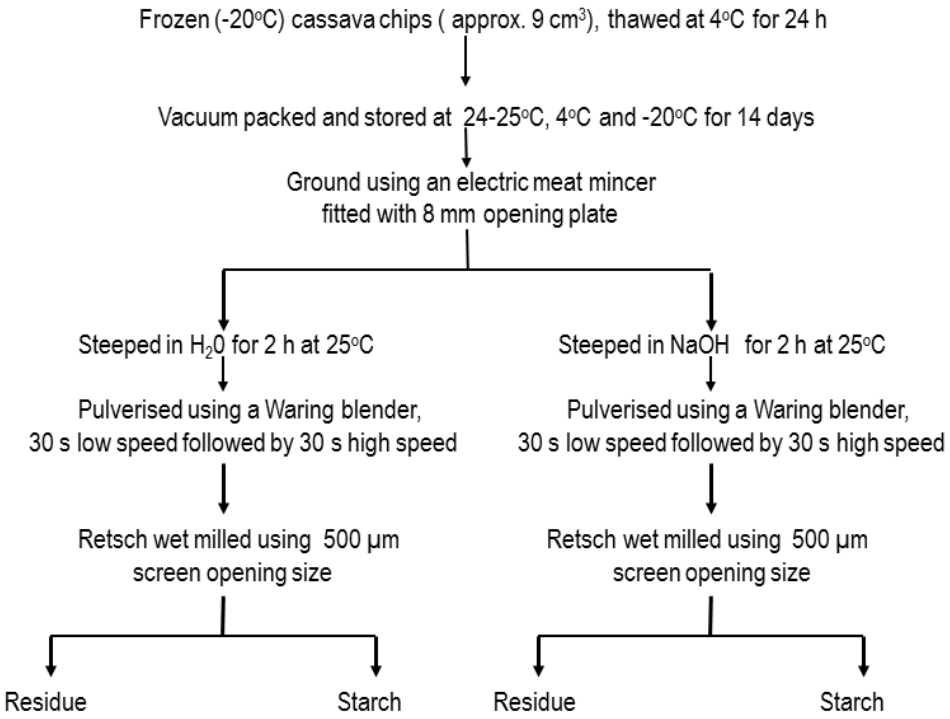

Figure 4.2.1 Flow diagram of the stages followed during cassava starch extraction in determining the effects of storage temperature and dilute NaOH steeping on starch yield (section 4.2.3.2.2)

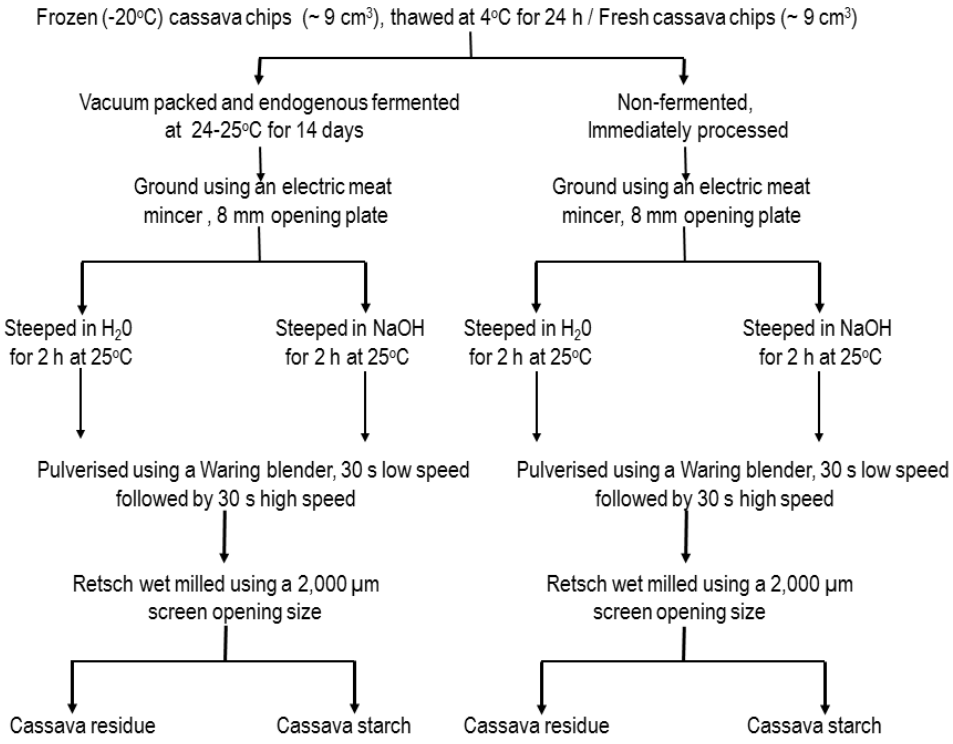

Figure 4.2.2 Flow diagram of the stages followed during cassava starch extraction in determining the effects of endogenous fermentation followed by dilute NaOH steeping on starch yield (section 4.2.3.2.3)

4.2.4 Preparation of cassava starch and residue fractions

Preparation of the cassava starch and residue fractions were carried out as described in Chapter 4.1. The milled fractions were stored in zip-lock type sealed polyethylene bags at 4^{o}C until assayed.

4.2.5 Analyses

4.2.5.1 Moisture

Moisture content was determined by 2-stage draught air oven drying, AACC Method 44-15A (AACC International, 2000).

4.2.5.2 pH and total titratable acidity

The pH and total titratable acidity (in lactic acid equivalents) of the ground cassava pieces was determined after diluting the pulp 1:2 with distilled water and thoroughly mixing.

4.2.5.3 Residue fraction particle size

The residue fraction particle size analysis was carried out as described in Chapter 4.1.

4.2.5.4 Microscopy

Cassava residue was suspended in distilled water and viewed using light transmission microscopy as described in Chapter 4.1.

4.2.5.5 Starch content

Starch content of the starch and residue fractions (prior to starch removal) were determined as described in Chapter 4.1.

4.2.5.6 Microbiological analysis

Ground cassava pieces (200 g) were incubated in 16 individually sealed multi-layered vacuum bags at 25^{o}C for 14 days with two bags taken every 48 h for analysis. Cassava pieces (25 g) were suspended in 225 ml Maximum Recovery Diluent (MRD) containing 0.1% (w/v) peptone and 0.85% (w/v) NaCl and then homogenized in a Stomacher for 30 s. From appropriate 10-fold dilutions, lactic acid bacteria (LAB) were enumerated on De Man,

Rogosa and Sharpe agar (Biolab, Johannesburg, South Africa) after incubation at 30^{o}C for 48 h. Yeasts and moulds were enumerated on Potato Dextrose Agar (Biolab) amended with 30 µg/ml chloramphenicol after incubation at 25°C for 96 h. At each sampling interval, where target organisms were detected, 10 colonies from the higher dilutions were randomly selected and purified.

For identification of the dominant fermentation microflora, isolates of presumptive lactic acid bacteria, yeasts and moulds were transferred directly to a Microflex LT^{TM} Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) target plate (Bremen, Germany) and overlaid with α-cyano-4-hydroxycinnamic acid matrix. Identification analysis was carried out as described by du Plessis, Duvenage and Korsten (2015) using the Biotyper automation software and library (Bruker). Score values between 1.999 and 1.700 were used to identify the microorganism genus and >2.300 for species identification.

4.2.5.7 Statistical analysis

All experiments were performed at least twice. Data were analysed by STATISTICA v10 software using one-way analysis of variance (ANOVA). Fisher's Least Significant Difference (LSD) test was used to determine significant differences between the treatments.

4.2.6 Results and discussion

4.2.6.1 Effects of endogenous fermentation and NaOH steeping on starch extraction

Vacuum storage of cassava pieces at 24-25^{o}C for 14 days resulted in a pH drop from 6.3 to 3.9 and increase in total titratable acidity from 0.1% to 0.6% lactic acid equivalents (Table 4.2.1). However, for cassava pieces stored at 4^{o}C and -20^{o}C, there was neither a decrease in pH nor increase in total titratable acidity. During incubation of cassava roots, a decrease in pH to approx. pH 3.9 has been associated with acid production by lactic acid bacteria (Kostinek, Specht, Edward, Pinto, Egounlety, Sossa, Mbugua, Dortu, Thonart, Taljaard and Mengu, 2007). Thus, the cassava pieces stored under vacuum packaging at 24-25^{o}C for 14 days seemed to have undergone endogenous fermentation, unlike the pieces stored at 4^{o}C and -20^{o}C.

Endogenous fermentation of the cassava pieces stored at 24-25^{o}C followed by water steeping resulted in greater starch extraction, an approx. 9% and 12% increase in starch yield compared to storage at 4^{o}C and -20^{o}C, respectively (Table 4.2.1). Importantly, fermentation of cassava pieces stored at 24-25^{o}C followed by water steeping did not result in a significant ($p \geq 0.05$) reduction in starch purity. With regard to endogenous fermentation of cassava pieces stored at 24-25^{o}C followed by NaOH steeping, a higher starch yield resulted, approx. 6% and 9% higher compared to storage at 4^{o}C and -20^{o}C, respectively. However, the 95.8% starch yield was not significantly ($p \geq 0.05$) more than the 96.3% achieved by endogenous fermentation followed by water steeping. Importantly, for the fermented cassava pieces steeped in dilute NaOH, there was no reduction in starch purity. For the non-fermented cassava, i.e. pieces stored at 4^{o}C and -20^{o}C, steeping in NaOH caused a significant ($p < 0.05$) increase in starch yield compared to water steeping. This finding is consistent with findings in Chapter 4.1 where steeping of ground cassava in 0.75% NaOH resulted in approx. 4% higher starch yield compared to the water treatment.

Table 4.2.1 Effects of storage of ground cassava pieces at different temperatures for 14 days followed by sodium hydroxide steeping on starch extraction by wet milling using a 500 µm opening screen (yield per 1 kg cassava roots)

Storage temp.	pH	Starch fraction wt. (g d.b[1].)		Starch fraction – starch purity (%)		Starch fraction – starch wt. (g d.b.)		Starch yield (%)	
		Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
24-25°C	3.9^{A}±0.0[2]	276.2^{D}±1.3	271.6^{D}±3.8	90.6^{AB}±0.1	90.8^{B}±0.4	250.2^{D}±1.5	246.5^{D}±2.3	96.3^{E}±0.6	95.8^{E}±0.1
4°C	6.2^{B}±0.0	254.4^{B}±0.6	261.6^{C}±2.3	89.8^{AB}±0.4	90.8^{B}±2.8	228.5^{B}±0.6	237.5^{C}±5.3	87.7^{C}±0.4	89.8^{D}±0.4
-20°C	6.3^{B}±0.0	239.7^{A}±1.7	255.5^{B}±1.5	91.3^{B}±0.6	87.8^{A}±0.2	218.9^{A}±1.7	224.2^{AB}±1.8	84.0^{A}±0.4	87.3^{B}±0.2

Storage temp.	**Total titratable acidity** (% lactic acid eqv.)	**Residue fraction wt. (g d.b.)**		**Residue fraction- starch content (%)**		**Residue fraction - starch wt. (g d.b.)**		**Total starch in both fractions (g d.b.)**	
		Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
24-25°C	0.6^{B}±0.0	23.4^{A}±1.1	23.4^{A}±0.4	45.2^{A}±4.0	47.7^{A}±0.8	10.6^{A}±1.4	11.2^{A}±0.3	260.8^{A}±0.0	257.7^{A}±2.6
4°C	0.1^{A}±0.0	53.4^{D}±0.6	45.7^{B}±0.9	58.3^{B}±1.8	57.4^{B}±2.8	31.1^{C}±1.3	26.2^{B}±1.8	259.6^{A}±1.9	263.7^{A}±7.1
-20°C	0.1^{A}±0.0	62.4^{E}±1.3	50.7^{C}±0.6	65.5^{C}±0.2	65.5^{C}±0.1	40.8^{D}±1.0	33.2^{C}±0.4	259.7^{A}±0.9	257.3^{A}±1.4

[1]d.b. = dry weight basis
[2]Means± standard deviations of two independent experiments
For each analysis, means of values with different letters are significantly different ($p<0.05$)

Cassava pieces stored at 4^{o}C and -20^{o}C were significantly ($p<0.05$) less disaggregated by wet milling in comparison to pieces stored at 24-25^{o}C (Table 4.2.2A). Furthermore, cassava pieces stored at 4^{o}C and -20^{o}C followed by NaOH steeping resulted in significantly ($p<0.05$) more disaggregation compared to the water steeping. Concerning the freezing of plant materials, especially fruits and vegetables, cell wall damage and subsequently tissue softening has been observed (Yamada, Kuroda, Jitsuyama, Takezawa, Arakawa and Fujikawa, 2002). However, the weakening of cassava cell walls as a result of lower temperatures appears insignificant in comparison to endogenous fermentation or NaOH steeping. This is probably because freeze damage induced tissue softening is dependent on the botanical origin and nature of plant organ (Yamada et al., 2002). Averre (1967) found that cassava roots could be stored satisfactorily under deep-freeze conditions in polyethylene bags. This probably explains why deep freezing is generally accepted as an effective method of storing fresh cassava roots (Ravi and Aked, 1996).

Light microscopy (Figure 4.2.3) showed that wet milling of fermented cassava pieces which had been stored at 24-25^{o}C resulted in more ruptured parenchyma root cells (Figures 4.2.3A,B), compared to cassava pieces stored at 4^{o}C and -20^{o}C, irrespective of whether the cassava pieces were subsequently steeped in NaOH or water. Thus, the combination of endogenous fermentation and NaOH steeping appears not to further increase the disaggregation of cassava cell walls. As expected, the more ruptured parenchyma root cells contained less starch granules (Figures 4.2.3A,B). With regard to the cassava pieces stored at 4^{o}C and -20^{o}C, light microscopy showed that NaOH steeping (Figures 4.2.3C,E) resulted in more ruptured parenchyma root cells, which contained less starch granules compared to the water treatment (Figures 4.2.3D,F). However, considering the limitations of light microscopy i.e. only a small area of material being examined, the disaggregation of cassava root parenchyma cell walls was further investigated by particle size analysis of the residue fractions in the bagasse.

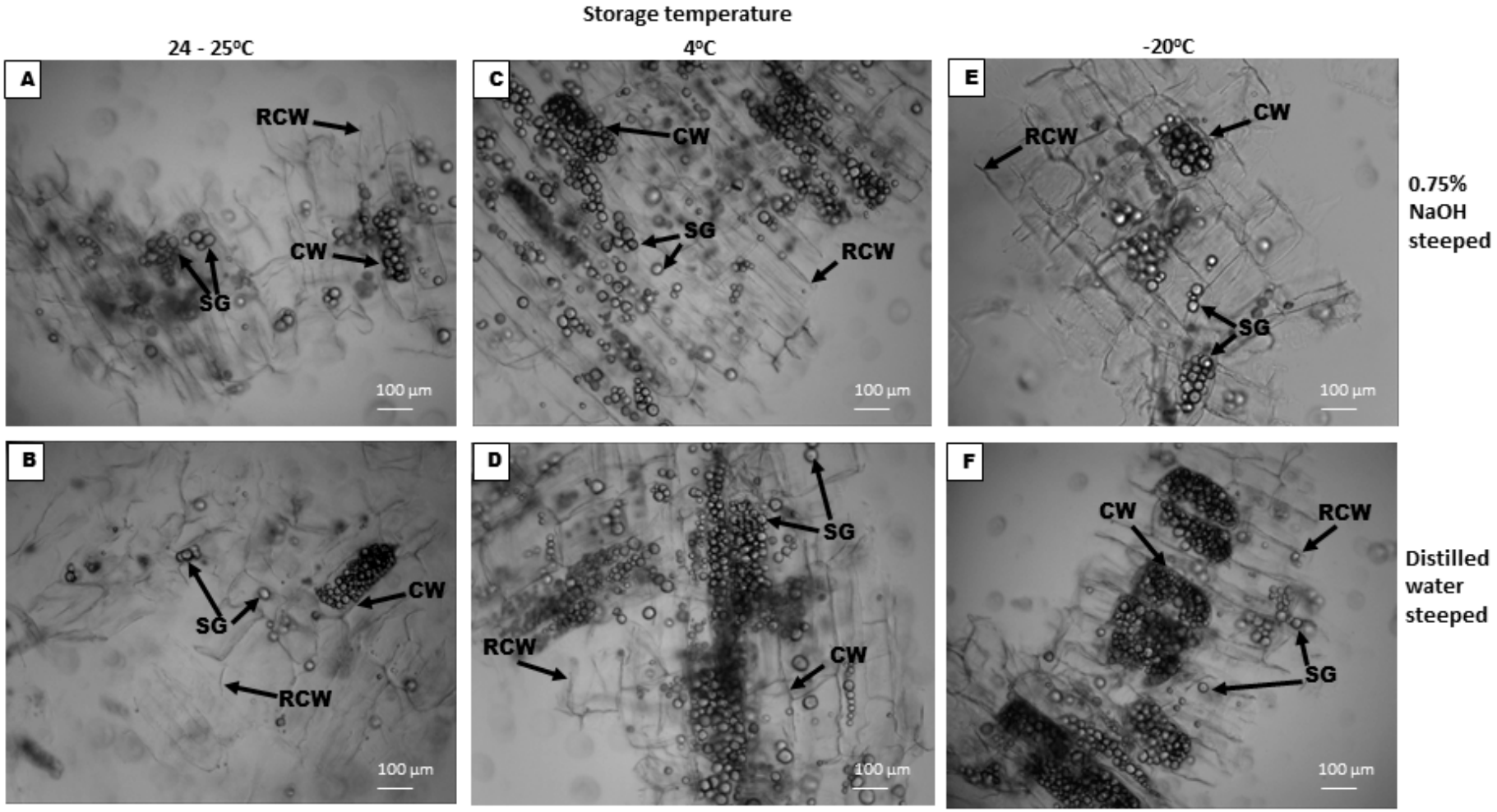

Figure 4.2.3 Light microscopy showing the effects of steeping ground cassava pieces from 14 day storage in distilled water and 0.75% NaOH followed by wet milling on disaggregation of cassava root parenchyma cells and subsequent release of starch granules (A) 0.75% NaOH and 24-25^{o}C, (B) Distilled H_2O and 24-25^{o}C, (C) 0.75% NaOH and 4^{o}C, (D) Distilled H_2O and 4^{o}C, (E) 0.75% NaOH and -20^{o}C, and (F) Distilled H_2O and -20^{o}C

CW: cell wall, **RCW**: Rupture in cell wall, **SG**: starch granules

Wet milling of the fermented (at 24-25^{o}C) cassava pieces followed by water steeping resulted in 49% and 41% less large root residue particles (diameters ≥ 250 µm) in comparison to the pieces stored at 4^{o}C and -20^{o}C, respectively (Table 4.2.2A). However, wet milling of the fermented cassava pieces followed by NaOH steeping resulted in 24% less and 1% more large root residue particles compared to the 4^{o}C and -20^{o}C treatments, respectively. With regard to cassava pieces stored at 4^{o}C and -20^{o}C (non-fermented) followed by NaOH steeping, there was a consistent ($p<0.05$) decrease in large root residue particles and increase in ruptured parenchyma root cells as compared to the water treatment (Table 4.2.2A and Figure 4.2.3). In contrast, steeping of fermented cassava pieces in dilute NaOH did not reduce the large root residue particles nor increase rupture of the parenchyma root cells in comparison to the water treatment. Mechanistically, in the weakening of plant cell walls, alkali primarily solubilises the soluble pectin fraction and also hemicellulosic constituents (Sun et al., 2016). Furthermore, both alkali and biological pre-treatments of plant cell walls remove the structure strengthening polymer, lignin (Sun et al., 2016). Therefore, the apparent inability of endogenous fermentation followed by NaOH steeping to improve cassava starch yield is probably due to similar cell wall weakening mechanisms. However, the ability of NaOH steeping to influence the cassava cell walls only in the absence of endogenous fermentation suggests that fermentation treatment is more dominant.

Considering starch fraction purity, Padmanabhan et al. (1992) observed that in excessively ground cassava roots, the separation of starch granules from the fibrous cell wall material was more difficult compared with coarser ground cassava roots. Significantly, in Chapter 4.1, 0.75% (w/v) NaOH steeping followed by wet milling with the coarser (1,000 µm and 2,000 µm) screen openings as would be used in industrial starch extraction, improved starch yield more than with the fine 500 µm screen size. Thus, increase in milling attrition (decrease in wet mill screen opening size) was observed to reduce the percentage increase by dilute NaOH steeping on starch yield. Therefore, using a coarse (2,000 µm) wet mill screen opening size, the combined effects of endogenous fermentation and NaOH steeping on cassava starch yield was further investigated.

Table 4.2.2 Effects of storage temperature, endogenous fermentation and NaOH steeping on the percentage of wet milled cassava residue particles collected over sieve mesh sizes 250 µm and 25 µm

A. Effects of storage temperature and NaOH steeping with wet milling using a 500 µm opening screen

	250 µm		25 µm	
Storage temperature	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
24-25°C	44.2^{A}±1.9	63.7^{B}±0.0	55.8^{E}±1.9	36.3^{D}±0.0
4°C	86.7^{E}±1.0	84.0^{D}±0.4	13.4^{A}±1.0	16.0^{B}±0.4
-20°C	75.0^{C}±1.3	63.0^{B}±0.6	25.0^{C}±1.3	37.0^{D}±0.6

B. Effects of endogenous fermentation of non-frozen roots and NaOH steeping with wet milling using a 2,000 µm opening

	250 µm		25 µm	
Fermentation duration (days)	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
0	93.8^{C}±0.2	60.7^{B}±0.9	6.3^{A}±0.2	39.4^{B}±0.9
14	64.8^{B}±0.3	53.0^{A}±3.4	35.2^{B}±0.3	47.0^{C}±3.4

C. Effects of endogenous fermentation of thawed roots and NaOH steeping with wet milling using a 2,000 µm opening screen

	250 µm		25 µm	
Fermentation duration (days)	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
0	78.4^{B}±1.0	33.6^{A}±1.4	21.6^{A}±1.0	66.4^{B}±1.4
14	77.1^{B}±0.7	76.7^{B}±0.4	22.9^{A}±0.7	23.3^{A}±0.4

[1]Means± standard deviations of two independent experiments
For each analysis, means of values with different letters are significantly different ($p<0.05$)

With regard to non-frozen cassava roots (Table 4.2.3), endogenous fermentation followed with water steeping substantially ($p<0.05$) increased the starch yield in comparison to the non-fermented and water steeped treatment, by approx. 17%. The increase in starch yield again appeared to be as a result of the substantial ($p<0.05$) reduction (approx. 31%) in the residue large particles, diameters ≥250 µm (Table 4.2.2B). Similar to the starch extraction with wet milling using a 500 µm opening screen (Table 4.2.1) there was no significant ($p\geq0.05$) decrease in the starch purity (Table 4.2.3). Considering dilute NaOH steeping of fermented cassava pieces, there was a slight ($p<0.05$) increase in starch yield compared to the non-fermented cassava pieces steeped in dilute NaOH. However, the increase in starch yield resulted in a significant ($p<0.05$) decrease in its purity. Similar to the finding in Chapter 4.1 when a 2,000 µm wet mill screen was used, steeping of non-fermented cassava pieces in dilute NaOH resulted in a significant ($p<0.05$) increase in starch yield as compared to the non-fermented cassava and water steeped treatment. Paradoxically, there was no significant ($p\geq0.05$) decrease in starch purity. Importantly, steeping of fermented cassava pieces in 0.75% NaOH increased starch yield less (by approx. 5%) compared to water steeping of fermented cassava pieces (approx. 17%) (Table 4.2.3). This is probably because NaOH steeping after endogenous fermentation did not result in a substantial further reduction in residue large particles, only approx. 13% (Table 4.2.2B).

For the thawed (after frozen storage at -20^{o}C for 11 months) cassava roots (Table 4.2.4), water steeping of the fermented cassava pieces significantly ($p<0.05$) increased the starch yield compared to the non-fermented cassava steeped in water. However, the increase (approx. 3%) was much less in comparison to the approx. 17% obtained when non-frozen cassava roots were utilised. The slight increase in starch yield was associated with an approx. 2% reduction in residue large particles, diameters ≥250 µm (Table 4.2.2C). However, the slight increase in starch yield was at the expense of a significant ($p<0.05$) reduction in starch purity. Considering dilute NaOH steeping of the fermented cassava pieces, there was a significant ($p<0.05$) decrease in starch yield in comparison to NaOH steeping of non-fermented cassava pieces. Furthermore, the approx. 0.7% increase in starch yield obtained by NaOH steeping of the fermented cassava pieces was negligible. Dilute NaOH steeping of the non-fermented cassava pieces also resulted in a significant ($p<0.05$) increase in starch yield in comparison to the water treatment.

Table 4.2.3 Effects of endogenous fermentation of non-frozen cassava pieces incubated at 24-25^{o}C in vacuum bags for 14 days followed by NaOH steeping on starch extraction by wet milling using a 2,000 µm opening screen (yield per 1 kg cassava roots)

Time (days)	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
	Starch fraction wt. (g d.b[1].)		**Starch fraction – starch purity (%)**		**Starch fraction – starch wt. (g d.b.)**		**Starch yield (%)**	
0	247.3^{A}±1.5[2]	269.8^{B}±0.2	90.0^{B}±0.2	88.8^{B}±0.0	222.5^{A}±2.0	239.6^{B}±0.2	78.9^{A}±0.0	84.7^{B}±0.4
14	299.0^{C}±0.5	297.0^{C}±2.9	88.6^{B}±0.0	85.6^{A}±1.7	264.7^{C}±0.6	254.2^{C}±7.5	92.6^{D}±0.1	88.9^{C}±2.9

Time (days)	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
	Residue fraction wt. (g d.b.)		**Residue fraction – starch purity (%)**		**Residue fraction – starch wt. (g d.b.)**		**Total starch in both fractions (g d.b.)**	
0	87.7^{D}±1.1	62.9^{C}±1.3	69.0^{C}±0.2	67.1^{B}±0.6	60.4^{D}±0.9	42.2^{C}±1.2	282.9^{B}±1.1	281.8^{AB}±1.4
14	34.7^{B}±0.2	30.7^{A}±1.0	61.1^{A}±0.9	61.3^{A}±0.5	21.2^{B}±0.4	18.8^{A}±0.8	285.9^{B}±1.0	273.1^{A}±6.7

[1]d.b. = dry weight basis
[2]Means± standard deviations of two independent experiments
For the effect of fermentation alone, for each analysis, means in the same column with different letters are significantly different ($p<0.05$)
For the effect of NaOH steeping alone, for each analysis, means in the same row with different letters are significantly different ($p<0.05$)

Table 4.2.4 Effects of endogenous fermentation of thawed cassava pieces incubated at 24-25^{o}C in vacuum bags for 14 days followed by NaOH steeping on starch extraction by wet milling using a 2,000 µm opening screen (yield per 1 kg cassava roots)

Time (days)	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
	Starch fraction wt. (g d.b[1].)		**Starch fraction – starch purity (%)**		**Starch fraction – starch wt. (g d.b.)**		**Starch yield (%)**	
0	246.0^{A}±2.4[2]	261.0^{B}±1.1	91.0^{B}±0.1	90.8^{B}±0.1	223.7^{A}±2.5	236.8^{C}±1.2	75.3^{A}±0.7	79.8^{D}±0.4
14	258.5^{B}±3.4	258.5^{B}±1.6	88.7^{A}±0.4	89.2^{A}±0.5	229.3^{B}±2.1	230.4^{B}±0.2	77.6^{B}±0.3	78.2^{C}±0.3

Time (days)	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH	Distilled H_2O	0.75% NaOH
	Residue fraction wt. (g d.b.)		**Residue fraction – starch purity (%)**		**Residue fraction – starch wt. (g d.b.)**		**Total starch in both fractions (g d.b.)**	
0	97.0^{C}±2.5	80.0^{A}±0.6	75.8^{A}±0.1	75.2^{A}±0.8	73.5^{C}±2.0	60.0^{A}±1.1	297.1^{A}±0.4	296.9^{A}±0.1
14	87.2^{B}±2.1	84.7^{AB}±0.6	75.9^{A}±1.1	76.0^{A}±0.6	66.1^{B}±0.6	64.3^{B}±1.0	295.4^{A}±1.5	294.8^{A}±0.8

[1]d.b. = dry weight basis
[2]Means± standard deviations of two independent experiments
For the effect of fermentation alone, for each analysis, means in the same column with different letters are significantly different ($p<0.05$)
For the effect of NaOH steeping, for each analysis alone, means in the same row with different letters are significantly different ($p<0.05$)

To better understand the apparent differences between the fermentation of non-frozen and thawed cassava roots, the nature of their endogenous microflora was studied. Fermentation of non-frozen and thawed cassava roots at 25^{o}C for 14 days resulted in a decrease in pH from pH 6.3 to 3.9 and 3.8, respectively (Figure 4.2.4A). As expected, the decrease in pH was closely associated with increase in total titratable acidity. With regard to fermentation of thawed cassava pieces, at day 0, MALDI-TOF analysis indicated the presence of the mould *Penicillium dierckxii* in addition to *Lactobacillus plantarum*, whereas from day 2 onwards the fermentation was dominated by *L. plantarum* (Figure 4.2.4B). Considering the fermentation of non-frozen cassava pieces, at day 0 the presence of yeasts and moulds in addition to lactic acid bacteria were found. In contrast to the fermentation of thawed cassava pieces, the yeasts were present until day 6 of incubation. Thereafter, the fermentation was dominated by lactic acid bacteria. Therefore, in fermentation of thawed and non-frozen cassava roots, lactic acid bacteria were the dominant species. However, the high lactic acid bacteria counts seemed not to have influenced the weakening of the cassava root parenchyma cell walls. Thus, it is probable that *P. dierckxii* and yeasts weakened the cassava cell walls through the action of extracellular acid-stable polygalacturonase (Shubakov and Elkina, 2002, Padonou, Nielsen, Akissoe, Hounhouigan, Nago and Jakobsen, 2010). Enzymatic weakening of the non-frozen cassava root cell walls by endogenous fermentation could explain the inability of alkaline steeping to weaken the cell walls.

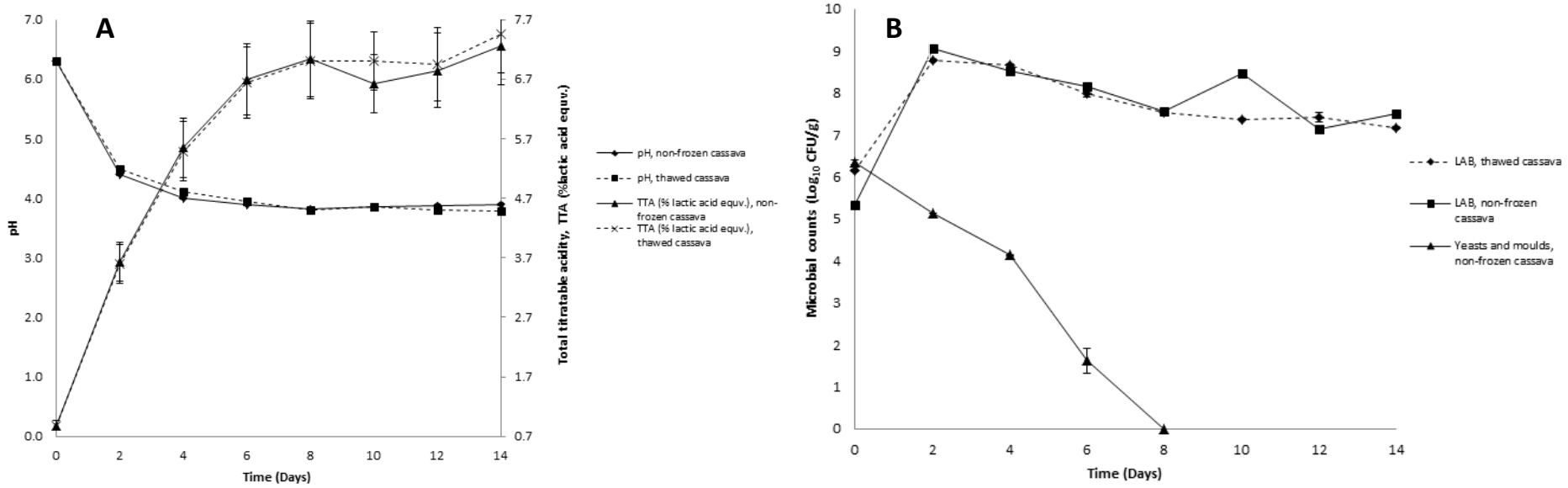

Figure 4.2.4 Fermentation of non-frozen and thawed cassava root pieces at 25°C for 14 days (A) effect on pH and total titratable acidity, (B) effect on lactic acid bacteria, yeasts and moulds
Error bars indicate standard deviation, n = 3

4.2.7 Conclusions

Separately, endogenous fermentation and 0.75% NaOH steeping of cassava pieces increase starch liberation and result in improved starch yield. Endogenous fermentation most likely improves cassava starch yield through hydrolysis of the parenchyma cell walls, probably through the action of extracellular cell wall degrading enzymes of endogenous yeasts and/moulds. As observed in Chapter 4.1, dilute NaOH steeping seems to cause solubilisation of the alkaline soluble cell wall pectin fraction and further weakens the cell walls through formation of micropores. However, in combination, endogenous fermentation followed by dilute NaOH steeping of cassava pieces did not further improve starch yield. Considering that improvement in cassava starch yield appears dependent on cell wall disaggregation, it is probable that in principle the mechanism by which endogenous fermentation weakens the cassava cell walls (primarily pectin hydrolysis) is similar to the mechanism by which dilute NaOH also weakens the cell walls (pectin solubilisation). Thus, the absence of an additive effect in the combination treatment is possibly because of their weakening effects on the cassava cell walls. Therefore, endogenous fermentation in combination with dilute NaOH steeping is not an appropriate technique for further weakening of plant cell walls.

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4.3 SOLID STATE CASSAVA ROOT FERMENTATION USING ALKALIPHILIC *BACILLUS AKIBAI*, B. *CELLULOSILYTICUS* AND *B. HEMICELLULOSILYTICUS* CULTURES ALONE, AND IN COMBINATION TO IMPROVE CELL WALL DISAGGREGATION

4.3.1 Abstract

Solid state fermentation of sodium carbonate wetted ground cassava root, surface pH of 10.2, using alkaliphilic *Bacillus akibai* + endogenous microflora (EM), *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM were investigated to determine whether and how they modify cassava cell walls. Gas chromatography data of cell wall constituent sugar composition and Fourier transform infrared (FTIR) data showed that alkaliphilic *Bacillus* spp. treatments reduced the levels of pectin, xyloglucan and cellulose in cassava cell walls. FTIR and wide-angle x-ray scattering spectroscopy also indicated that alkaliphilic *Bacillus* spp. treatments increased the residual cellulose relative crystallinity. Scanning electron microscopy and enzyme assay indicated that alkaliphilic *Bacillus* spp. production of extracellular cellulase and polygalacturonase caused formation of micropores through the cell wall and exposed the embedded fibrillar cellulosic network. Light microscopy revealed that these modifications increased disaggregation of cassava parenchyma cells. Fermentation using alkaliphilic *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM resulted in decreased large residue particle sizes in the bagasse and subsequently greater starch yield. Therefore alkaliphilic *Bacillus* spp. fermentation can improve the effectiveness of wet milling in disintegrating cell walls through hydrolysis of pectin, xyloglucan and cellulose, thereby reducing cell wall strength.

4.3.2 Introduction

Cassava (*Manihot esculenta* Crantz L.) fermented food products are generally made from its tuberous starch-containing roots (Kobawila, Louembe, Keleke, Hounhouigan and Gamba, 2005). Fermentation of cassava roots is important in several foods consumed worldwide (Almeida, Rachid and Schwan, 2007). In Africa, the roots may be fermented into food products such as *gari, lafun*and *fufu* and in South America, *cauim, calugi* and *yakupa* (Ramos, Sousa, Ribeiro, Almeida, Santos, Abegg and Schwan, 2015).

Fermentation of cassava roots aids reduction of postharvest losses, degradation of toxic cyanogenic glycosides, enhancement of root palatability and protein content (reviewed by Ray and Sivakumar, 2009). In cassava root fermentation, two different approaches are adopted, i.e. solid state and submerged (Ramos et al., 2015). Solid state fermentation involves growth of microorganisms on moist solid substrates free of flowing water, while submerged fermentation involves the presence of excess water (Singhania, Sukumaran, Patel, Larroche and Pandey, 2010; Behera and Ray, 2016). Importantly, solid state fermentation has several advantages over submerged fermentation including higher yield of extracellular enzymes (Ray, Shetty and Ward, 2008). This is probably because in nature, the growth and cellulose utilisation by aerobic cellulolytic microorganisms is more of a solid-state than submerged process (Zhu, Pan, Wang and Gleisner, 2009).

In Chapter 4.1, 0.75% (w/v) NaOH steeping of cassava root pieces at pH 11.5 was found to chemically modify and subsequently weaken the parenchyma cell walls. Mechanistically, dilute NaOH seemed to solubilise the alkaline soluble cassava cell wall pectin fraction through β-elimination reactions, hence weakening the structure. Thus, it may be hypothesized that combining spontaneous fermentation and alkaline steeping could further weaken cassava root parenchyma cell walls. However, even dilute (0.175%) NaOH soln. is reported to exert significant antimicrobial activity (Capita, Alonso-Calleja, del Camino García-Fernández and Moreno, 2002). Thus, a relatively weaker base such as, sodium carbonate (Na_2CO_3) is desirable in such an application. Sodium carbonate, at 50 mM concentration, can solubilise highly-branched pectic polysaccharides from cassava cell walls (Favaro et al., 2008). The solubilised cell wall fraction was found to comprise mainly galactose and arabinose residues in rhamnogalacturonan I (RG-I) stabilising side chains (Favaro et al., 2008, Cosgrove, 2016). Further, the solubilised fraction also contained rhamnose, which is an important backbone

component in RG-I (Brummell, 2006; Favaro et al., 2008). Thus, Favaro et al. (2008) demonstrated that Na_2CO_3 induced cassava cell wall weakening aids tissue disaggregation.

Alkaliphiles are microorganisms with growth optima pH 10 or higher (Horikoshi, 1999). Alkaliphilic *Bacillus* spp. produce several alkaline-stable extracellular enzymes of industrial importance (Nogi et al., 2005). Specifically, these alkaline stable enzymes include: cellulases, xylanases, α-amylases and pectinases among several others (Horikoshi, 1999). Due to the stability of alkaline enzymes at extremes of salinity, pH and ionic strength, biocatalysis using alkaliphiles and their enzymes is of significant industrial interest (Gomes and Steiner, 2004).

The effect of alkaliphilic *Bacillus* spp. fermentation on cassava cell wall structure and disaggregation has not been investigated. The aim of this study was to test the hypothesis that alkaliphilic *Bacillus* spp. capable of producing cell wall hydrolysing enzymes would weaken cassava parenchyma cell walls and as a consequence result in effective disaggregation of the parenchyma cells during wet milling.

4.3.3 Materials and methods

4.3.3.1 Materials

Cassava roots (sweet variety '*South Africa'*) were obtained as described in Chapter 4.1. *Bacillus akibai* (ATCC 43226) was procured from the American Type Culture Collection (Manassas, Virginia, USA). *Bacillus cellulosilyticus* (JCM 9156) and *Bacillus hemicellulosilyticus* (JCM 9152) were procured from the Japan Collection of Microorganisms (Koyadai, Japan). Carboxymethylcellulose – sodium salt (high viscosity) was procured from British Drug Houses (BDH) Chemicals (Poole, England). Pectin AS 509 was procured from Tate & Lyle (London, UK).Viscozyme L (produced from *Aspergillus aculeatus*) and Pectinex Ultra SP-L were kindly donated by Novozymes South Africa (Johannesburg, South Africa).

4.3.3.2 Methods

4.3.3.2.1 Cassava root transport and storage

Directly after harvesting, the roots were transported, processed and stored as frozen chips at the University of Pretoria, as described in Chapter 4.1.

4.3.3.2.2 Cassava chip size reduction

As described in Chapter 4.1, the frozen chips were thawed at 4^{o}C for 24 h, then ground into smaller pieces using an electric rotary meat mincer fitted with an 8 mm opening plate.

4.3.3.2.3 Preparation of alkaliphilic Bacillus spp. inocula and culture conditions

B. akibai was cultured on ATCC 1513 PY-CMC agar (carboxymethyl cellulose 10 g, Becton and Dickinson (BD) 211910 polypeptone peptone 5 g, yeast extract 5 g, NaCl 5 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 2 g, agar 15 g, distilled water 1 L and 100 ml 10% (w/v) Na_2CO_3 soln. added aseptically after autoclaving) and incubated aerobically at 30°C for 18 h. *B. cellulosilyticus* and *B. hemicellulosilyticus* were cultured on Horikoshi I agar (glucose 10 g, BD 211910 polypeptone peptone 5 g, yeast extract 5 g, K_2HPO_4 1 g, $MgSO_4.7H_2O$ 0.2 g, agar 15 g, distilled water 900 ml and 100 ml 10% Na_2CO_3 soln. added aseptically after autoclaving) and incubated aerobically at 37°C for 18 h.

Harvested *Bacillus* spp. cells were suspended into 8 ml sterile Maximum Recovery Diluent (MRD) (0.1% peptone and 0.85% NaCl) and centrifuged at 2,400 g for 20 minutes, 4°C. Concentrated *Bacillus* spp. cells were standardised to 9.0 McFarland standard, using a DEN-1 McFarland densitometer (Grant Instruments, Cambridgeshire, U.K.). In preparing *B.* spp. in combination inocula, equal portions of standardised single spp. inocula were combined. Inocula viable cell counts were confirmed by enumerating appropriate dilutions onto corresponding growth media. For *B.* spp. in combination inocula, Horikoshi I agar was used.

4.3.3.3 Effect of alkaliphilic Bacillus spp. inocula on cassava cell walls

Ground cassava pieces (200 g), in 1 L plastic buckets, were wetted using 40 ml sterile 10% (w/v) Na_2CO_3 soln. The sodium carbonate soln. was added, 5 ml aliquots, and each time vigorously mixed using a flame sterilised spatula. Standardised 8 ml inocula of *B. akibai*, *B. cellulosilyticus*, *B. hemicellulosilyticus* and *B.* spp. in combination were added. The inocula were also added in aliquots (1 ml) and each time vigorously mixed. To the controls, 8 ml sterile Maximum Recovery Diluent (MRD) was added. Addition of Na_2CO_3 raised the cassava surface pH to pH 10.2 and also introduced sodium ions, which are vital in cytoplasmic pH homeostasis by growing aerobic alkaliphilic *Bacillus* spp. above pH 9.5 (Krulwich, 1995). The plastic buckets were covered using aluminium foil and incubation was at 30^{o}C for 72 h. After incubation, fermented ground cassava pieces were pulverised in a Waring blender for 1 min. using low and high speed, each for 30 s. The pulp was suspended in 5x its volume distilled water and wet milled at 12,000 rpm using a Retsch EZ200 wet mill (Haan, Germany) fitted with a 2000 µm opening size screen.

4.3.3.3.1 Preparation of cassava starch and residue fractions

Preparation of the cassava starch and residue fractions were carried out as described in Chapter 4.1. The milled fractions were stored in zip-lock type sealed polyethylene bags at 4^{o}C until assayed.

4.3.3.3.2 Starch removal from the cassava residue fraction

Starch in the residue fraction was removed by enzymic hydrolysis as described in Chapter 4.1 to enable characterization of the insoluble cell wall material. The residual material after starch removal, referred to as cell wall material (CWM), was freeze dried, milled using an air-cooled, knife-type laboratory mill (IKA, Staufen, Germany) and stored at 4^{o}C in zip-lock type polyethylene bags until required.

4.3.3.4 Analyses

4.3.3.4.1 pH

The pH of ground fermented cassava was determined after diluting 1:2 with distilled water and thoroughly mixing.

4.3.3.4.2 Starch content

Starch content of the starch and residue fractions (prior to starch removal) were determined as described in Chapter 4.1.

4.3.3.4.3 Residue fraction particle size

Residue fraction particle size was determined by sieving 5 g wet residue through 250 µm, 106 µm and 25 µm opening sieve sizes using 2 L distilled water. Retained particles on the sieves were dried separately using AACC Method 44-15A (AACC International, 2000).

4.3.3.4.4 Microscopy

Preparation, mounting and viewing of cassava residue fractions using light transmission microscopy and scanning electron microscopy (SEM) were carried out as described in Chapter 4.1.

4.3.3.4.5 ATR-FTIR and WAXS spectroscopy

For ATR-FTIR and WAXS, residual cassava CWM preparation, conditioning and measurement were carried out as described in Chapter 4.1.

4.3.3.4.6 Gas chromatography characterization of residual cassava CWM

Gas chromatography (GC) analysis of the neutral sugar composition of residual cassava CWM and spectrophotometric analysis of uronic acid content were carried out by Englyst Carbohydrates (Southampton, UK) as described in Chapter 4.1.

4.3.3.4.7 Microbiological analysis and extracellular enzyme assays

4.3.3.4.7.1 Growth kinetics of endogenous microflora and alkaliphilic Bacillus spp.

Ground cassava pieces (25 g), in 100 ml plastic tubs, were wetted with 5 ml sterile 10% (w/v) Na_2CO_3 soln., final pH 10.5. One ml standardised bacteria inocula was added. For the control (endogenous microflora), 1 ml sterile MRD was added. The plastic tubs were covered with aluminium foil. The treatments were incubated at 30^{o}C for 72 h in triplicate. At 0 h, and after every 12 h, triplicate samples (25 g) of each treatment were suspended in 225 ml MRD and homogenized using a Stomacher for 30 s.

From appropriate dilutions, samples were plated on growth media, as follows: *B. akibai* on ATCC 1513 PY-CMC agar, incubated at 30°C for 48 h; *B. cellulosilyticus* and *B. hemicellulosilyticus* on Horikoshi I agar, incubated at 37°C for 48 h; Endogenous (control) and *Bacillus* spp. in combination on Horikoshi I agar, incubated at 30°C for 48 h. Plates were enumerated and the log_{10} transformed data were fitted to the bacteria growth model of Baranyi and Roberts (1994) using DMFit version 3.5 MS Excel add-in software (Institute of Food Research, Norwich, UK).

4.3.3.4.7.2 Alpha-amylase

Alpha-amylase production was assayed by inoculating 0.1 ml standardised inocula in triplicate onto alkaline starch agar, pH 10.2 (Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, $MgSO_4$ 0.24 g, $CaCl_2$ 0.01 g, peptone 3 g, 1% soluble starch, agar 15 g, distilled water 900 ml and 100 ml sterile 1 M Na_2CO_3 soln. added aseptically after autoclaving) (Burhan, Nisa, Gökhan, Ömer, Ashabil and Osman, 2003). Alpha-amylase was detected by flooding inoculated plates after incubation at 30^{o}C for 72 h using Lugol's iodine (Saxena, Dutt, Agarwal, Nayyar, 2007). The flooded plates were scanned using a flatbed scanner. Alpha-amylase action was estimated by the ratio of unstained (hydrolysed starch) plate area to the total (iodine stained and unstained) plate area.

4.3.3.4.7.3 Beta-1,4-endoglucanase activities and endo-polygalacturonase

Ground cassava pieces (50 g) were moistened with 10 ml sterile 10% (w/v) Na_2CO_3 soln., final pH 10.2. Two ml standardised bacteria inocula was added. For the control (endogenous microflora), 2 ml sterile MRD was added. The treatments were incubated at 30^{o}C for 72 h in triplicate. At 0 h, and after every 24 h, triplicate samples of each treatment were centrifuged

at 10,000 g for 15 min. 4^{o}C. The cell-free supernatant was immediately assayed. Alkaline β-1,4-endoglucanase and endo-polygalacturonase activities of alkaliphilic *Bacillus spp*. were assayed by reacting the cell free enzyme extracts with 1% (w/v) carboxylmethylcellulose (high viscosity) and 5% (w/v) pectin AS 509, respectively. Substrate solutions of 1% carboxylmethylcellulose and 5% pectin AS 509 were prepared using 0.1 M glycine-NaOH buffer (pH 10.5). Aliquots, 20 ml, buffered substrate soln. were equilibrated for 5 min. at 37^{o}C and 160 s^{-1} shear rate using an Anton Paar Physica MCR 101 rheometer (Graz, Austria). Immediately, 0.2 ml enzyme extract (maintained at 37^{o}C using a water bath) was added. The enzyme extract: buffered substrate reaction mixture was incubated for 10 min. at 37^{o}C and 160 s^{-1} shear rate in the rheometer. For controls (enzyme blanks), 0.2 ml sterile 10% (w/v) Na_2CO_3 solution was added. Enzyme activities were determined by the decrease to the reaction mixture viscosity (η) where the rheometer was fitted with the paddle tool. Beta-1,4-endoglucanase and endo-polygalacturonase activities were recorded as percentage viscosity reduction (% η-reduction) of the respective reaction mixtures after incubation for 10 min. at 37^{o}C and 160 s^{-1} shear rate (Ramírez-Tapias et al., 2015).

To quantify these activities, serial enzyme dilutions, 10^{-3} (1000 ppm) to 10^{-5} (10 ppm), of Viscozyme L (β-1,4-endoglucanase activity) and Pectinex Ultra SP-L (endo-polygalacturonase activity) were prepared using 0.1 M sodium actetate (pH 5.0) buffer. Beta-1,4-endoglucanase and endo-polygalacturonase activities were assayed by reacting different concentrations of Viscozyme L and Pectinex Ultra SP-L with 1% (w/v) carboxylmethylcellulose and 5% (w/v) pectin AS 509, respectively. The substrate solutions of 1% carboxylmethylcellulose and 5% pectin AS 509 were prepared using 0.1 M sodium actetate (pH 5.0). Percent η-reduction by different Viscozyme L or Pectinex Ultra SP-L concentrations under similar assay conditions were recorded. From the data, standard curves of % η-reduction against enzyme concentrations for Viscozyme L and Pectinex Ultra SP-L were plotted. The linear ranges, 50–700 ppm, were used to convert enzyme extract % η-reduction data into Viscozyme L and Pectinex Ultra SP-L activity equivalences.

4.3.3.4.8 Statistical analyses

All experiments were replicated at least twice. Microbiological analyses and extracellular enzyme assays were replicated at least three times. Data were analysed using one-way analysis of variance (ANOVA). Fisher's Least Significant Difference (LSD) test was used to determine significant differences between means.

4.3.4 Results and discussion

4.3.4.1 Growth of endogenous microflora and alkaliphilic Bacillus spp. on alkaline ground cassava

Increase in incubation time from 0 h to 48 h resulted in substantially higher microbial counts in the endogenous microflora (no inoculation control), *B. akibai, B. cellulosilyticus, B. hemicellulosilyticus* and *B.* spp. in combination (Figure 4.3.1A) treatments. At 48 h, the counts for endogenous microflora, *B. akibai, B. cellulosilyticus, B. hemicellulosilyticus* and *B.* spp. in combination were approx. 10.8, 11.3, 11.0, 10.9, and 10.6 $\log_{10}$ CFU/g, respectively. However, further incubation to 72 h caused a slight decrease in the microbial counts to 9.1, 10.6, 10.9, 10.6 and 10.4 $\log_{10}$ CFU/g, respectively. In spite of the low count at 0 h (4.4 $\log_{10}$ CFU/g) for the endogenous microflora treatment, after 24 h of incubation, there were substantially more microbial counts (> 9 $\log_{10}$ CFU/g). Considering that the Na_2CO_3 wetted ground cassava (surface pH 10.5) was not sterile, microbial colonies isolated on Horikoshi agar (pH 10.0) from the endogenous microflora treatment were considered presumptive alkaliphiles. Horikoshi (1996) found that only alkaliphilic microorganisms possessed the ability to grow well on Horikoshi agar, pH 10.0. Working with cassava, Ogbo (2003) found that rinsing peeled roots using 0.6 M NaOH soln. inhibited growth of some but not all endogenous microorganisms. Importantly, in enumerating microbial counts, the distinct morphological differences between the endogenous microflora and alkaliphilic *Bacillus* spp. colonies enabled reliable counts. Significantly, the presence of presumptive alkaliphilic endogenous microflora appeared not to hinder the growth of *B. akibai, B. cellulosilyticus, B. hemicellulosilyticus* and *B.* spp. in combination inocula (Figure 4.3.1A). Considering that several alkaliphilic microorganisms have been isolated from the soil (Horikoshi, 1996), the endogenous microflora probably originated from cassava root contamination by soil.

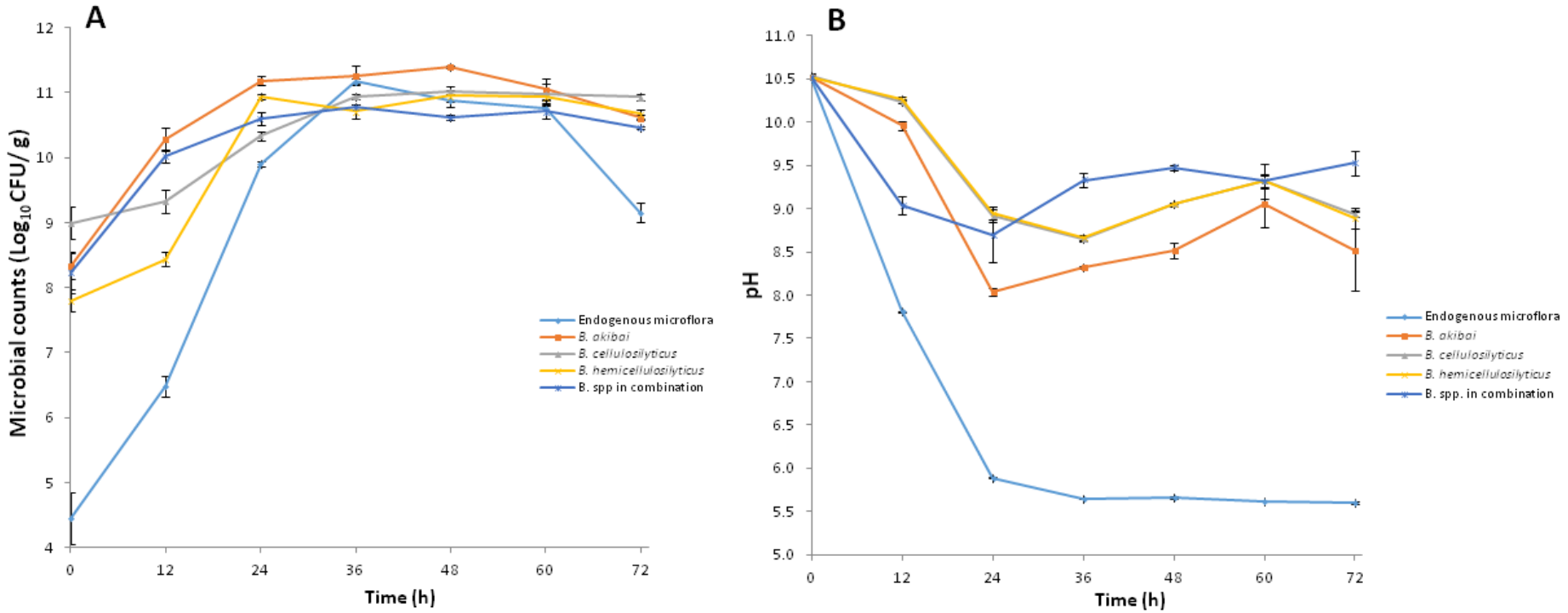


Figure 4.3.1 Growth of endogenous microflora and alkaliphilic *Bacillus* spp. on ground cassava pieces (A) and pH change (B). Error bars indicate standard deviation, n = 3

The increase in microbial counts with the endogenous microflora, *B. akibai*, *B. cellulosilyticus*, *B. hemicellulosilyticus* and *B.* spp. in combination treatments appeared to be associated with a general decrease in pH (Figure 4.3.1B). Excluding endogenous microflora and *B.* spp. in combination treatments, the decrease in pH followed a similar pattern, i.e. an initial decrease followed by a slight increase and then a final decrease. Importantly, throughout the fermentations using alkaliphilic *B. akibai*, *B. cellulosilyticus*, *B. hemicellulosilyticus* and *B.* spp. in combination, the pH did not drop below pH 8.0. In contrast, during endogenous microflora fermentation, the pH dropped below 7.0 after only 24 h of incubation. This was probably as a result of more acid production by the endogenous microflora in the absence of competition with the alkaliphilic *Bacillus* spp. inocula. This idea is supported by the fact that Paavilainen, Helisto and Korpela (1994) observed that during growth of alkaliphilic *Bacillus* spp. on carbohydrate substrates, pH decrease occurred mainly due to production of acetic, isobutyric, isovaleric and phenylacetic acids.

Considering the inocula growth kinetics, the Baranyi and Roberts (1994) model revealed the relative growth rates as *B. hemicellulosilyticus* > endogenous microflora > *B. akibai* > *B.* spp. in combination > *B. cellulosilyticus* (Table 4.3.1). The model's reliability (coefficient of determination) was high i.e. 0.99, 0.98, 0.98, 0.91and 0.89 for *B. cellulosilyticus*, *B. hemicellulosilyticus*, *B.* spp. in combination, *B. akibai*, and endogenous microflora, respectively. With *B. hemicellulosilyticus* there was a significantly ($p<0.05$) longer lag phase compared to *B. cellulosilyticus* and the endogenous microflora. For *B. akibai* and *B.* spp. in combination treatments, the 12 h sampling interval was too long for determination of the lag phase. With regard to biomass at the end of fermentation (yEnd), the model predicted significantly ($p < 0.05$) more biomass production by *B. akibai* (11.10 $\log_{10}$ CFU/g) compared to 10.97, 10.84, 10.63 and 10.49 $\log_{10}$ CFU/g for *B. cellulosilyticus*, *B. hemicellulosilyticus*, *B.* spp. in combination and endogenous microflora, respectively. However, the increase of 4.7 $\log_{10}$ CFU/g within 72 h with the endogenous microflora fermentation was significantly ($p<0.05$) higher than the 2.3, 1.9, 2.9 and 2.2 $\log_{10}$ CFU/g for *B. akibai*, *B. cellulosilyticus*, *B. hemicellulosilyticus, and B.* spp. in combination, respectively. This is probably because the endogenous microflora was better adapted to growing on cassava than the alkaliphilic *Bacillus* spp. Raimbault (1998) found that during fermentation, microbial growth was positively related to metabolic processes, including extracellular enzyme production. Furthermore, Roussos, Raimbault, Prebois and Lonsane (1993) observed a strong positive correlation between microbial growth and production of extracellular amylases, cellulases

and pectinases. Thus, in this present work, it was hypothesised that the observed differences in microbial growth parameters would probably influence production of extracellular cell wall hydrolysing enzymes.

4.3.4.2 Effect of alkaliphilic Bacillus spp. fermentation on cassava CWM

Fermentation of Na_2CO_3 wetted ground cassava by the endogenous microflora (i.e. without inoculation control (EM)) yielded 2.7 g CWM /100 g root pieces (fresh wt. basis), significantly ($p<0.05$) more than the 1.7 g, 1.5 g, 1.5 g, and 1.4 g for *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM, respectively (Table 4.3.2). The lower CWM yield with the alkaliphilic *Bacillus* spp. treatments suggests that part of the cassava CWM was hydrolysed into its component soluble sugars. However, the endogenous microflora CWM yield was higher than values of between 1.3 and 1.7 g/100 g (fresh wt. basis) reported for cassava cell walls by other workers (Salvador et al., 2000; Favaro et al., 2008). With regard to the study by Favaro et al. (2008), the harvested cassava roots were 7 months old, younger than the 11 months old roots used in the present study. Thus, the higher CWM yield in this present work was probably due to greater root cell wall lignification (Adetunji et al., 2016).

Generally, the neutral sugar composition of the hydrolysates from the fermented cassava CWM comprised glucose, xylose, galactose, arabinose, mannose, fucose and rhamnose in decreasing order (Table 4.3.3). Total NSP sugar recovery from the alkaliphilic *Bacillus* spp. fermented cassava CWMs were significantly ($p < 0.05$) lower in comparison to that from the endogenous microflora. However, in all treatments, total NSP sugars recovery was generally low, approx. 46-38% (Table 4.3.2). This can also be attributed to the residual cassava CWM being contaminated with cell wall lignin (Adetunji et al., 2016). With regard to lignin, this is primarily from the cassava root xylem and central vascular fibre tissue cell walls (Figueiredo et al., 2015). Importantly, glucose, xylose and galactose were the major neutral sugars in the endogenous microflora and alkaliphilic *Bacillus* spp. fermented cassava CWM (Table 4.3.3). This is indicative that cellulose and probably xyloglucans were the major NSP in the residual cassava CWM.

Table 4.3.1 Growth kinetic parameters, estimated by the primary model of Baranyi and Roberts (1994) for endogenous microflora and alkaliphilic *Bacillus* spp. on ground cassava pieces

Fermentation type	yDatMin	yDatMax	rate	lag	y0	yEnd	se(fit)	R^2
Endogenous microflora	4.68^{a}±0.03[1]	11.17^{c}±0.05	0.31^{c}±0.01	6.54^{a}±0.48	4.68^{a}±0.03	10.49^{a}±0.02	0.91	0.89
B. akibai	8.33^{c}±0.20	11.41^{d}±0.01	0.17^{b}±0.03	nd	8.33^{c}±0.19	11.10^{e}±0.01	0.31	0.91
B. cellulosilyticus	8.98^{d}±0.25	11.06^{b}±0.09	0.09^{a}±0.02	8.95^{b}±0.75	8.99^{d}±0.26	10.97^{d}±0.06	0.08	0.99
B. hemicellulosilyticus	7.80^{b}±0.17	11.00^{b}±0.02	0.66^{d}±0.03	11.11^{c}±0.40	7.80^{b}±0.17	10.84^{c}±0.03	0.17	0.98
B. spp. in combination	8.23^{c}±0.31	10.80^{a}±0.06	0.15^{b}±0.03	nd	8.23^{c}±0.31	10.63^{b}±0.05	0.13	0.98

[1]Means± standard deviations of three independent experiments
nd – lag phase was not determined by the 12 h sampling interval
Means of values in the same column but with different letters are significantly different ($p<0.05$)

Table 4.3.2 Residual cell wall material yield and sugar composition after fermentation of ground cassava using endogenous microflora and alkaliphilic *Bacillus* spp

Fermentation type	**Residual cell wall material** (g/ 100 g root pieces, fresh wt. basis)	**NSP sugar recovery** (g / 100 g cell wall material)
Endogenous microflora (EM)	$2.7^{c}\pm0.1$[1]	$38.3^{a}\pm1.3$
B. akibai + EM	$1.7^{b}\pm0.2$	$40.6^{ab}\pm0.2$
B. cellulosilyticus + EM	$1.5^{a}\pm0.0$	$46.4^{c}\pm0.5$
B. hemicellulosilyticus + EM	$1.5^{ab}\pm0.1$	$41.0^{b}\pm0.5$
B. spp. in combination + EM	$1.4^{a}\pm0.0$	$42.2^{b}\pm1.4$

[1]Means± standard deviations of three independent experiments
Means of values in the same column but with different letters are significantly different ($p<0.05$)

Table 4.3.3 Constituent sugar composition of residual cassava root cell wall material after fermentation of ground cassava with endogenous microflora and alkaliphilic *Bacillus* spp. (mg sugar/100 g root pieces, fresh wt. basis)

Fermentation type	Rhamnose	Fucose	Arabinose	Xylose	Mannose
Endogenous microflora (EM)	5.3^{b}±0.3[1]	10.3^{b}±2.3	54.0^{c}±2.3	134.8^{b}±15.1	32.7^{b}±4.8
B. akabai+ EM	3.7^{a}±0.7 (-30.2%)[2]	6.2^{a}±0.0 (-39.8%)	23.6^{b}±1.4 (-56.3%)	95.8^{a}±8.2 (-28.9%)	20.2^{a}±1.6 (-38.2%)
B. cellulolyticus + EM	2.8^{a}±0.6 (-46.8%)	7.5^{a}±0.7 (-27.7%)	18.5^{a}±1.9 (-65.7%)	94.8^{a}±2.1 (-29.7%)	20.7^{a}±1.6 (-36.9%)
B. hemicellulolyticus + EM	2.5^{a}±0.8 (-52.8%)	7.1^{a}±0.6 (-30.8%)	17.9^{a}±0.0 (-66.8%)	87.0^{a}±3.9 (-35.4%)	20.1^{a}±0.6 (-38.5%)
B. spp. in combination + EM	2.9^{a}±0.2 (-45.7%)	6.7^{a}±0.3 (-35.2%)	20.1^{ab}±0.7 (-62.7%)	83.9^{a}±2.9 (-37.8%)	17.2^{a}±0.4 (-47.5%)

Fermentation type	Galactose	Glucose	Galacturonic acid	Total NSP sugars
Endogenous microflora (EM)	68.1^{c}±4.4	602.4^{b}±50.2	108.1^{c}±2.6	1015.8^{b}±82.0
B. akabai + EM	38.1^{b}±0.2 (-44.1%)	438.4^{a}±50.5 (-27.2%)	76.5^{b}±6.4 (-29.3%)	702.6^{a}±66.3 (-30.8%)
B. cellulolyticus + EM	32.8^{ab}±0.2 (-51.9%)	433.8^{a}±4.6 (-28.0%)	66.8^{a}±2.5 (-38.2%)	677.7^{a}±7.8 (-33.3%)
B. hemicellulolyticus + EM	32.6^{a}±1.5 (-52.2%)	397.8^{a}±10.2 (-34.0%)	68.4^{ab}±2.5 (-36.8%)	633.5^{a}±17.7 (-37.6%)
B. spp. in combination + EM	33.7^{ab}±1.3 (-50.5%)	363.2^{a}±2.3 (-39.7%)	65.6^{a}±0.8 (-39.3%)	593.2^{a}±1.2 (-41.6%)

[1]Means± standard deviations of three independent experiments
Means of values in the same column but with different letters are significantly different ($p<0.05$)
[2]Column values in parentheses are percentage differences between the means of endogenous microflora and *Bacillus* spp. treatments

In general, fermentation of ground cassava with *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM substantially ($p < 0.05$) reduced all CWM NSP sugars in comparison with endogenous microflora (Table 4.3.3). The percentage reduction in arabinose content was most substantial, followed by galactose. Importantly, among the *Bacillus* spp. treatments, significant ($p < 0.05$) differences in the reduction of galacturonic acid, galactose and arabinose occurred. In cassava cell walls, pectin is composed mainly of galacturonic acid and galactose residues, while the hemicellulosic fraction comprises mainly xylose, galactose and glucose (Salvador et al., 2000). Generally, fermentation using alkaliphilic *Bacillus* spp. caused a decrease in all these sugars. Therefore, fermentation using alkaliphilic *Bacillus* spp. appears to have solubilised CWM pectin, hemicellulosic and cellulose fractions.

The cassava cell wall pectin fraction comprises two major domains: homogalacturonan (HG) which is exclusively comprised of galacturonic acid residues, and rhamnogalacturonan I (RGI) composed of a main chain of alternating galacturonic acid and rhamnose residues substituted by side chains of neutral sugars (Ngea et al., 2016). These side chains are attached to rhamnose residues in RGI and are mainly composed of β-1,4 linked-D-galactose and α-1,5 linked arabinose residues (Huang et al., 2016). Thus, the reduction in galactose, arabinose and rhamnose CWM constituents in alkaliphilic *Bacillus* spp. fermented cassava roots suggests that modification of pectin side-branches did occur.

4.3.4.3 Cassava cell wall modifications by alkaliphilic Bacillus spp. fermentation

Considering the FTIR spectroscopy data (Figure 4.3.2), the average normalised IR spectra of cassava CWM from endogenous microflora (no inoculation) and *Bacillus* spp. treatments revealed peaks within the 1800–400 cm^{-1}, the carbohydrate region (Szymanska-Chargot and Zdunek, 2013). The IR spectral peaks were located at; 3330 cm^{-1}, 2900 cm^{-1}, 1610 cm^{-1}, 1420 cm^{-1}, 1362 cm^{-1}, 1315 cm^{-1}, 1235 cm^{-1}, 1150 cm^{-1}, 1080 cm^{-1}, 898 cm^{-1} and 852 cm^{-1} wavenumbers. In general, the IR spectra of CWM from *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM revealed two IR spectra differences in comparison to endogenous microflora treatment. Specifically, IR spectral differences were at 1080 cm^{-1} and 898 cm^{-1}. At 1080 cm^{-1}, the peak vibration intensity was prominent in the CWM from endogenous microflora, *B. akibai* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM treatments but was diminished in *B. cellulosilyticus* +

EM fermentation. Further, at 898 cm^{-1}, the CWM shoulder vibration intensity was more prominent in the endogenous microflora and *B. akibai* + EM treatments but less prominent in the *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM treatments and absent in the *B. cellulosilyticus* + EM treatment.

Szymanska-Chargot and Zdunek (2013) used FTIR to study sequentially extracted fractions of residual potato parenchyma CWM. These authors determined the FTIR vibration wavenumbers for the cell wall pectin, xyloglucan and cellulose polymers. Based on this work, with this present work the IR spectra peak data at 1080 cm^{-1}, (C-O stretching, C-C stretching, xyloglucan ring), suggest that *B. cellulosilyticus* + EM resulted in more hydrolysis of xyloglucans compared to the endogenous microflora, *B. akibai* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM treatments. The spectra data at 898 cm^{-1}, (C1-H bending, xyloglucan/cellulose β-anomeric link), suggest *B. cellulosilyticus* + EM caused more hydrolysis of cellulose compared to endogenous microflora, *B. akibai* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM treatments. This finding is different from Chapter 4.1, where FTIR revealed that dilute NaOH steeping solubilised mainly cell wall pectin as indicated by the reduced 1737 cm^{-1} vibration ascribed to C=O stretching of methyl esterified uronic carboxyl groups (Oliveira et al., 2016) and 1234 cm^{-1} vibration ascribed to the stretching vibration of C-O methyl ester groups (Lim et al., 2014).

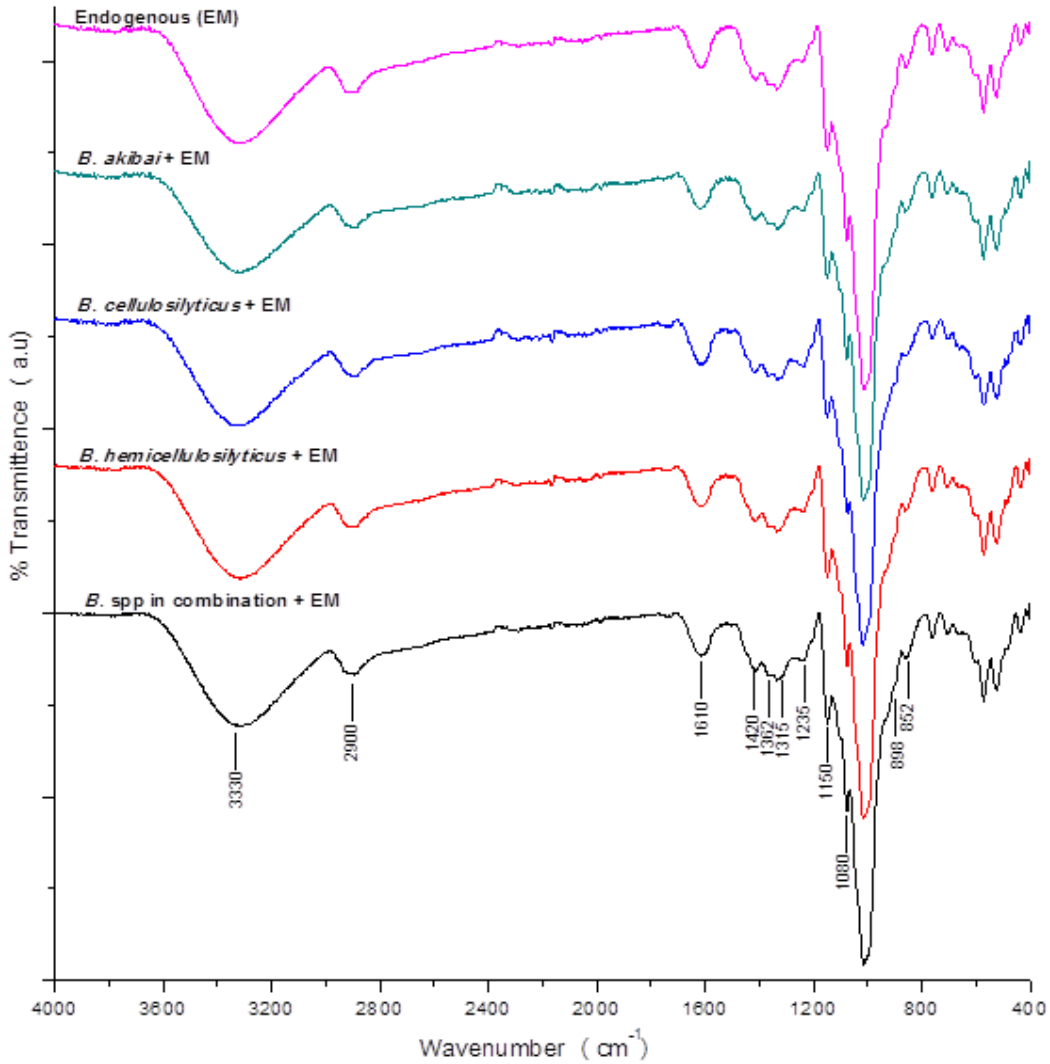


Figure 4.3.2 ATR FTIR spectroscopy showing the effect of endogenous microflora and alkaliphilic *Bacillus* spp. fermentation of ground cassava on the average normalised FTIR spectra of the residual cell wall material

The above apparent modifications to cassava root parenchyma cell walls as denoted by differences in cellulose and xyloglucan ascribed FTIR vibration peaks were further investigated using WAXS. In determining cellulose crystallinity, XRD evaluates contributions of amorphous and crystalline cellulose to the entire spectra area (Park et al., 2010). Thus, it provides a more accurate assessment of cell wall cellulose crystallinity than FTIR (Park et al., 2010). WAXS diffraction spectra of the CWM from all the fermentations revealed three planes (denoted by small lines) at 2θ approx. 15.5°, 22.5° and 37.7° (Figure 4.3.3). The peak at 2θ approx. 22.5° has been assigned to the (0 0 2) plane of cellulose I, the broad peak at 2θ approx. 15°–17° to the overlap between (101) and (10-1) planes of cellulose I, and the peak at about 37.7° has also been associated with cellulose I (Liao et al., 2011; Leite et al., 2017). Fermentation with *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM caused a significant ($p < 0.05$) increase in CWM relative crystallinity compared to endogenous microflora treatment. Furthermore, *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM caused increased peak (0 0 2) intensity compared to the endogenous microflora fermentation. An increase in diffraction peak (0 0 2) intensity is indicative of higher crystalline cellulose molecular chain regularity (Chen, Yu, Liu, Chen, Zhang and Hai, 2011). Thus, cell wall degrading extracellular enzymes produced by *B. akibai*, *B. cellulosilyticus*, *B. hemicellulosilyticus* and *B.* spp. in combination may have partially hydrolysed amorphous cassava cell wall fractions and caused their solubilisation. This finding is consistent with Chapter 4.1 where dilute NaOH treatment of cassava milled with a 2000 µm screen opening size also caused an increase in the CWM peak intensity (002) and its narrowing.

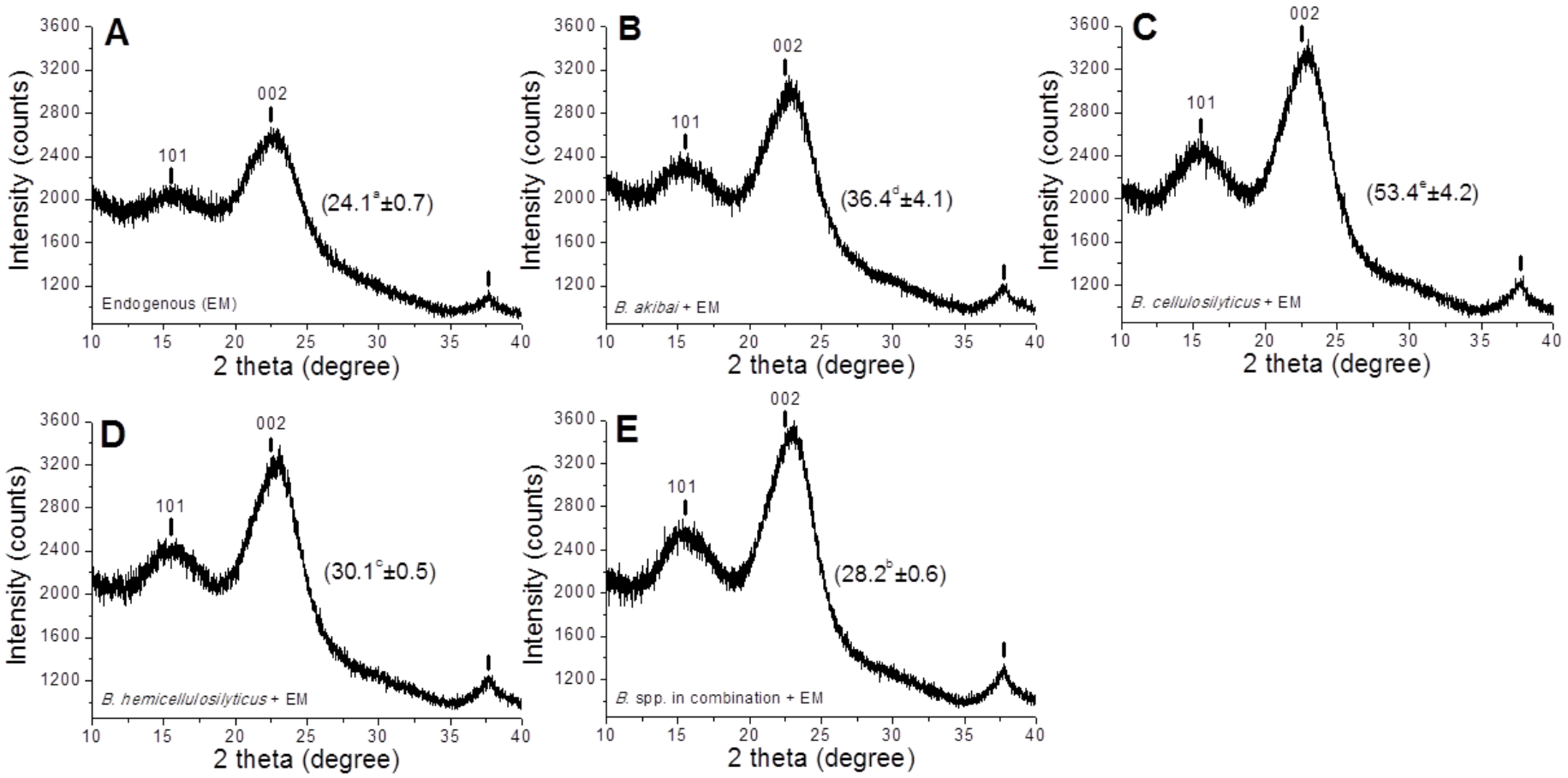


Figure 4.3.3 WAXS showing the effect of endogenous microflora and alkaliphilic *Bacillus* spp. fermentation of ground cassava on the X-ray diffraction spectra of the residual cell wall material; (A) endogenous (EM), (B) *B. akibai* + EM, (C) *B. cellulosilyticus* + EM, (D) *B. hemicellulosilyticus* + EM, (E) *B.* spp. in combination + EM.
*Values in parentheses are mean relative crystallinity (%) ± SD, n = 2. Means with different letters are significantly different ($p<0.05$)

Scanning electron microscopy (Figure 4.3.4) revealed that fermentation with *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM (Figures 4.3.4B,C,D,E,) resulted in cell wall rupturing (arrows labelled RCM) and the appearance of fibrils (arrows labelled CMF), features absent with endogenous microflora fermentation (Figure 4.3.4A). Cell wall breakdown through fibril formation (exposing embedded crystalline cellulose) has been associated with reduction in cellulose relative crystallinity (Hoeger, Nair, Ragauskas, Deng, Rojas and Zhu, 2013). Furthermore, with *B. akibai* + EM fermentation, SEM revealed the presence of cell wall perforations (micropores, arrows labelled MP), approx. 2-6 µm across. SEM also revealed the presence of what was probably bacteria biofilms (arrow labelled LBF) adhered onto the *B. cellulosilyticus* + EM cassava CWM. In cassava root parenchyma cell walls, micropore formation has been reported in microwave-heated, acid pre-treated CWM (Cheng et al., 2015). These authors attributed the formation of such micropores to weakened cell walls due to hydrolysis of hemicellulosic and lignin constituents within the cellulose-hemicellulose-lignin polymer matrix. With regard to fibril formation, this is thought to involve breakdown in the lignin cross linkages, degradation of cellulose crystals and shortening of cellulose chains (Wang, Zhu, Gleisner, Kuster, Baxa and McNeil, 2012; Hoeger et al., 2013).

4.3.4.4 Effect of alkaliphilic Bacillus spp. fermentation on starch yield

Light microscopy (Figure 4.3.5) revealed that in the residue fractions from *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM fermentations (Figures 4.3.5C,D,E) the root parenchyma cells had more dispersed starch granules (DSG) compared to the endogenous microflora and *B. akibai* + EM treatments (Figures 4.3.5A,B). Furthermore, the residue fractions from the endogenous microflora and *B. akibai* + EM treatments had closely packed starch granules (PSG) within some apparently intact cells. The dispersed nature of the starch granules in the *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM treatments was probably due to greater cell wall disintegration. The apparent reduction in intact cell walls was probably a result of greater microbial/enzymic hydrolysis. However, considering the limitations of light microscopy, i.e. only a small area of material examined, the disaggregation of cassava root parenchyma cell walls was further investigated by particle size analysis of the residues from the various treatments.

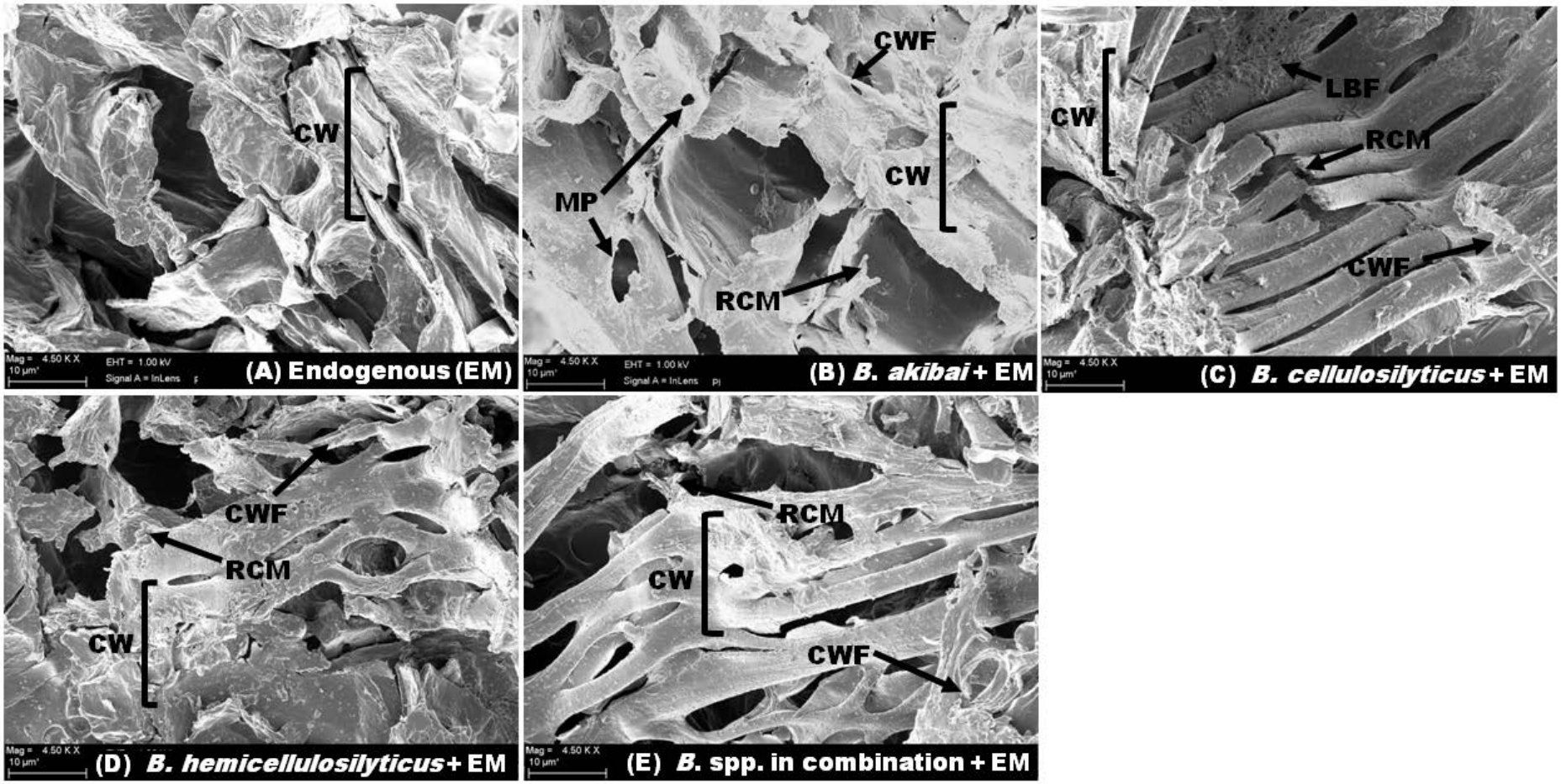


Figure 4.3.4 Scanning electron microscopy showing the effect of endogenous microflora and alkaliphilic *Bacillus* spp. fermentation of ground cassava on the root parenchyma cell walls; (A) Endogenous, (B) *B. akibai* + EM, (C) *B. cellulosilyticus* + EM, (D) *B. hemicellulosilyticus* + EM and (E) *Bacillus* spp. in combination + EM.
CW = cell wall material, MP = micropore, CWF = cell wall fibrils, RCM = ruptured cell wall material, LBF = lyophilized bacteria biofilm

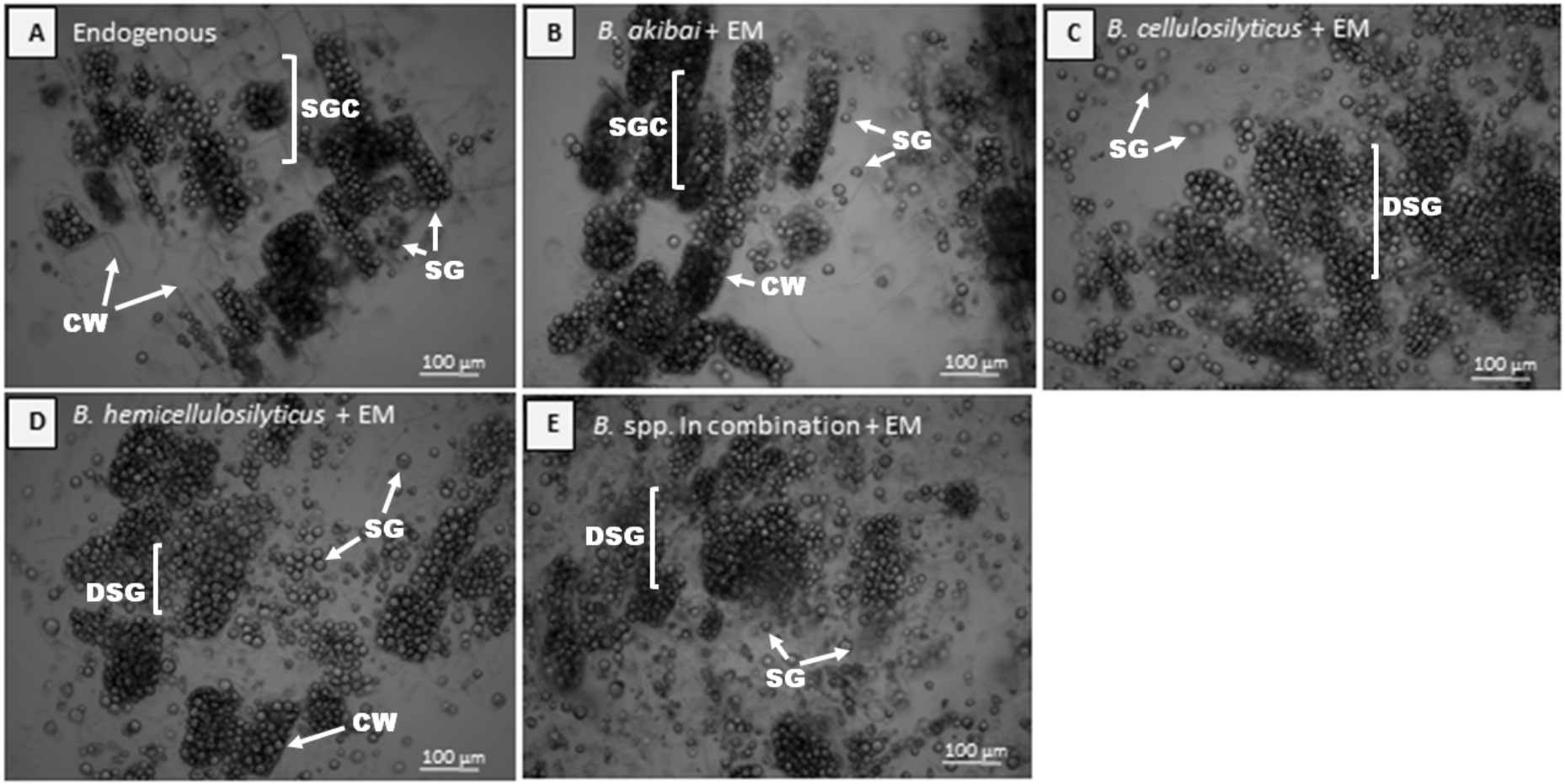


Figure 4.3.5 Light microscopy showing the effect of endogenous microflora and *Bacillus* spp. on cassava root parenchyma cell wall disaggregation and starch granule; (A) Endogenous, (B) *B. akibai* + EM, (C) *B. cellulosilyticus* + EM, (D) *B. hemicellulosilyticus* + EM and (E) *Bacillus* spp. in combination + EM.
CW = cell wall, SG = starch granule, DSG = dispersed starch granules, SGC = starch granule clump

Fermentation using alkaliphilic *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM resulted in a substantial ($p < 0.05$) decrease in large residue particles (diameters ≥ 250 μm) by approx. 51%, 54% and 37%, respectively, compared to endogenous microflora treatment (Table 4.3.4). Fermentation with *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM resulted in a great ($p < 0.05$) increase in smaller residue particles (diameters ≤ 106 μm to > 25 μm) by approx. 3540% and 3240%, respectively, compared to endogenous microflora. *B. akibai* + EM and *B. cellulosilyticus* + EM treatments had no significant ($p \geq 0.05$) effect on smaller residue particles (diameters ≤ 106 μm to > 25 μm). These findings support the concept that microbial/enzymic hydrolysis was involved in the cassava cell wall disaggregation, as indicated by SEM (Figure 4.3.4).

Fermentation of ground cassava using alkaliphilic *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM resulted in significantly ($p < 0.05$) higher starch yields, by approx. 20%, 23%, 17% and 22%, respectively, compared to endogenous microflora fermentation (Table 4.3.5). Alkaliphilic *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM treatments caused a significant ($p < 0.05$) reduction in total starch content by approx. 3.9%, 4.4% and 5.0%, respectively. However, in spite of the reduction, fermentation with *B. cellulosilyticus* + EM and *B.* spp. in combination + EM resulted in the highest starch yields, approx. 90%. Together, the XRD, SEM, residue fraction particle size analysis and the starch yield data suggest that fermentation with *Bacillus* spp. weakened cassava root parenchyma cell walls, which in turn caused more cell wall disaggregation during wet milling and consequently released more starch granules.

Table 4.3.4 Effect of endogenous microflora and alkaliphilic *Bacillus* spp. fermentation of ground cassava on the residue fraction percentage particle size

Fermentation type	Retained particle size fraction (%)		
	> 250 µm	**≤ 250 µm to > 106 µm**	**≤ 106 µm to > 25 µm**
Endogenous microflora (EM)	91.9^{c}±0.9[1]	8.1^{a}±0.9	0.0^{a}±0.0
B. akibai + EM	75.3^{bc}±6.1 (-18.1%)[2]	20.1^{ab}±5.2 (+148.1%)	4.7^{a}±1.7 (+470%)
B. cellulosilyticus + EM	45.0^{a}±19.9 (-51.0%)	43.3^{b}±25.7 (+434.6%)	11.7^{ab}±6.5 (+1170%)
B. hemicellulosilyticus + EM	42.1^{a}±14.5 (-54.2%)	22.5^{ab}±11.3 (+177.8%)	35.4^{c}±25.7 (+3540%)
B. spp. in combination + EM	57.7^{ab}±22.2 (-37.4%)	9.9^{a}±15.6 (+22.2%)	32.4^{bc}±9.6 (+3240%)

[1]Means± standard deviations of three independent experiments
Means of values in the same column but with different letters are significantly different ($p<0.05$)
[2]Column values in parentheses are percentage differences between the means of endogenous microflora and *Bacillus* spp. treatments

Table 4.3.5 Effect of endogenous microflora and alkaliphilic *Bacillus* spp. fermentation on starch extraction yield from cassava pieces wet milled using a 2 mm screen opening size (yield per 1 kg cassava roots)

Fermentation type	Starch fraction wt. (g d.b[1].)	Starch fraction, starch purity (%)	Starch fraction, starch wt. (g d.b.)	Starch yield (%)
Endogenous microflora (EM)	223.5^{a}±1.0[2]	89.9^{a}±2.1	201.0^{a}±5.6	73.4^{a}±1.2
B. akibai + EM	267.8^{b}±7.9 (+19.8%)	90.3^{a}±1.4 (+0.4%)	241.8^{bc}±10.4 (+20.3%)	88.3^{bc}±3.8 (+20.3%)
B. cellulosilyticus + EM	278.9^{c}±1.2 (+24.8%)	88.9^{ab}±0.8 (-1.1%)	247.9^{c}±3.2 (+23.3%)	90.5^{c}±1.2 (+23.3%)
B. hemicellulosilyticus + EM	269.8^{b}±4.0 (+20.7%)	87.6^{ab}±1.0 (-2.6%)	236.3^{b}±4.6 (+17.6%)	86.2^{b}±1.7 (+17.4%)
B. spp. in combination + EM	283.9^{c}±1.6 (+27.0%)	86.8^{b}±1.9 (-3.5%)	246.5^{bc}±4.5 (+22.6%)	90.0^{bc}±1.6 (+22.6%)

Fermentation type	Residue fraction wt. (g d.b.)	Residue fraction, starch content (%)	Residue fraction, starch wt. (g d.b.)	Total starch in both fractions (g d.b.)
Endogenous microflora (EM)	98.0^{c}±0.9	74.5^{c}±3.4	73.0^{c}±3.4	273.9^{c}±5.4
B. akibai + EM	45.0^{b}±8.3 (-54.1%)	60.6^{b}±3.9 (-18.7%)	27.4^{b}±6.2 (-62.5%)	269.2^{bc}±4.3 (-1.7%)
B. cellulosilyticus + EM	30.2^{a}±0.9 (-69.2%)	50.8^{a}±2.6 (-31.8%)	15.4^{a}±1.2 (-78.9%)	263.2^{ab}±3.8 (-3.9%)
B. hemicellulosilyticus + EM	40.0^{b}±2.0 (-59.2%)	63.7^{b}±0.2 (-14.5%)	25.5^{b}±1.3 (-65.1%)	261.7^{ab}±4.0 (-4.4%)
B. spp. in combination + EM	27.5^{a}±0.3 (-71.9%)	49.8^{a}±1.9 (-33.2%)	13.7^{a}±0.4 (-81.2%)	260.2^{a}±4.9 (-5.0%)

[1]d.b. = dry weight basis
[2]Means± standard deviations of three independent experiments
Means of values in the same column with different letters are significantly different ($p<0.05$)

4.3.4.5 Extracellular enzyme activities of the endogenous microflora and Bacillus spp.

As enzymic hydrolysis of the cassava starch granules with the *Bacillus* spp. treatments was indicated (Table 4.3.5), the production of α-amylase by the inocula was investigated. Pure cultures of *B. akibai*, *B. cellulosilyticus* and *B. hemicellulosilyticus* were observed to produce clear (de-stained) areas on alkaline starch agar (Figures 4.3.6A,B,C), indicative of extracellular α-amylase activity. Furthermore, the levels of α-amylase activity differed between the cultures, as revealed by the area of de-staining. The *B. akibai* cleared area (58%) was substantially ($p < 0.05$) larger in comparison to *B. cellulosilyticus* (23%) and *B. hemicellulosilyticus* (22%). Nogi et al. (2005) reported that alkaliphilic *B. akibai* and *B. cellulosilyticus* are amylolytic, while *B. hemicellulosilyticus* is non-amylolytic. This presumably explains the higher extracellular α-amylase activity of the *B. akibai* culture. Furthermore, the higher α-amylase activity of the *B. akibai* probably also explains its ability to substantially ($p < 0.05$) grow better than *B. cellulosilyticus* and *B. hemicellulosilyticus* (Table 4.3.1). Interestingly, the extracellular α-amylase activity of *B. hemicellulosilyticus* was contrary to its reported non amylolytic characteristic (Nogi et al., 2005).

With regard to cellulase activity (Figures 4.3.7 A1,2), fermentation of cassava using *B. akibai* + EM, *B. cellulosilyticus* + EM, *B.* spp. in combination + EM, *B. akibai* + EM and *B. hemicellulosilyticus* + EM gave significantly ($p < 0.05$) higher cellulase activity in comparison to the endogenous microflora. The principal enzyme activity of Viscozyme L is endo-β-glucanase with minor activities of xylanase, cellulase, and hemicellulase (Novozymes, 2014). In all treatments, fermentation time resulted in higher cellulase production. At 72 h, *B. cellulosilyticus* produced the highest concentration of cellulase, 628 ppm (Viscozyme L equiv.). Adetunji et al. (2016) found that 250 ppm Viscozyme L in combination with lactic acid bacteria fermentation hydrolysed 1,4-glycosidic linkages mainly in the cassava cell wall hemicellulosic fraction. The authors also observed an associated reduction in large residue fraction particle sizes. Thus, it is likely that the extracellular *Bacillus* spp. cellulase hydrolysed cassava cell walls polymers and consequently weakened the cell walls. This probably explains why the cell wall residue fractions of the *Bacillus* spp. treatments generally had less large residue particles (diameters ≥ 250 μm) in comparison to endogenous microflora fermentation (Table 4.3.4).

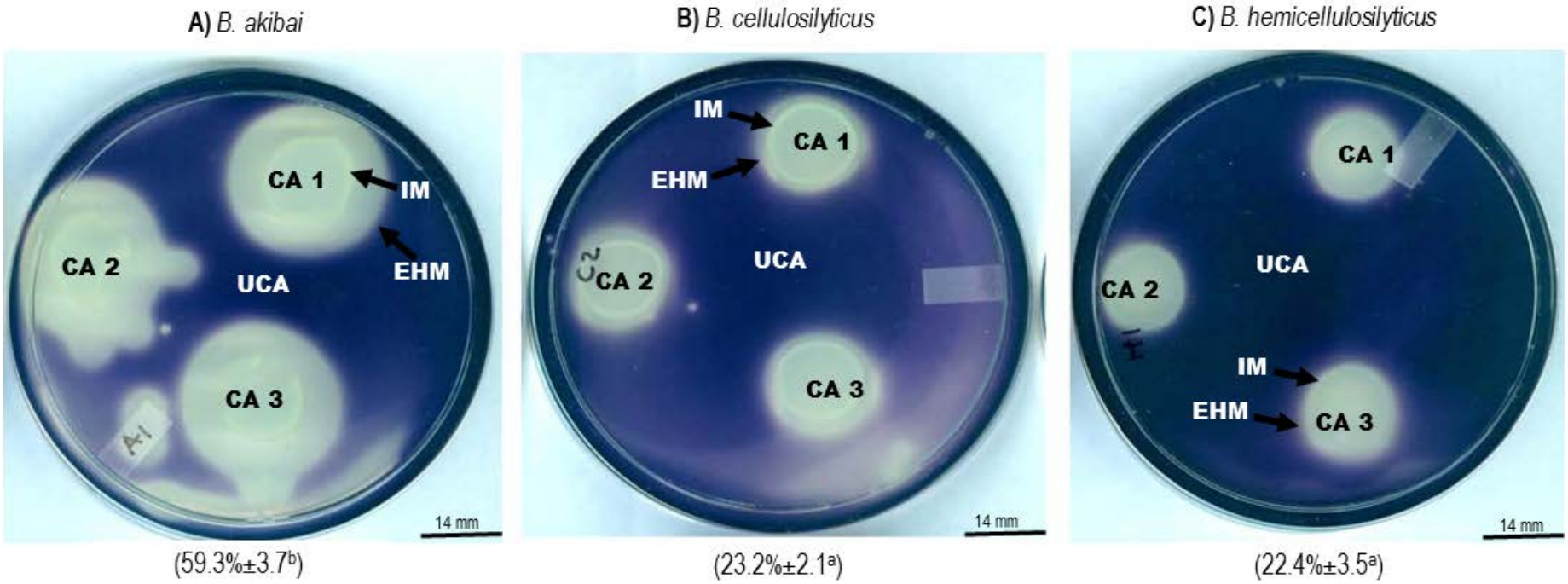
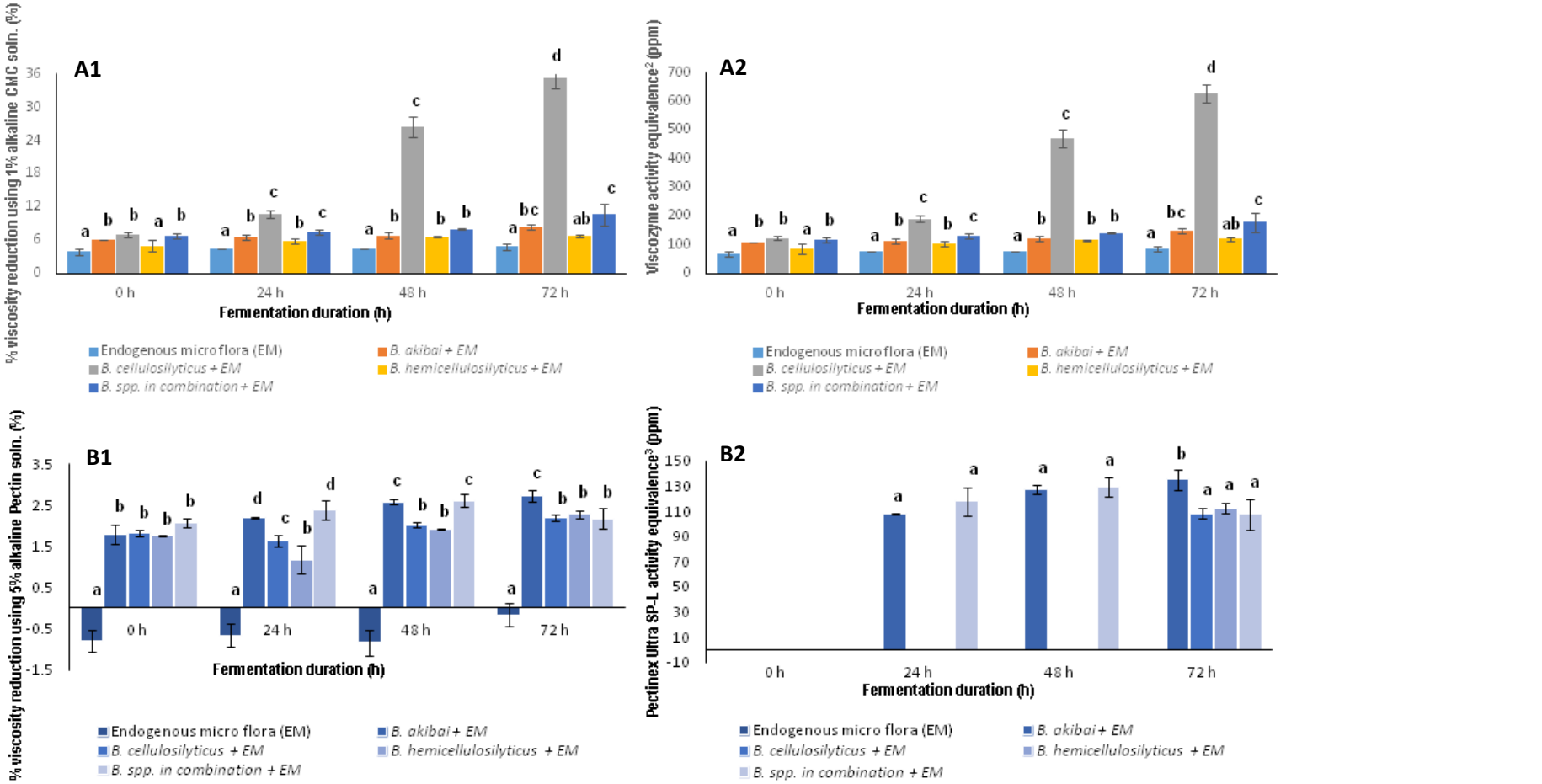


Figure 4.3.6 Images showing the effect of alkaliphilic *Bacillus* spp. α-amylase on the clearing of alkaline starch agar.
CA (1-3) = α-amylase cleared areas, triplicates, UCA = uncleared area, IM = inoculum margin, and EHM = enzyme hydrolysis margin. Values in parentheses are means ± standard deviation of cleared area (%), n = 3. Means with different letters are significantly different ($p<0.05$).



[1]Means± standard deviations of three independent experiments
[2]Regression between % viscosity reduction and Viscozyme L activity equiv.; y = 0.0558x + 2.42
[3]Regression between % viscosity reduction and Pectinex Ultra SP-L activity equiv.; y = 0.0199x + 2.09. For % viscosity reduction values less than 2.09 (the graph y-intercept at x=0, i.e. enzyme blank, polygalacturonase activity equiv. was regarded as not detectable (ND), 0 ppm.
Means of values in the same fermentation duration time cluster but with different letters are significantly different ($p<0.05$)

Figure 4.3.7 Effect of endogenous microflora and alkaliphilic *Bacillus* spp. fermentation on production of extracellular cellulase (A1,2) and pectinase (B1,2)

With regard to endo-polygalacturonase production (Figures 4.3.7 B1,2) at 72 h, the *B. akibai* + EM fermentation had slight but significantly ($p < 0.05$) more polygalacturonase activity compared to the *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM fermentations. The major enzyme activity in Pectinex Ultra SP-L is polygalacturonase (Novozymes, 2015). Polygalacturonase production was not detectable throughout the endogenous microflora fermentation. Importantly, with the *B. cellulosilyticus* + EM and *B. hemicellulosilyticus* + EM fermentations, polygalacturonase activity was not detectable until 72 h incubation. Mead, Drinkwater and Brumm (2013) reported that *B. cellulosilyticus* does not possess pectate lyase (PL) or GH28 family members genes, which is indicative of the microorganism's inability to produce extracellular pectinases. In a genomic study by Yuki, Oshima, Suda, Oshida, Kitamura, Iida, Hattori and Ohkuma (2014), the presence or absence of PL or GH28 family member genes in *B. akibai* and *B. hemicellulosilyticus* were not indicated. Thus, possibly interactions between the endogenous microflora and alkaliphilic *Bacillus* spp. aided polygalacturonase production.

4.3.5 Conclusions

Fermentation of alkali wetted ground cassava roots using *B. akibai* + EM, *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM hydrolyses the cell wall pectin, xyloglucans and cellulose. The concomitant loss in cell wall structural integrity probably enables *Bacillus* spp. cellulases to access and hydrolyse embedded amorphous cellulose fibrils, causing an increase in cell wall cellulose relative crystallinity. Together with hydrolysis of cross-linking pectin side branches and xyloglucan, partial hydrolysis of cellulose microfibril structures results in structural collapse, manifested as cell wall micropores and exposure of the embedded fibrillar cellulosic network. Cumulatively, these modifications weaken the cell walls, enabling increased disaggregation of starch-containing parenchyma cells during wet milling. Thus, fermentation of cassava using alkaliphilic *Bacillus* spp. starter cultures has potential for application in processes requiring hydrolysis/weakening of plant parenchyma cell walls.

4.3.6 References

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5 GENERAL DISCUSSION

This general discussion is divided into three sections. The first is a critical review of the methodological challenges faced in analysing the residual cassava CWM. The second section focuses on the main research findings concerning modifications to the cassava cell walls by dilute NaOH steeping and microbial fermentation for improved cellular disaggregation. This section also proposes mechanisms that could be responsible for cell wall weakening as a result of solubilisation/hydrolysis of the cell wall constituents. The final section discusses the potential use of dilute NaOH steeping and alkaliphilic *Bacillus* spp. fermentation in cassava root processing and the further research work required.

5.1 METHODOLOGICAL CONSIDERATIONS CONCERNING ANALYSES OF THE RESIDUAL CASSAVA CWM

In Chapter 4.1, dilute (0.75% w/v) NaOH steeping of ground cassava pieces was studied with the aim of establishing the mechanism involved in cassava cell wall disaggregation. Three different wet mill opening sieve sizes were used and the residue fraction starch granules were enzymatically hydrolysed to isolate the residual cassava CWM. In this part of the study, interpreting the few peak differences between the FTIR spectra of NaOH and water steeped residual CWM was problematic. This was because the signal strengths were not clearly differentiated from probable background noise. The signals were mainly within wavenumbers associated with lignin (Thumanu et al., 2015). Thus, utilisation of near-IR Raman spectroscopy which is better adapted for studying non-polar polymers like lignin than FTIR (Agarwal, 2014) may have resolved this challenge. Furthermore, near-IR Raman spectroscopy in combination with FTIR is reported to offer a more detailed assessment of the specific cell wall polymers than each technique alone (Pereira, Amado, Critchley, Van de Velde and Ribeiro-Claro, 2009). Additionally, analysis using ^{13}C cross-polarization, magic-angle spinning, solid-state nuclear magnetic resonance (^{13}C CP MAS ssNMR) spectroscopy, a technique able to clearly differentiate lignin signals from other cell wall components (Fu, McCallum, Miao, Hart, Tudryn, Zhang and Linhardt, 2015), could probably provide detailed information on lignin modifications Thus, the inclusion of either Raman or ^{13}C CP MAS ssNMR spectroscopy in the present study could have reduced the number of non-ascribed wavenumbers.

In both Chapters 4.1 and 4.3, improved release of starch granules from the cassava root parenchyma cells was associated with increased cellular disaggregation. FTIR, GC and WAXS were used to determine the chemical modifications to the cassava root cell walls, which were probably responsible for weakening the microstructure. Structural weakening of the cassava cell walls was inferred from the degree of cellular disaggregation after wet milling by using microscopy and residue fraction particle size. Jarvis and McCann (2000) proposed a more direct approach for determining cell wall strength comprising application of a known force followed by analysis of the resultant stress and deformations. However, because of the complex nature of plant cell walls, current techniques attempting such an approach still face several challenges. For example, some workers have attempted to deduce the mechanical attributes of plant cell walls by use of models derived from synthetic materials (Flores and Friswell, 2012). However, such attempts are limited by some of the assumptions made to simplify the cell wall structure, assumptions which may not hold true *in vivo* (Braybrook and Jönsson, 2016). Furthermore, cell wall functional properties (e.g. strength) are dependent on the fine details of cell wall macromolecular structure, its conformations and architecture; information that is partially lost during classical cell wall analyses (Jarvis and McCann, 2000). Thus, to date, the detailed functional roles of pectin, hemicelluloses and cellulose in cell wall mechanics have not been fully established (Kozioł, Cybulska, Pieczywek and Zdunek, 2017). Importantly, plant cell walls do not only influence the mechanical behaviour of the individual cells but also regulate the behaviour of tissues, since neighbouring cells are tightly bound together (Bidhendi and Geitmann, 2015). Thus, in the present study, linking mechanical weakening of the cassava cell walls to modifications of specific cell wall polymers by the different treatments was challenging.

In Chapter 4.3, fermentation of ground cassava pieces using alkaliphilic *Bacillus* spp. + endogenous microflora fermentation (EM) was evaluated to determine the ability of the *Bacillus* spp. to disaggregate cassava root cell walls. Despite the similarity in CWM yields in this present study and those found by other workers on cassava root cell walls (Favaro et al., 2008; Ngea et al., 2016), the present study identified a lower (46-38%) percentage of cassava CWM constituents. In cassava root cell walls, the identification percentage of CWM constituents appears to be influenced by age of the roots and state of processing (Favaro et al., 2008; Ngea et al., 2016). With regard to age, a higher identification percentage of CWM constituents is associated with younger roots, e.g. up to 7 months (Favaro et al., 2008). Thus, the low identification percentage of CWM constituents in the present study was likely due to

lignification of the in the root xylem and central fibrous tissue in the older (11 months) roots utilised. However, it is unlikely that the low identification percentage of the cassava CWM constituents impaired the inferences made concerning modifications to the cassava root cell walls. This is because higher cell wall lignin content may cause an apparent decrease in the percentage of CWM neutral and acidic sugars assayed but is unlikely that their mole fraction distributions are affected, which were the bases for the inferences.

In Chapters 4.1, 4.2 and 4.3, the fresh cassava root pieces utilised as raw material in the study were not characterised with regard to starch, NSP and lignin contents. In harvested cassava roots, only the xylem cell walls of the central vascular fibres are lignified but not the walls of the starchy parenchyma cells (Figueiredo et al., 2015). Importantly, modifications to the lignified cell walls of the central vascular fibres have obviously no effect on the release of starch during wet milling as this part of the root does not contain starch. Thus, the root lignin content is probably not a reliable indicator for studying the weakening and disaggregation of starch containing parenchyma cells and the subsequent release of starch granules during wet milling. However, in retrospect, a more detailed characterisation of the cassava roots would probably have enriched the discussion in Chapter 4.1.

In Chapter 4.2, immediate processing of cassava roots was considered as the control (no fermentation) to endogenous fermentation. Therefore, it could be argued that the immediately processed cassava pieces did not undergo a similar incubation time period of 14 days as compared to the endogenous fermentation treatment. Probably, the use of low temperatures (e.g. -20^{o}C) could have been used to incubate the control (no fermentation) samples for 14 days, thus, enabling a more appropriate comparison with the endogenous fermented samples. However, such an approach could equally be critiqued given the principle that freezing of plant cell walls results in cell wall damage and subsequently tissue softening. This occurs primarily because in freezing plant tissues, water migrates out of the cell protoplasm as a result of the differences between intracellular super cooled water and extracellular ice crystals (Yamada et al., 2002). Thus, freezing of plant cell walls has been reported to cause cytorrhysis i.e. the collapse of the cell wall around the shrinking protoplasm (Carpita, Sabularse, Montezinos and Delmer, 1979). Yamada et al. (2002) found that the shrinking sensitivity of the plasma membrane during freezing was mainly linked to denaturation of the membrane proteins. Furthermore, the authors reported that the freezing sensitivity of plant cell walls was dependent on their botanical and organ characteristics. However, although

cassava roots are believed to store well in the frozen state, there is inadequate scientific literature on the detailed molecular changes that possibly occur within the root cell walls (Ravi and Aked, 1996).

5.2 SUMMARY OF THE MAIN RESEARCH FINDINGS

In the present study, investigation of treatment of cassava root parenchyma cell walls, using: i) dilute NaOH steeping followed by wet milling, ii) endogenous fermentation followed by dilute NaOH steeping, and iii) alkaliphilic *Bacillus* spp. + endogenous microflora fermentation led to a number of significant research findings (Table 5.1).

Table 5.1 Summary of the main research findings on cassava root cell wall modification by dilute NaOH and microbial (alkaliphilic *Bacillus* spp. and endogenous microflora) fermentation

Research Chapter	Summarised research findings
4.1	Dilute NaOH steeping solubilised mainly the cassava cell wall pectin fraction.
4.1	NaOH steeping with fine milling reduced cell wall cellulose crystallinity slightly.
4.1	NaOH steeping weakened the cassava cell walls through formation of micropores.
4.1	Dilute NaOH steeping increased cell wall disintegration in ground cassava pieces.
4.1	NaOH steeping effect was additive to milling attrition in increasing starch yield.
4.2	Endogenous fermentation weakened cassava cell walls by the action of extracellular enzymes from yeasts and/or moulds.
4.2	Endogenous fermentation followed by NaOH steeping did not further weaken the cassava cell walls.
4.3	*Bacillus* spp. + EM (endogenous microflora) fermentation hydrolysed the pectin, hemicellulosic and cellulose fractions of cassava cell walls. Hydrolysis of the pectin likely involved modifications to rhamnogalacturonan I side-branches.
4.3	*B. cellulosilyticus* + EM fermentation substantially hydrolysed the xyloglucan and cellulose cell wall fractions.
4.3	*Bacillus* spp. + EM fermentation increased the cassava cell wall cellulose relative crystallinity by partially hydrolysing the amorphous cell wall fractions.
4.3	*Bacillus* spp. + EM fermentation caused the disintegration of cassava cell walls by action of extracellular cellulase and polygalacturonase

Table 5.1 *Continued*

Research Chapter	Summarised research findings
4.3	*Bacillus* spp. + EM fermentation of ground cassava pieces increased cell wall disaggregation.
4.3	*Bacillus* spp. + EM fermentation weakened the cassava root cell walls through perforation (micropores) of the micro-structure and exposure of the embedded fibrillar cellulose network to cellulase action.
4.3	*Bacillus* spp. + EM fermentation of ground cassava pieces increased starch yield.

5.3 PROPOSED MECHANISMS FOR WEAKENING OF CASSAVA ROOT PARENCHYMA CELL WALLS

5.3.1 Chemical (alkaline) weakening of cell walls by dilute NaOH steeping

Alkaline (0.5 M KOH) weakening (decreased cohesion) of cassava root cell walls, mainly by modifications to pectic and hemicellulosic polymers, has been linked to improved cellular disintegration (Favaro et al., 2008). A possible mode of action of dilute NaOH steeping in weakening cassava root cell walls is illustrated in Figure 5.1. Figure 5.1a presents the cell wall structural model of Cosgrove (2005). Cell walls are envisioned as reticulated networks comprising cellulose-cellulose junctions referred to as "biochemical hotspots", cross-linked by pectin and hemicelluloses into a quasi-homogenous load bearing mesh (Dick-Pérez, Zhang, Hayes, Salazar, Zabotina and Hong, 2011; Park and Cosgrove, 2012). These intertwined polymers act collectively as key control points in the mechanical behaviour of individual cell walls (Ulvskov et al., 2005; Zhang, Zheng and Cosgrove, 2016). At the molecular level, cell wall mechanical properties are thought to be influenced by selective loosening and shifting of load-bearing linkages between the cellulose micro fibrils (Cosgrove, 2005). Given the random distribution of "biochemical hotspots" at the μm scale, mechanically, it is probable that cell wall stiffness and adhesiveness are non-homogenous (Zhang et al., 2016). The cellulose microfibril structures are tightly bound by xyloglucan chains, while xyloglucan chains are loosely held by the arabinan/galactan polymers (Talbott and Ray, 1992). In such a structure, the presence of mechanically stronger domains suggests the likely existence of comparably less strongly bound domains.

As shown in Figure 5.1b, it is proposed that NaOH solubilises the accessible homogalacturon (HG) fractions of the cassava cell wall pectin, probably through β-elimination depolymerisation reactions. In pectin, the HG backbone is thought to be covalently cross-linked via rhamnose units to rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG-II) backbones, resulting into a macromolecular network (Willats, McCartney, Mackie and Knoxl, 2001; Caffal and Mohnen, 2009). Thus, dilute NaOH solubilisation of HG likely resulted in fragmentation of the pectin macromolecular network. Consequently, given the strengthening role of complex pectic cross-linkages in cell walls (Caffal and Mohnen, 2009), fragmentation of the network likely contributed to weakening of the cell wall cohesion.

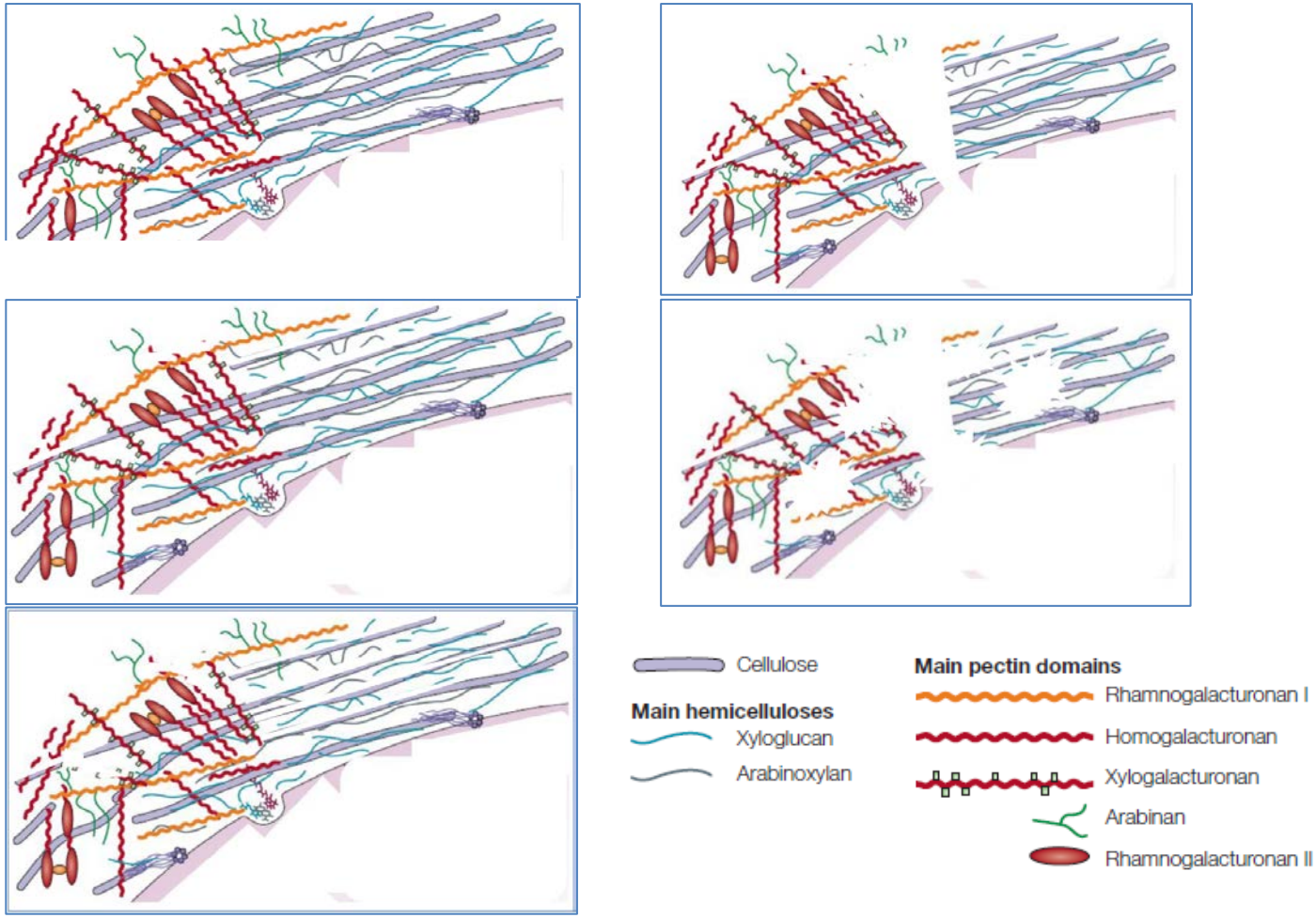

Figure 5.1 Illustration of the proposed effect of NaOH steeping on weakening of the cassava root cell walls.
a: Spatial arrangement of cell wall structural polymers (adapted from Cosgrove, 2005); b: Dilute NaOH solubilises the accessible homogalacturon pectin; c: Cell wall opening up by NaOH and fine milling exposes more cellulose myofibrils and cellulose-xyloglucan junctions to alkaline solubilisation; d: amorphous cellulose solubilisation disrupts surface anchored cellulose-xyloglucan junctions causing localised structural collapse, micropores; e: application of mechanical milling to the weaken cell walls causes increased structure disaggregation. The main pectin domains are excluded from the right-side of the schematic diagram for clarity.

Ulvskov et al. (2005) suggested that in cell walls the rhamnogalacturonan I fraction of pectin contributes to mechanical stability by cushioning the transmission of stresses onto the load-bearing cellulose microfibril structures. The authors also observed that small changes to pectin rheological properties had large consequences with respect to cell wall biophysical properties. This was probably because in cell walls there are far more pectin–cellulose interactions than xyloglucan-cellulose cross-linkages (Dick-Perez et al., 2011; Wang et al., 2012, 2015). Thus, dilute NaOH fragmentation of the pectin macromolecular network likely caused a decrease in the cell wall cohesion, i.e. cell wall weakening.

Consequently, as illustrated in Figure 5.1c, solubilisation of the accessible HG pectin fraction further exposed the embedded cellulose microfibril structures to dilute NaOH. In combination with fine (intense milling attrition) wet milling, more of the embedded cellulose microfibril structures are exposed to dilute NaOH. Opening up of the cell wall probably also exposes the relatively inaccessible xyloglucan-cellulose junctions to dilute NaOH solubilisation, which are critical for structural integrity (Park and Cosgrove, 2012). Mechanical pre-treatment is known to make cell wall cellulose microfibril structures more susceptible to react with NaOH as compared to native cellulose (Cuissinat and Navard, 2006). Thus, increased chemical reactiveness between dilute NaOH and the cellulose microfibril structures likely opens up the cell wall further, as manifested by decrease in the CWM cellulose relative crystallinity (Chapter 4.1). Structurally, cellulose microfibril structures are composed of a crystalline core and less organized (amorphous) peripheral domains (Earl and Vander-Hart, 1981). The peripheral domains (surface) of cellulose microfibril structures are known to occasional make contact with xyloglucan chains, which are thought to bind together the cellulose microfibril structures (McCann, Wells and Roberts, 1990; Levy, York, Struike-Prill, Meyer and Staehelin, 1991; Whitney, Brigham, Darke, Reid, Gidley, 1995).

Thus, as illustrated in Figure 5.1d, partial solubilisation of the surface (amorphous fraction) domains of cellulose microfibril structures possibly also dislodges the anchoring xyloglucan-cellulose junctions, further weakening the cell walls. In the less strongly bound cell wall domains (areas far removed from the “biochemical hotspots” and xyloglucan-cellulose junctions), it is possible that relatively more cell wall weakening occurred. The more weakened and localised cell wall domains then experienced complete structural collapse upon the application of a mechanical stress (wet milling attrition), as observed by the formation of micropores (Chapter 4.1). In the “biochemical hotspot” cell wall model, mechanical

properties are thought to depend on more than just strength of individual junctions but also the spacing between junctions as well as thickness and flexibility of the polymers (Cosgrove, 2015). Therefore, the formation of micropores likely disrupted the orientation of cellulose fibrils and interactions between the different polymers, further weakening the cell wall structure. Consequently, as illustrated in Figure 5.1e, application of a mechanical stress (wet milling attrition) to the dilute NaOH weakened cassava root cell walls resulted in increased cellular disaggregation, as manifested by the decrease in residue large ($\geq$ 250 μm) particle size (Chapter 4.1). Increased disaggregation of the cassava root cell walls consequently resulted in less entrapment of the starch granules, and thus improved starch release/yield (Chapter 4.1).

5.3.2 Microbial/enzymic weakening of cassava cell walls by *Bacillus* spp. cultures

With regard to the alkaliphilic *Bacillus* spp. weakening of cassava root cell walls, a possible mode of action is illustrated in Figure 5.2. Figure 5.2a again presents the cell wall structural model of Cosgrove (2005). As illustrated in Figure 5.2b, wetting of the ground cassava using 10% (w/v) Na_2CO_3 soln. partially solubilised the alkaline soluble pectin fraction. In cassava root cell walls, sodium carbonate (0.05 M) treatment solubilises the highly branched cell wall pectin (Favaro et al., 2008), probably through de-esterification reactions. Concerning the pectic polymers, a high degree of branching is indicative of the RG I and RG II fractions (Caffal and Mohnen, 2009). Therefore, dilute Na_2CO_3 soln. likely solubilised the RG-I and RG-II pectin fractions of the cassava root cell walls, which can occur through de-esterification of alkali-labile ester bonds (Brummell, 2006; Favaro et al., 2008). In cell walls, the neutral side chains of RG-I pectin are occasionally cross linked to xyloglucan chains (Keegstra, Talmadge, Bauer and Albersheim, 1973). Thus, it is probable that Na_2CO_3 wetting of the ground cassava also disrupted the cross-linkages responsible for stabilising xyloglucans chains, which are important for maintaining the packing between cellulose microfibril structures (Zhang et al., 2016). Also, as discussed earlier (Section 5.3.1), the inter-connected pectin linkages between RG I backbones to the RG-II and HG domains (Caffal and Mohnen, 2009), were likely disrupted. Thus, dilute Na_2CO_3 solution, like dilute NaOH, likely caused fragmentation of the cassava cell wall pectin network. The pectin network is stabilized by several types of cross-linkages (backbone glycosidic linkages, calcium and borate ester crosslinking, and covalent linkages to phenolic compounds) (Caffal and Mohnen, 2009), that were likely also disrupted and as a consequence weakened the cassava cell wall pectin.

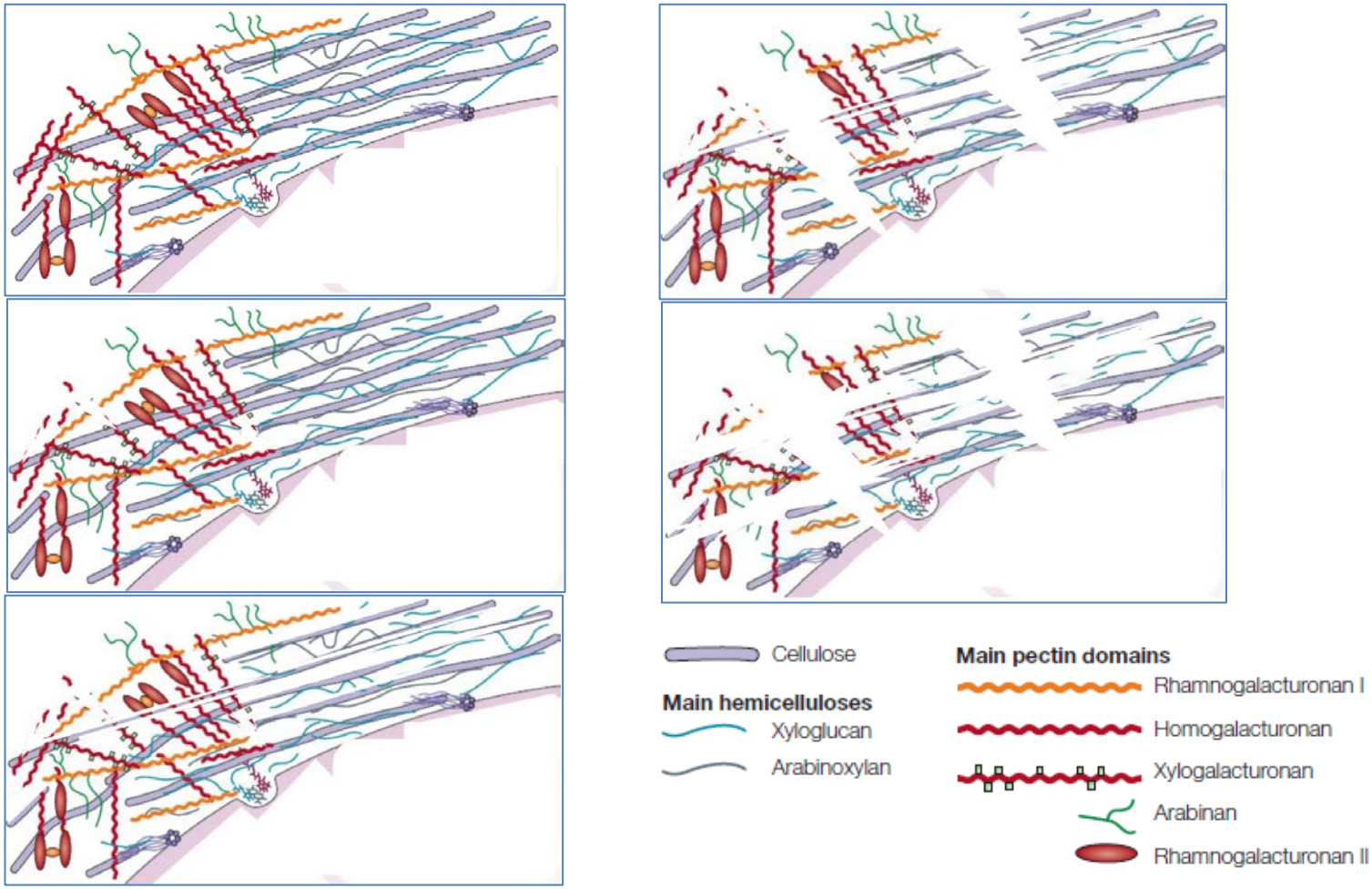

Figure 5.2 Illustration of the proposed effect of crude cellulase on weakening of the cassava root cell walls.
a: Spatial arrangement of the cell wall (adapted from Cosgrove, 2005); b: dilute Na_2CO_3 solubilises RG-I and RG-II pectin fractions by de-esterification of alkali-labile ester bonds (Brummell, 2006; Favaro et al., 2008); c: cellulase hydrolyses amorphous cellulose and xyloglucan-cellulose junctions, shortening the microfibril structures; d: hydrolysis of these cross-linkages results in localised structural collapse i.e. the micropores; e: weakening of the cell walls results in extensive disruption after wet milling exposing the embedded cellulose fibrillar network.

In plant cell walls, endogenous (polygalacturonase, pectate lyase and pectin methyl esterase) enzymes affect the mechanical properties of the cell wall through modifications to pectin and hemicelluloses linkages (Kozioł et al., 2017). In contrast, the cellulose framework remains stable and rigid due to the absence of cellulolytic activity in the endogenous cell wall degrading enzymes (Whitney, Gothard, Mitchell and Gidley, 1999). As determined by enzymic assay, solid-state cassava fermentation using alkaliphilic *Bacillus* spp. cultures + endogenous microflora produced extracellular cellulase and polygalacturonase. Endo-glucanases are thought to act by reducing the number of cellulose/cellulose interactions and xyloglucan/cellulose junctions, which subsequently weaken the cell wall (Chebli and Geitmann, 2017).Thus, as illustrated in Figure 5.2c, the hydrolytic action of extracellular cellulase most likely hydrolysed the peripheral (amorphous) fraction of the cell wall cellulose microfibril structures, as manifested by increase in the CWM cellulose relative crystallinity. Peripheral hydrolysis of the amorphous cellulose likely shortened the cellulose microfibril structures and fractured the xyloglucan-cellulose junctions. These modifications likely reduced the cell wall mechanical strength given its dependence on the integrity of polymer bundles and degree of spacing between the cross-linked junctions (Cosgrove, 2015). Furthermore, weakening of plant cell walls also occurs when constituents of the embedded xyloglucan-cellulose junctions are hydrolysed (Cosgrove, 2016). Importantly, this hydrolytic action is thought to be possible by only enzymes exhibiting both xyloglucanase and cellulase activities (Cosgrove, 2016).

As illustrated in Figure 5.2d, polymer hydrolysis (shortening) of the exposed cellulose microfibril structures and xyloglucan-cellulose junctions, likely resulted in mechanical weakening of the cell wall. As discussed earlier (Section 5.3.1), it is possible that the less strongly bound areas of the cell wall were weakened to a greater extent, leading to localised structural collapse, resulting in micropores (Chapter 4.3). The perforations (micropores) of the cell wall structure indicate that the rigid cellulosic framework was disrupted by cellulolytic action. The implication is that, as discussed earlier (Section 5.3.1), the cassava cell wall polymer packing and adhesiveness were likely also disrupted. This is because the cellulose framework is linked by non-covalent interactions to the matrix pectin, where the strength of the association determines most of the physical properties of the cell wall (Cosgrove, 2005).

Considering that in cell walls, xyloglucans are believed to bind onto the surface of cellulose microfibril structures and cross-link the adjacent microfibril structures (Cosgrove, 2016), it is possible that perforations (micropores) of the cell wall structure likely disrupted the intertwined xyloglucan-cellulose polymers. The change in spatial positioning would in turn have dislodged and exposed the galactose and arabinose residue side chains of xyloglucan to enzymatic action as revealed by the percentage reduction in the neutral sugar mole fractions measured by GC (Chapter 4.3). These side chains are believed to modulate the binding of xyloglucan to cellulose microfibril structures and also strengthen the cellulose-xyloglucan junctions (Jia, Cash, Darvill and York, 2005; Caffal and Mohnen, 2009). Thus, as illustrated in Figure 5.1e, formation of perforations (micropores) through the cell walls, shortening of the cellulose microfibril structures and disruption of the cross-linkages extensively weakened the microstructure. As a result, wet milling caused extensive cell wall disruption and exposed of the embedded fibrillar cellulose network (Chapter 4.3). Therefore, application of milling stress to the cellulase weakened cassava root cell walls resulted in increased cellular disaggregation and subsequently improved starch release/yield (Chapter 4.3).

With regard to polygalacturonase, its primarily action involves degradation of the cell wall pectin fraction (Kozioł et al., 2017). However, Cosgrove (2016) found that treatment of cell walls with polygalacturonase resulted in stiffening of the structure, rather than weakening. In view of this apparent contradiction, discussion of the possible mechanistic role of the alkaliphilic *Bacillus* spp. extracellular polygalacturonase in this present study is problematic.

5.4 POTENTIAL APPLICATION OF DILUTE NAOH AND ALKALIPHILIC *BACILLUS* SPP. IN CASSAVA ROOT UTILISATION

A process comprising dilute NaOH steeping followed by wet milling should improve the disintegration of cassava root parenchyma cell walls by wet milling. Consequently, dilute alkaline treatment of cassava roots would reduce the amount of residue fraction (bagasse) generated. Dilute NaOH steeping could also aid in weakening the cell walls in the cassava residue, thus, improving the fermentation process used to produce value-added products such as animal feeds (Ubalua, 2007). However, there will be a need for further studies on the health safety and nutritional characteristics of such feeds. Thus, dilute alkaline treatment has the potential to promote increased industrial utilisation of cassava roots for various food and non-food production processes, by improving cassava processing economic viability through

biorefining the bagasse. Biorefining is the processing of lignocellulosic (cell walls) biomass into bio-based products (food, feed, chemicals, and materials) and bioenergy (biofuels, power and/ heat) (Kim, Lee and Kim, 2016). However, utilisation of dilute NaOH steeping at an industrial scale would most likely generate a lot of alkaline waste effluent, which must be neutralised by acid treatment before discharge into the environment. The recent development of highly efficient re-cycling technologies that recover and re-use the unconsumed alkali to reduce NaOH consumption by approx. 60% (García-Torreiro, Pallín, López-Abelairas, Lu-Chau and Lema, 2016), should improve the potential viability of the dilute NaOH steeping process. For example, highly efficient re-cycling processes would theoretically reduce the alkali waste water concentration from 0.75% to 0.30% NaOH (w/v). Concerning such waste waters, biological treatments exist that could further minimise the risks to humans and the environment (Baccella, Cerichelli, Chiarini, Ercole, Fantauzzi, Lepidi, Toro and Veglio, 2000).

With regard to the process of microbial/enzymic solid-state fermentation using alkaliphilic *Bacillus* spp. cultures it could also improve the disintegration of cassava root parenchyma cell walls by wet milling. However, it is likely that the process would be only appropriate for specific industrial processes. For example, adopting the process for industrial starch extraction will be difficult. This is due to the long fermentation duration which is inappropriate for such a continuous production operation. The long duration is mainly because of the lag phase during alkaliphilic *Bacillus* spp. growth.

However, with regard to freeing starch granules when brewing or producing bioethanol using cassava (Adetunji et al., 2016), the process will likely improve the bioethanol brewing efficiency. With regard to the continuous processes, the fermentation duration could be shortened by using purified alkaline-stable cell wall degrading enzymes. The approach could probably be more effective than most current commercial enzymes which act at mild acid or neutral pH and do not benefit from alkaline-induced cell wall weakening. In contrast to continuous production systems, for the batch production of second generation bioethanol which utilises non-edible cellulosic wastes and crops (Nigam and Singh, 2011), the process should be appropriate. Furthermore, with regard to pollution concerns, the organic acids produced by alkaliphilic *Bacillus* spp. inocula during fermentation could aid the neutralisation of alkaline waste effluent. Furthermore, combined pre-treatment approaches e.g. combination of *Chinodontium taxodii* and dilute H_2SO_4, are promising techniques to

improve the efficiency of cell wall hydrolytic degradation and shorten the process duration (Mood, Golfeshan, Tabatabaei, Jouzani, Najafi, Gholami and Ardjmand, 2013).

5.4.1 Future research work

There is need to more specifically characterise how the different cell wall polymers are altered in the cassava cell walls by dilute NaOH steeping. Currently, plant cell wall architecture is understood at a superficial level and recent research advances have even found some earlier preconceptions to be incorrect (Braybrook and Jönsson, 2016). This is even more so with regard to specific plant cells and tissues, such as cassava root cell walls. With regard to establishing the exact polymer modifications responsible for mechanical weakening of cell walls, research should focus on the decrease in the adhesiveness between the different cell wall polymers. Atomic force microscopy (AFM), a technique capable of pico-newton force resolution, has been used to quantify the magnitude of interactive forces between cell wall biomolecules (Willemsen, Snel, Cambi, Greve, De Grooth and Figdor, 2000). In combination with fluorescent probe labeling which involves the use of small molecules with specific binding affinity for particular cell wall polymers (Altartouri and Geitmann, 2015), the approach could aid the understanding of how modifications to specific cell wall polymers weakens the cassava cell walls. The information obtained from these studies could be useful in optimising NaOH weakening of the cassava cell walls.

In addition, analytical methods that involve minimal sample preparation e.g. 2D and 3D magic-angle-spinning (MAS) solid-state Nuclear Magnetic Resonance (SSNMR) could provide more realistic (*in situ* conditions) information on how physical modifications to specific structures and intermolecular interactions relate to cell wall weakening (Dick-Pérez et al., 2011). In the present study, chemical solubilisation followed by GC analysis was used to quantify the cell wall constituents. However, this approach is limited by the partial and non-specific nature of bond cleavage involved, and the destruction of subtle physical interactions within the cell wall (Dick-Pérez et al., 2011).

With regard to studies on the presumptive alkaliphilic endogenous microflora, the approach of Kazeem, Shah and Baharuddin (2017) could be used, which involves single colony purification followed by DNA extraction and molecular phylogenetic analysis. This would enable the identification of the microbial spp. in the presumptive alkaliphilic endogenous

microflora and their possible role in cassava root cell wall hydrolysis. Also a detailed understanding about the possible interactions between the *Bacillu*s spp. and the endogenous microflora during non-sterile cassava fermentation is required. This information is important in achieving better control of the fermentation process which in turn could aid further improvement to the effectiveness of the technique. The research could be conducted using the BIOLOG microbial identification system. The BIOLOG system is able to distinguish closely related bacterial spp., aid the understanding of their metabolic differences, and assist in the modelling of their metabolic behaviour (Shea, Wolcott, Daefler and Rozak, 2012).

There is also need for more understanding about the differences between the extracellular *Bacillus* spp. enzymes in weakening of the cassava root cell walls. This will require establishing the complete profile of cell wall degrading enzymic activities present in the different crude enzyme extracts. The research should focus on isolation, purification and characterisation of the different *Bacillus* spp. and endogenous microflora crude enzymes. It is probable that a wide range of cellulolytic and hemicelluloytic enzymes with differing bond cleavage activity are present in the crude preparation. But it is also possible that not all enzymatic activities present are directly involved in weakening of the cassava cell walls. Furthermore, considering that in a multi-enzyme system not all enzyme activities have the same degree of substrate potency (Reyes-Sosa, Morales, Gómez, Crespo, Zamorano, Rocha-Martín, Molina-Heredia and García, 2017), the potency of the different enzymic activities on cellulose, hemicelluloses and pectin hydrolysis should be determined. The information would aid in the optimisation of fermentation conditions to promote specific enzyme production. The approach could further improve the effectiveness of alkaliphilic *Bacillus* spp. fermentation in cassava cell wall disaggregation.

The effects of combining dilute NaOH steeping and alkaliphilic *Bacillus* spp. fermentation on disaggregation of cassava root cell walls could also be studied with the aim of further improving root starch extraction.

6 CONCLUSIONS

Steeping of ground cassava in dilute NaOH solubilises the parenchyma cell wall pectin, and in combination with fine milling affects the xyloglucan polymers. The concomitant loss in cell wall structural integrity, especially with fine milling, enables NaOH to access the embedded cellulose microfibril structures and causes a slight reduction in cellulose crystallinity. Together with solubilisation of pectin and xyloglucan polymers, the loss in cellulose crystallinity results in localised structural collapse, manifested as formation of micropores in the cassava cell walls. It appears that these cell wall micropores in turn weaken the cell walls, enabling increased disaggregation of the starch-containing parenchyma cells with wet milling. Thus, the effect of NaOH steeping in weakening cassava root cell walls appears to primarily involve the solubilisation of the alkaline soluble fraction of cell wall pectin. The exact mechanism probably involves β-elimination depolymerisation reactions in fragmenting the cell wall pectin network. Thus, the solubilisation of pectin by dilute NaOH steeping can facilitate greater disintegration of cassava parenchyma cell walls in the development of more efficient starch extraction processes for the roots.

Microbial fermentation of dilute sodium carbonate wetted ground cassava roots using *B. akibai* + endogenous microflora (EM), *B. cellulosilyticus* + EM, *B. hemicellulosilyticus* + EM and *B.* spp. in combination + EM hydrolyse the pectin, xyloglucan and cellulose fractions of the cassava root cell wall. The concomitant loss in cell wall structural integrity probably enables *Bacillus* spp. cellulases to access and hydrolyse the embedded amorphous cell wall constituents, causing an increase in cellulose relative crystallinity. It is proposed that together with hydrolysis of cross-linking rhamnogalacturonan I side branches and xyloglucan, partial hydrolysis of the amorphous cellulose microfibril fraction results in localised structural perforations, manifested as cell wall micropores. Peripheral hydrolysis of the cassava root cell wall cellulose microfibril structures enables wet milling to expose the embedded fibrillar cellulose network. Cumulatively, these modifications weaken the cell walls, enabling increased disaggregation of the starch-containing parenchyma cells by wet milling. Thus, fermentation of cassava using alkaliphilic *Bacillus* spp. starter cultures has the potential for application in industrial batch type processing where breakup of the parenchyma cell walls is required to optimise starch hydrolysis, e.g. brewing and bioethanol production.

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8 PUBLICATIONS, PRESENTATIONS AND POSTERS BASED ON THIS RESEARCH

Odoch, M., Buys, E.M. and Taylor, J.R.N., 2017. Mechanism of cassava tuber cell wall weakening by dilute sodium hydroxide steeping. *Food Chemistry*, 228, 338-347.