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IMPACT OF BIOPROCESSING ON THE PROPERTIES OF SORGHUM AND COWPEA FLOURS

NUTRIFOODS Leap-Agri Project

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Abstract

The aim of this study was to assess the impact of enzymatic treatment and fermentation on the functional properties of Sorghum and Cowpea flours intended to be used in baking applications. Both crops are known as African Climate-Smart Food Crops which are not utilised to their fullest potential due to inaccessibility of the part of the nutrients and unsatisfactory sensory evaluation. Thesis is a part of NUTRIFOODS Project the aim of which is to increase demand in local sustainable crops. The aim of sorghum treatment was to solubilise insoluble dietary fibre, which contributes to the poor taste of sorghum-based products, and to hydrolyse insoluble proteins, which limit the nutritional value of the flour. The aim of cowpea treatment was to increase the protein solubility.

For sorghum, cell wall degrading enzymes were used to solubilise dietary fibre. The results were promising, Viscozyme L (1%, 4h, 50 °C) solubilised 23% more dietary fibre compared to the control sample. The effectiveness of enzymes was increased with longer incubation time. Proteases had minor effect on the protein hydrolysis, the highest increase was from 6.3% (control, 4h, 50 °C) to 9.3% (FlavourSEB, 0.5%, 4h, 50 °C). At pH 4 and 5 protein solubility decreased. Combination of proteases, cell wall degrading enzymes, phytase and amylase did not have a significant difference in protein solubility. The proteases treatment of cowpea increased protein solubility from 29% (control) to 48%, (FlavourSEB, 0.5%, 4h, 50 °C). The combination of protease with cell wall degrading enzyme, phytase, amylase did not improve protein solubility compared to FlavourSEB alone. However, combination protease+phytase (FlavourSEB 0.5%+1% UltraBio, 4h, 50 °C) increased protein hydrolysis to 55.4% when measured at pH4. Protein solubility of control and sample treated only with protease was lower at pH 4 and 5 than at native pH (~ 6.2). Fermentation of both flours with lactic acid bacteria did not have a significant effect on protein solubility.

Selected enzymes will be used with fermentation trials and further tested in bread baking. Thesis results can assist in decision making in future development of sorghum and cowpea functional and nutritional characteristics. Improvement of nutritional and functional characteristics of sorghum and cowpea flours can increase the acceptability of bakery goods, thereby decreasing demand in wheat flour.

Keywords

Sorghum, cowpea, enzymatic treatment, protein solubility, dietary fibre.

CONTENTS

APPENDICES

Appendix 1. Results of protein solubility and reducing sugars analyses

NOMENCLATURE

AACC American Association of Cereal Chemists

AA amino acids

CSA climate-smart agriculture

CSFC climate smart food crops

CWD cell wall degrading

DC dry content

DH degree of hydrolysis

DM dry matter

DF dietary fibre

LAB lactic acid bacteria

PS protein solubility

RS reducing sugars

SSA Sub Saharan Africa

1 INTRODUCTION

Climate-smart agriculture (CSA) is defined as an agricultural system which aims to maintain fertility of the soil, conserve the environment while supporting food production on the economically feasible level. Crops cultivated within this system are called climate-smart crops. Factors which affect the success rate of climate-smart agriculture system include crop rotation frequency, crop's resilience and adaptability, correctly adapted management system.

Application of CSA is extremely important in the regions where the increase of food production volumes is a necessary outcome. However, not all climate-smart crops can provide sufficient level of nutritional value due to their chemical characteristics. Therefore, these crops are modified or processed after harvesting in order to liberate unavailable essential nutrients and to meet the desired sensory properties.

The thesis work is part of Leap-Agri NUTRIFOODS project, the aim of which is to increase the use of underutilised African Climate Smart Food Crops (CSFC). NUTRIFOODS project is funded by LEAP-Agri, Europe-Africa Research and creation Innovation (R&I) Partnership related to Food and Nutrition Security and Sustainable Agriculture (FNSSA) (LEAP-Agri no date). Crops species which are being studied in the project are *Sorghum Bicolor (L.) Moench* and *Cowpea (Vigna Unguiculata (L.) Walp.])* cultivar *Bechuana white*. These crops are native to Sub-Saharan Africa (SSA) region. Being an excellent source of nutrition, both crops are not utilised at fullest potential due to inaccessibility of part of the nutrients, presence of antinutrients and especially the unsatisfactory sensory evaluation.

The aim of the thesis is to assess the impact of bioprocessing on the solubilisation of dietary fibre and proteins from sorghum and cowpea flours. The solubilisation of these compounds is related to the improvement of the nutritional and functional properties of sorghum and cowpea flours. The outcomes of the thesis are intended to be used in sorghum and cowpea baking applications.

Bioprocessing techniques used for this study include enzymatic treatments and fermentation.

Research was held under the supervision of VTT Technical Research Centre of Finland Ltd, Department of Industrial Biotechnology and Food Solutions. In parallel with the experimental part described in the thesis, additional research was conducted by the commissioner, the outcomes of which are used for the continuous research.

1.1 NUTRIFOODS project background

The goal of NUTRIFOODS project is to increase the use of underutilised African CSFC in baked goods. Preconditions for the project initiation are malnutrition in SSA region, growing demand in wheat production in SSA and population suffering from celiac disease.

Food insecurity in the Sub Saharan Africa region

According to the statistics, 239 million people out of 925 million of the worldwide population suffering from the undernourishment are citizens of Sub-Saharan Africa region (Schönfeldt and Hall, 2012; Fanzo, 2012). Malnutrition is the main reason to deaths among children under 5 years. The main reasons of malnourishment in SSA are low energy intake, protein deficiency and its low quality. (Schönfeldt and Hall, 2012.)

Growing demand in wheat production in baking industry

Wheat-based baked products are gaining popularity amongst citizens of more urbanized areas of SSA. According to Tadesse et al. (2018) around 17.5 million tons of wheat consumed in SSA is imported, which corresponds to 70% of the total wheat demand. Wheat is not native to SSA region and its importation from other countries leaves local crops unutilised. Wheat is not climate adaptive crop, meaning that its growth depends heavily on the weather conditions. Wheat

production will decrease on 6% on every Celsius degree increase. Taking into consideration that Africa already suffers from worsened weather conditions, there is an increased chance of food insecurity if farmers repurpose their lands to wheat production. (Asseng et al. 2015.)

Celiac disease population worldwide

Celiac disease is an immune disease which is characterised by damaging the small intestine as a response to gluten consumption (MedlinePlus, no date). Crops which are studied in this research are gluten-free which makes them a promising alternative to products available on the market which are mostly high in starch, fats and low in dietary fibre and proteins.

2 LITERATURE SURVEY

Sorghum (cereal) and cowpea (legume) are sustainable crops native to SSA region. Both sorghum and cowpea are adapted to harsh weather conditions, which makes them a reliable food, feed and revenue source supporting local economy. (FAO, 2007; Jayathilake, 2018.) Increasing the demand of these crops will improve the environmental performance of agricultural sector of SSA.

2.1 Sorghum

Sorghum is the genus of flowering plants of Poaceae family which grow in semi-arid tropic regions on all six continents (Figure 1). They are widely used as fodder for livestock, human nutrition and bioenergy source. Production rate in the world is around 59 million metric tons and exceeded only by wheat, rice, barley and maze production. (FAOSTAT no date.) It is a part of staple diet for about 750 million people in Africa, Asia and Latin America (FAO 2007).

Figure 1. Sorghum plant *(ISAAA.org*, *2019*)

Sorghum species which is commonly used for human or livestock nutrition purposes is called *Sorghum Bicolor (L.) Moench*, also referred as sweet sorghum, great millet, guinea corn, kafir corn, dura, mtama, jowar or kaoliang depending on the region (Queroz 1991).

Grain sorghum is a gluten-free cereal rich in nutrients which is consumed in a form of baked bread, porridge, couscous, etc (Kulamarva et al. 2009). The main health-promoting advantages of sorghum include high fibre content (contributes to gastrointestinal health), slowly digestible starch (low glycemic index), antioxidant activity (contributes to the pathogenesis of chronic diseases), unsaturated fatty acids in lipid fraction (prevent dyslipidemia), vitamins and minerals (Stefoska-Needham et al. 2015). Over the past few decades, sorghum gained attention among food scientists due to its nutritional properties and cheaper production costs (Lemlioglu-Austin et al. 2012, cited in Teixeira et al. 2016).

2.1.1 Role in CSA

With the global warming Africa will suffer from rainfall shortage and longer heat waves. These factors will impact sorghum yields. However, negative effect will be cushioned by increased CO_2 level, which allows sorghum to use water more proficiently (University of Queensland 2015.). Sorghum is considered to be a

drought resistant crop due to extensive branched root system spreading deep into soil, hence providing the access to hidden water reservoirs. The leaves and stems of sorghum are covered by waxy bloom which prevents the absorption of water but drains it to the roots increasing water content in the soil. Wax also prevents insects from damaging the plant. (Ayyangar et al. 1937.)

Sorghum Bicolor is mostly grown in harsh conditions by small-scale farmers who have limited access to pesticides, high quality soil, improved quality seeds and water (FAO 2007). In comparison with maize, which has similar physicochemical properties, sorghum delivers more yields and absorbs water, light and soil nitrogen more efficiently in normal and stressed conditions. (Danalatos & Archontoulis, 2009). Sorghum allows agriculturists to earn more revenue by planting less seeds. These advantages are favourable for small scale farmers, whose economic stability strongly depends on the yields (CCAFS 2015).

2.1.2 Sorghum chemical composition and structure

Sorghum cultivars are more diverse in chemical compositions than other crops due to sorghum's capability to grow under different climate conditions which results in different combinations of characteristics (Rooney 1996). The nutrient composition of sorghum is presented in table 1. Sorghum bicolor nutrient parameters are similar to the other cereals, like wheat or corn. Despite various beneficial properties, however, the human consumption rate of sorghum lag significantly behind wheat or maize corn.

Table 1 Sorghum, wheat, corn nutrient composition per 100 g of DM. (Nutrition facts for Sorghum grain, no date; USDA, 2019)

Proximates	Sorghum	Wheat	Corn
Energy (kcal/kJ)	329/1377	339/1418	265/1527
Protein (g)	10.62	13.68	9.42
Total lipid (fat) (g)	3.46	2.47	4.74
Carbohydrate (g)	72.9	71.13	74.26
Fibre (g)	6.7	10.7	7.3

Kernel structure

The main components of sorghum kernel are endosperm (storage tissue), pericarp (outer covering), testa (interlayer between pericarp and endosperm) and germ. Figure 2 illustrates the structure and components of sorghum kernel.

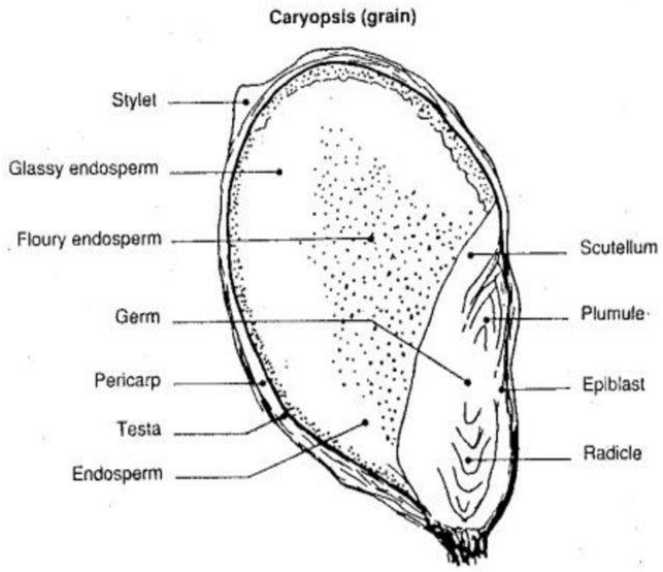

Figure 2. The structure of sorghum kernel (FAO 2007).

Endosperm stores energy in a form of starch, and proteins for a germ growth. It is low in ash, mineral content and oil, but contributes to 80% of kernel protein, and 94% of starch. (FAO 1995 cited in Kulamarva et al. 2009).

Testa layer is responsible for the protection of germ from outer environmental stressors. It also controls the biological and physical factors in order to support the germination stages (Debeaujon et al 2000). Sorghum testa contains tannins and condensed tannins, as well as polyphenolic compounds. (FAO 2007; Stefoska-Needham et al. 2015.)

Pericarp is the outer envelope of kernel which consist of three layers called epicarp, mesocarp and endocarp. The pericarp layer of sorghum grain is relatively low in protein, but rich in dietary fibre. (FAO. no date b; Guindo et al. 2016).

Germ's two most important parts are embryonic axis and scutellum. Scutellum absorbs nutrients located in endosperm needed for germination. It is rich in lipids, proteins, enzymes and minerals. Around 68% percent of minerals and 75% of oil in sorghum kernel are located in the germ. (Hubbard et al. 1950 cited in FAO, no date a).

2.1.3 Starch

Starch is a polysaccharide composed from glucose monomers bound by α 1→4 linkages. There are two types of starch constitutes:

- amylopectin, water soluble branched polymer consisting of linear chain of glucose units bond by α-1, 4 linkages and branches of glucose units bond by α-1, 6 linked side chains.
- amylose, water insoluble straight-chain polymer consisting of glucose units bond by α-1, 4 glycosidic bonds.

(Mehta and Satyanarayana, 2016.)

As the most of the cereal grains, sorghum is high in starch. Its content in dried sorghum mass is equal to 71%. Depending on the variety and growing environment, 70-80% of sorghum starch consist of amylopectin, on 20-30% of amylose in non-waxy species and much lower in waxy species (Deatherage et al. 1955 cited in Fao.org, 2019). The proportion of amylopectin to amylose also impacts the rheological properties of sorghum flour. (Morais Cardoso et al. 2017.)

The digestibility of sorghum starch is significantly low (33-48%) in comparison with similar grains, like corn (53-58%) (Sikabbubba 1989 cited in Patil, no date). The reason could be due to the fact that sorghum starch is stored in granules bound by complex alkalisoluble glutelin and alcohol-soluble prolamin protein matrix in the endosperm, which significantly decreases starch digestibility (Ezeogu et al. May 2008).

Resistant starch

The prevailing part of starch is digested in the small intestine, the part of gastrointestinal tract which digests most of the nutrients derived from food. Starch which cannot be digested by enzymes in upper digestive tract, is called resistant starch. Resistant starch is hydrolysed in larger bowel by intestinal enzymes, which makes its effect similar to dietary fibre. (Yue & Waring 1998 cited in Teixeira et al. 2016.) Resistant starch is beneficial in obesity combat due to its indigestibility and ability to affect the secretion of gut peptides responsible for satiety. (Higgins 2014). The colonic fermentation of the resistant starch releases short chain fatty acids which contribute to the prevention of colorectal cancer (Key et al. 2014).

Among urbanised population, which has an unlimited access to food, the indigestibility of starch is desirable as diets in these areas are excessive in energy. Among food in-secure population with low energy diet, the retention of indigestible starch is considered as a negative impact. Therefore, the digestibility rate of resistant starch can be differently interpreted depending on the region.

Resistant starch present in sorghum have a positive effect on the intestinal microbiota and the synthesis of leptin, protein hormone responsible for satiety signalling, and adiponectin, protein regulating glucose level (Shen et al. 2015 cited in Teixeira et al. 2016). The percentage of resistant starch to dry matter of sorghum ranges from 0.31% to 65.66% depending on the sorghum genotype (Teixeira et al. 2016).

2.1.4 Amino acid profile

The nutritional value of food directly depends on the protein composition and their digestibility. Protein macromolecules consist of amino acids (AAs) which are divided into three functional groups: essential, nonessential and conditional essential (table 2). Human body cannot produce essential amino acids by itself, therefore, they need to come from food. Nonessential AAs are produced by organism even if human consumes these amino acids with food. Third type of

AAs, conditional essential, are not essential unless body experiences stress or illness. (Stump 2015.)

Table 2. 22 amino acids participating in protein building (Li et al. 2009).

Essential AA	Nonessential AA	Conditionally essential AA
Arginine	Alanine	Cysteine
Histidine	Asparagine	Glutamine
Isoleucine	Aspartate	Hydroxyproline
Leucine	Glutamate	Proline
Lysine	Glycine	Taurine
Methionine	Serine	
Phenylalanine	Tyrosine	
Threonine		
Tryptophan		
Valine		

Limiting amino acids

Food can contain broad variation of the amino acids, however, some AAs can limit the digestibility of the rest of AAs depending on their amount. Such kind of amino acids are called limiting amino acids. (Kansas State University 2019.) Amount of the limiting AAs indicates the nutritional quality of food. In cereals, main limiting AA is lysine, in legumes most common limiting AAs are methionine, cysteine and tryptophan. (FAO no date b).

Sorghum is low in lysine and methionine, but richer in tryptophan comparing with other cereals (table 3). Lysine content varies from 71 to 212 mg/g of nitrogen. (Belay 2018). Low amount of mentioned limiting amino acids decrease the nutritional value of sorghum.

Table 3. Essential amino acid composition (mg/g) of sorghum proteins. (FAO no date b)

Grain	Isoleucine	Leucine	Lysine	Methionine	Cystine	Phenylalanine	Tyrosine	Threonine	Tryptophan	Valine
Sorghum	245	832	126	87	94	306	167	189	63	313

2.1.5 Storage proteins

Plants store nitrogen and carbon used for germination in storage proteins. They are divided on 4 groups depending on the solubility properties: albumin (water soluble), globulin (soluble in diluted salt solution), prolamin (soluble in alcohol) and glutelin (extractable in dilute alkali or acid solutions). (FAO, 1995.)

Proteins found in the sorghum grain are 15 - 18% albumin globulin, 23 - 43% prolamin and 36 - 44% glutelin (Vidyapeeth et al. 2018 cited in FAO no date c). The AAs compositions of globulin and albumin fraction are comparatively high in lysine and tryptophan, however, they contribute only to 15% of the proteins. Prolamin present in sorghum grain is known as kafirin. (FAO no date b). Kafirins contain high percentage of the conditionally essential AAs proline and glutamine, but relatively low amount of the essential AAs such as arginine, lysine and histidine (Britannica 1998).

Protein digestibility

In vivo digestibility level of sorghum proteins is low (45-46%) comparing to other cereals (66-81%) due to the unique complex structure of proteins in endosperm (Wall 1971 cited in Rom 1992). Proteins which are located inside of organelles are called protein bodies. Protein bodies are captured in protein matrix, forming a complex structure. Most of protein bodies in the sorghum are prolamins which are encapsulated by glutelins linked by strong disulphide bonds. (Ratnavathi and Komala 2016; Rooney 1996).

Kafirin digestibility

The prolamins of sorghum were reported to be the main factor affecting sorghum protein digestibility (FAO no date b). Kafirin is the most hydrophobic prolamin variety which significantly contributes to low sorghum protein digestibility (Taylor and Taylor 2018). Prolamin is hardly digested due to strong disulphide bonds in monomeric proteins (intrachain bond) and both intrachain and interchain bonds in

polymeric groups (figure 3). Disulphide bonds allow compact packing of proteins, decreasing the exposure of proteins to enzymes. (Kanerva 2011.)

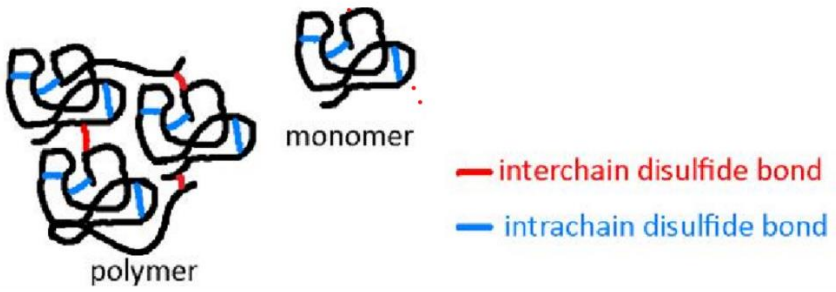

Figure 3. Difference between interchain and intrachain disulphide bonds (Kanerva 2011).

Kafirin digestibility lowers with the heat, as new disulphide bonds are created under higher temperature. New bonds change the structure of protein groups, intrachain bonds become interchain which leads to larger protein agglomerates with low digestibility. (Schofield et al. 1983 cited in Kanerva 2011).

2.1.6 Dietary fibre

Dietary fibre (DF) is a group of carbohydrates which are not hydrolyzed by the endogenous enzymes in the small intestine being fermented in the large intestine. The consumption of DF provides many health benefits. Numerous researches proved that it reduces the risk of heart diseases, hypertension, obesity, diabetes and several gastrointestinal disorders. (Anderson et al. 2009.) Most of the DF is stored in pericarp and endosperm cell walls in three different forms: soluble, insoluble fibre and resistant starch. Soluble DF includes pentosans, gums, pectins and mucilage, whereas insoluble consist of the cell wall components such as cellulases, hemicellulases and lignin. (Chawla and Patil 2010.)

Insoluble DF causes unfavourable bitter flavour which decrease the acceptability of some foodstuff (Aravind et al. 2012). Therefore, there is a great interest in various modification techniques to transform insoluble DF to soluble DF. These

techniques include enzymatic and chemical treatment, mechanical processing, fermentation, etc. (Yang et al. 2017).

Cell wall

The dietary fibre consist of plant cell walls (figure 4). Cell wall is a complex matrix composed from polysaccharides responsible for plant's nutrient circulation, protection from various pathogens, shape formation and rigidity. (Jayasekara and Ratnayake 2019.)

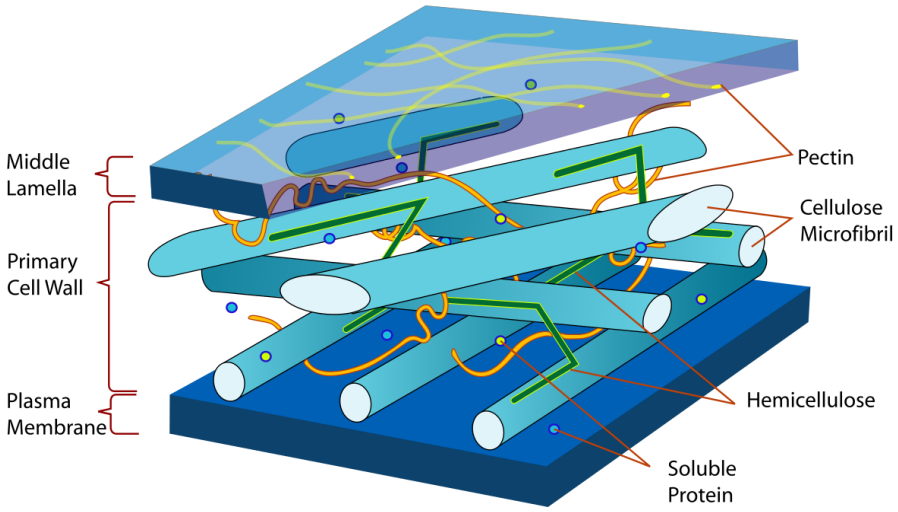

Figure 4. Cell wall structure and composition (Wikipedia no date).

Cell wall consists of two or three layers:

- primary cell wall, composed from cellulose, pectin and hemicellulose. It is responsible for plasticity and strength during cells' growth (Biology Online Dictionary no date).
- middle lamella, outer layer connecting cell to the neighbouring cells. Rich in pectin (Britannica no date a).
- often, cells have additional layer, called secondary wall, thick, waterproof layer formed inside primary wall to harden the cell wall when it is fully grown. It consist of 35 - 50% cellulose, 20 - 25% xylan, 10 - 25% lignin. (Biology Online Dictionary no date).

Cellulose

Cellulose is the most prevalent plant polysaccharide. It is a linear unbranched polymer composed from D-glucose subunits connected together with β-1,4-

glycosidic linkages (Jayasekara and Ratnayake 2019). Glucose molecules are connected through hydroxyl groups which form strong hydrogen bonds. These bonds are the main reason for poor cellulose solubility. (Lakna 2018.)

Hemicellulose

Hemicellulose is a short branched polymer consisting from sugar units, such as xylose, rhamnose, galactose, arabinose and mannose (Lakna 2018). Wide group of hemicelluloses can be categorised by the most common sugar residue in the backbone into xyloglucans, xylans, glucomannans or linkage β-glucans (Hayes, Mylotte and Swift 2017). Xylan is the commonest plant polysaccharide after cellulose (Hsieh and Harris 2019). In annual farm crops majority of the hemicellulases are (1 → 4)-β-D-xylopyranosyl units with side chains of α-L-arabinofuranosyl, D-galactopyranosyl or 4-O-methyl-β-D-glucuronopyranosyl (Whistler 2012).

Dietary fibre in sorghum

Sorghum is rich in dietary fibre, its content in sorghum is around 6-15% (Stefoska-Needham et al. 2015; Vila-Real et al. 2017). Around 75-90% of the DF in sorghum grain content is insoluble which is known to improve intestinal microbiota, bowel function and constipation symptoms (Ajiboye et al. 2014). Prevailing part of sorghum DF is hemicellulose, followed by cellulose and lignin. Depending on the cultivar, concentrations might differ, especially the one of the lignin fraction. According to the several studies, hemicellulose concentration is 5.3 - 14.7 g/100g , cellulose 4.33-4.37 g/100g, lignin 0.8-4.33g/100g (Wang et al. 2013; Holmes et al. 2013)

In cereals, arabinoxylans, the type of hemicellulose, are a major source of insoluble dietary fibre. Arabinoxylans are composed from pentose sugars, xylose and arabinose. (Cui, Wu and Ding 2013). In sorghum, it contributes to 1.8% - 4.6% of sorghum grain weight with the arabinose/xylan ratio 0.9 (Hashimoto 1987 as cited in Parameswaran et al. 2019). Sorghum arabinoxylans are formed

from (1→ 4)- β -D-xylan backbone, substituted by arabinose and uronic acid (Verbruggen 1996). In cereals, arabinoxylans are strongly bound to the cell walls, which makes them harder to solubilise (Bader et al. 2019).

2.1.7 Anti-nutrients

Two main anti-nutrients present in sorghum are polyphenolic compounds and tannins (Hariprasanna et al. 2015). Phenolic compounds greatly affect the protein digestibility. In sorghum, phenolic compounds are present in a form of flavonoids and tannins (Hahn, Rooney and Earp 1984). Phenolic compounds slow down the effect of carbohydrate-hydrolyzing enzymes (Axtell et al. 1981; Barros et al. 2012).

Tannin is able to bind with proteins forming insoluble complexes using hydrogen bonding and non-polar hydrophobic associations. In high tannin sorghum variety tannin content equal to 2 - 4%, which is able bind with most of the protein content (around 10%). Protein electrophoresis test conducted by Butler et al (1984) showed that the insoluble residue in high tannin variety mostly consist of kafirins.

Phytic acid and phytates

Plants need phosphorous for the germination and growth. Seeds contain phosphorous in a form of phytic acid or phytates. Phytic acid is a myo-inositol hexakisphosphate, in which phosphorous is stored in 6 phosphorous groups bound to inositol, vitamin with 6-carbon ring structure. Phytates are formed when phytic acid interacts with minerals. (Composition of Commercial Flour, 2016.) Phytic acid is present both in cereal and legume grains. However, human organism cannot digest phytic acid and often considered to be an “anti-nutrient” due to its bonding properties. During digestion, phytic acid acts as chelating agent and forms insoluble compounds with several health-beneficial minerals such as iron, zinc, calcium, magnesium. Phytic acids can interact with proteins forming insoluble complexes, thereby decreasing the digestibility of food. (Urbano et al. 2000). Besides, it can bind to proteases and amylases. In sorghum grain, the concentration of phytic acid varies from 2.40 mg/g to 6.7 mg/g. (Chitra et al.

1996). Its amount can be significantly decreased by fermentation. (Ratnavathi 2018) Cooking can decrease phytic acid content on ~ 60% (Arvanitoyannis and Stratakos 2010).

2.1.8 Studies conducted on sorghum flour

Several studies conducted to assess the digestibility of sorghum show that cooking significantly decreases the solubility of sorghum proteins. (Axtell 1981; Rom 1992). Axtell (1981) conducted test using pepsin as digesting agent to evaluate the digestibility of sorghum proteins before and after cooking. Sorghum was ground, mixed with pepsin solution and incubated for 2 hours at 37°C. The digestibility of proteins in uncooked kernels was ~ 50% higher in comparison with cooked kernels. Authors assumed that the majority of pepsin digested proteins were kafirins.

Another study was conducted by Rom (1992). Uncooked sorghum flour was either used “as is” or soaked for 12h at 4°C in reducing agent ($NaHSO_3$). To cook the flour, it was mixed with water and sodium bisulphite solution and placed in boiling water for 20 minutes. After, both uncooked and cooked flours were incubated in solution prepared from phosphate buffer and pepsin. The highest digestibility rate, 96%, was observed in uncooked, soaked sorghum flour incubated for 2 hours. Soaking flour in reducing agent clearly increased digestibility in both cooked and uncooked flour. Author suggested, that it could be explained by the fact that sodium bisulphate is capable of breaking down the disulphide bonds which form protein matrix in sorghum, thereby exposing protein bodies to enzymes. Similar study was conducted by Hamaker et al. (1987) who analysed the influence of another reducing agent, 2-mercaptoethanol, on the in vitro digestibility of the sorghum flour. They reported that, if soaked in 2-mercaptoethanol for 12 hours at 4°C, the digestibility of cooked flour increases on 25.1% and on 11.1% if the flour is uncooked. This was explained by the same fact as on previous research.

Sensory evaluation of bread prepared with sorghum flour

The acceptance rate of bread prepared from 50% of sorghum flour mixed with wheat flour and gluten was evaluated by Carson et al. (2000). The bread gained score 6.9 out of 9 by the Hedonic scale, indicating that most of the testers were satisfied with the taste. The conclusions of this research are in line with the findings of another study where the different ratio of sorghum and wheat flours were used for bread baking. Authors reported, that the increase of sorghum content decreases the acceptance score, where 100% is a fully acceptable bread prepared only from wheat flour. Breads prepared only from sorghum flour, were accepted on 45% and were described as too sticky and chewy. Samples prepared from 40% sorghum and 60% wheat received 73.2% score. (Keregero and Mtebe, 1994)

2.2 Cowpea

Cowpea, *Vigna unguiculata,* also known as field pea, crowder pea, southern pea, black-eyed pea is an annual legume which belongs to the family of flowering plants, *Fabacae* (Britannica no date b). Cowpea is native to the semi-arid tropic regions of SSA and several regions of Americas (Abberton 2018). It is grown for human consumption, animal fodder and cover crop for soil composition replenishment. (Britannica no date b) In 2017, Africa harvested 7.1 million tons of cowpea, which contributes to 96% of global production. 48% of African yields were harvested in Nigeria. Most of the legume yields are not exported or imported. (IITA no date.)

Cowpea is called “the meat of the poor” thanks to its rich nutritional properties. Cowpea is a versatile source of human nutrition (figure 5). The leaves of the legume are often consumed as spinach. While grains are not fully grown, they are used as common green beans and when fully grown, seeds can be boiled or canned. (Jayathilake et al. 2018a.)

Figure 5. Cowpea plant and separated seeds (*Queen Anne Blackeye Pea Southern Pea (Cowpea)* no date)

2.2.1 Role in CSA

Cowpea is considered to be an African climate smart food crop thanks to the number of properties such as drought and heat resistance, well adaptivity to biotic stresses and capability to recover soil (D.W. Davis et al. 1991). It is one of the most drought resistant food legume in Africa (Dadson et al. 2005). Cowpea is tolerant to poor soil quality. As other legumes, cowpea has high nitrogen fixation rate, it does not use the soil resources of nitrogen and phosphorous, supporting the soil fertility (Quin 1997 cited in Dadson et al. 2005). Being remarkably shade tolerant, it is often intercropped with high crops, such as sorghum, which increases the volume of yields harvested in total (Gómez no date). Moreover, cowpea ripens at the “hunger period” at Africa, when other crops are not delivering yields (Abberton 2018). The legume’s growth greatly dependant on the irrigation. If region suffers from water shortage, cowpea still delivers harvest, but in significantly lower amounts. Nevertheless, cowpea yields are significantly higher in comparison with similar peas such as green peas. While the yields of latter species varies from 1400 to 2200 kg per hectare, cowpea can deliver 2900 - 5000 kg. (Brandenberger et al. no date.)

Dadson et al. (2005) evaluated the impact of water shortages on 10 different cultivars of cowpea and reported that 2 cultivars showed higher production rates in water stressed conditions than in non-water stressed, making it a sustainable source of nutrition for population in dry regions. Ajetomobi and Abiodun (2010) studied the impact of climate change on cowpea production in Nigeria, where it is considered as an essential part of local population's diet. Authors reported that the consequences of climate change varies from region to region. Positive effect can be seen in Adamawa, Bauchi, Jigawa, Kogi states and negative in 5 northern states.

Cowpea cultivation plays an important role in population wealth, being the most economically important legume in Africa (Langyntuo et al. 2003). Aside income gained from grain production, farmers also sell leftover stems and leaves as hay for animal consumption. Cowpea has its weakness in the face of diseases and insects, however, International Institute of Tropical Agriculture (IITA) cultivated several cowpea varieties resistant to the most common weeds and parasites. These cultivars are approved by Nigerian Biosafety Management Agency and already in farmers' use. (IITA no date).

Legumes play an important role in balancing nutrition of African low-income population whose cereal-based diets lead to lysine deficiency (Suri et al. 2014). The deficiency of limiting amino acids in cereals is complemented by legume proteins, which are rich in lysine, leucine and valine (Margier et al. 2018).

2.2.2 Cowpea chemical composition and structure

Cowpea's chemical composition is valued as a source of healthy nutrition thanks to the high content of soluble and insoluble dietary fibre, protein, folate, calcium, potassium, vitamin A and other bioactive nutrients. (Jayathilake et al. 2018 b; Brandenberger et al. no date.) If compared with similar legumes, cowpea contains less fats but more proteins (table 4).

Table 4. Nutritional Facts of Cowpea and Green pea seeds per 100 g (Nutritional Value 2020)

Proximates	Cowpea	Green peas
Energy (kcal)	343	364
Protein (g)	24	23
Total lipid (fat) (g)	2.1	3.9
Carbohydrate (g)	60	62
Fibre (g)	11	22

Kernel structure

The cowpea seed consists of seed coat, two cotyledons, hypocotyl-radicle axis and plummule (figure 6) (Karmas and Harris 1988 cited in Fabbri and Crosby 2016). Proteins in cowpea are distributed along the cotyledon.

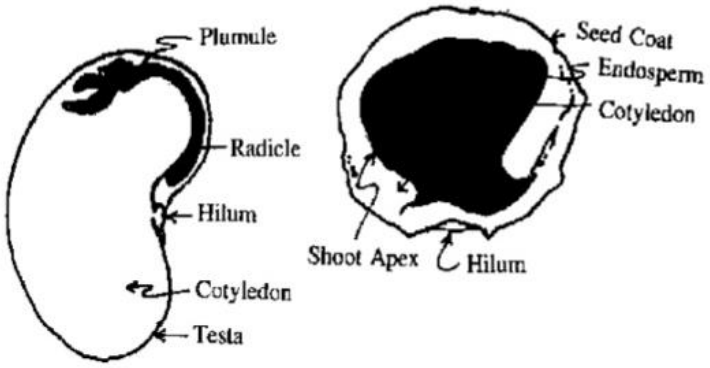

Figure 6. Cowpea Seed Structure (Budhani, 2017)

The shape of cowpea seed can vary on the different stages of germination. When growing, seed has a shape of kidney, but after certain size, the growth is restricted by the pea pod and seed is reshaped to globular shape. The seed can be of black, white, brown, green, red or cream colours. Some cultivars are predominantly white, but with black coloration around the hilum, therefore are referred as “black-eyed” pea (Bradenberger no date).

Starch

The starch content of cowpea is around 38 - 40%, of which 60 to 70% is amylopectin and 20 to 30% is amylose. (Thorne et al. 1983; Ashogbon and

Akintayo 2013). However, the percentage of amylose content could reach even 39 - 43%. The in vitro digestibility of cowpea starches ranges from 40 to 43 % depending on the cultivar (Ratnaningsih et al. 2016). Starch degradation rate is lower than in other legumes, such as soy bean or horse gram (Eashwarage 2017). Lindeboom et al. (2004) mentioned that the physical properties of starch granules can significantly affect their hydrolysis. The large size of granules and their smooth surface decrease the exposure of starch to enzymes, thereby decreasing the starch degradation. Cowpea starch granules are comparatively larger in size (10 - 20 µm) than, for example, granules of soybean (0.7 - 4 µm) (Stevenson et al. 2006; Ashogbon and Akintayo 2013). The surface of cowpea starch granules is smooth which also can contribute to low starch digestibility (Ratnavathi and Komala 2016). Cowpea starch digestibility is increased by cooking, extruding, germinating and dehulling (Rivas-Vega et al. 2009).

Resistant starch

Several studies reported that resistant starch accounts for 4.59 - 12.1% of total cowpea weight (Rengadu et al. 2019). According to the findings of Sasanam et al. (2011) study, cowpea's resistant starch can be considered prebiotic as it stimulated the growth of health promoting bacteria Bifidobacteria and Lactobacillus.

Dietary fibre

Cowpea's DF content ranges from 12 to 35% depending on the cultivar and growth condition (Carvalho et al. 2012; Jayathilake 2018). The amount of insoluble DF is approximately 4.5 times higher than the amount of soluble DF. Cowpea DF is 44% cellulose, 28% hemicellulose, 15% lignin and 13% lignin. (Khan et al. 2007.)

2.2.3 Protein profile

The protein percentage of cowpea is around 23-32% of legume's dry basis. Protein storages present in cowpea are globulins (50-70%), albumins (8.2%-

11.9%), glutelins (14.4–15.6%) and prolamins (2.3-5%). (Jayathilake 2018; Gupta et al. 2010.) Prolamins of cowpea are rich in proline and glutamine amino acids (Jayathilake 2018). The AA's composition of cowpea was reported to contain at least 17 different AAs, majority of which are essential (table 5) (Hussain and Basahy March 1998).

Table 5. Amino acid profile of cowpea, g per 100g (Gupta et al. 2010).

Essential amino acid	Amount in cowpea (g 100 g^{-1} protein)
Histidine	1.85–2.47
Cysteine	0.84–1.08
Methionine	1.28–2.06
Isoleucine	4.17–5.46
Leucine	6.45–8.5
Threonine	3.89–5.12
Lysine	7.3–8.74
Tryptophan	1–1.33

Cysteine, methionine and tryptophan are the most limiting amino acids in cowpea cultivars (Gupta et al. 2010). However, the combination of legumes and cereals can eliminate this problem, i.e diet including both sorghum and cowpea is complete in essential AAs as crops complement limiting acid amount in each other.

Digestibility of cowpea proteins

Aside the numerous nutritional advantages of cowpea, the digestibility of legume proteins are still significantly lower than of animal products. The in vitro digestibility of cowpea proteins ranges between 30 to 40% (Sosulski et al. 1988 cited in Carvalho et al. 2012). Legume proteins are poorly digestible due to the presence of antinutrients such as trypsin inhibitors. The negative effect of trypsin inhibitors is decreased with the increase of temperature. However, heating does not improve the digestibility of proteins as expected. On the other hand, isolated proteins are highly digestible, meaning that the proteins are not initially indigestible. The reason for digestion resistance in cowpea could be in their interaction with other components of seed structure. (Grant et al. 2003.)

The prevailing fraction of insoluble proteins are globulins, which consist of legumins and vicilins (Jayathilake et al. 2018b). The globulin proteins of legumes are less digestible if heated. This could be caused by the formation of larger low digestible globulin polymers which are less susceptible for intestinal enzymes. (Grant et al. 2003.)

Park et al. (2010) characterised albumins found in peas as proteolytic resistant proteins. Besides, they contain protease inhibitor proteins, lipoxygenase, lectins and phytocystatins which have been shown to decrease the protein digestibility of cowpea (Gonçalves et al. 2016). Lectins are proteins which bind to carbohydrate degrading enzymes, thereby decreasing the overall digestibility of legume. It can prevent the nutrient absorption by binding to digestive tract surface's cells. Lectins also can decrease the absorption of minerals such as zinc, phosphorous and calcium. (Harvard T.H. Chan School of Public Health no date.)

Bioprocessing techniques used to improve digestibility of cowpea protein

Segura-Campos et al. (2012) analysed the impact of two commercially available enzymes, Alcalase® (endo-protease) and Flavourzyme® (peptidase) on the solubility of cowpea protein isolates by assessing the degree of hydrolysis (DH), i.e the percentage of protein peptide bonds cleaved during the treatment (pH 8, 50°C) (Merz et al. 2015; University of Reading no date). The DH was significantly higher in cowpea protein treated with Alcalase. The highest DH value, 23.6% was observed after 1 hour treatment with Alcalase. Flavourzyme was not as effective, 1 hour treatment showed only 7.27% DH.

Oliveira et al. (2004) researched the in vitro digestibility of pepsin and trypsin treated globulin and albumin isolates. Authors reported that both fraction were highly digestible if treated with pepsin, but in case with trypsin, the rates of hydrolysis were negligible. These finding , however, differ from those obtained by Araüjo et al. (2002) who researched the impact of pepsin, trypsin, and chymotrypsin on native and heated globulin isolates. According to the results, globulins treated by trypsin were more hydrolysed than by pepsin.

2.2.4 Antinutrients

The antinutrients found in cowpea are phytic acid, trypsin inhibitors, lectins, tannins, hemagglutinins, cyanogenic glucosides, oxalic acid, dihydroxyphenylalanine and saponins. They are considered to be antinutrients because of their ability to bind with proteins and minerals, thereby decreasing their absorption. (Jayathilake et al. 2018.) In cowpea, phytic acid content ranges from 6.86 - 5.11 g/mg (Olivera-Castillo et al. 2007).

Several cooking techniques were proven to decrease the negative effect of antinutrients or their amount in cowpea (Goya 2016). Tannins and trypsin inhibitor activity in cowpea were reduced through the soaking in sodium bicarbonate solution (Vadivel and Pugalenthi 2009). Other treatments such as germination and fermentation were reported to reduce phytic acid content in cowpea. Fermentation reduces its content on 26-39%, while germinated cowpea can decrease the amount of phytates on 60%. Through decreasing the percentage of studied antinutrients, both methods significantly increase in vitro protein digestibility. (Chitra et al. 1996.)

Diouf et al. (2019) reported that sprouting decreased the antinutrients content of cowpea, showing 33-72% decrease in tannins and 96% in phytic acid. Steam precooking also was tested in the study, showing the reduction of phytates on 56%.

2.2.5 Sensory evaluation of bread prepared with cowpea flour

As cowpea is considered to be a health promoting additive to cereal-based foods, there is an interest in its possible use in bread making. Ahmed and Campbell (2012) evaluated the flavour and the overall acceptability of baked wheat breads with the different percentage of added cowpea flour. Bread prepared with the higher percentage of cowpea flour was less acceptable than bread prepared only from the wheat due to the unfavourable bread flavour. These results suggested that cowpea flour would need to be functionalized before being used in bakery products.

2.3 Enzymes

Enzymes are a group of proteins which catalyse biochemical reactions, but are not modified themselves. Substance which is not yet modified by enzyme is called substrate. Substrate binds with an enzyme and altered, releasing the product, the desired outcome of enzymatic treatment (figure 7). Enzymes are able to alter the structure of molecules thanks to the combination of shape and charge properties. Therefore, they are highly specific in their activity. (Robinson 2015.)

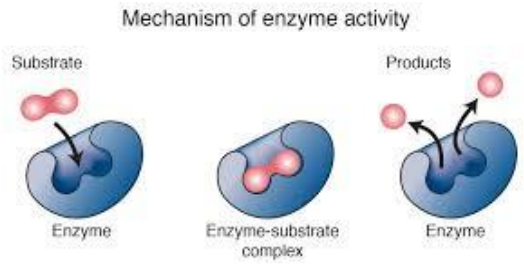

Figure 7. The mechanism of enzymatic reaction (Talking Glossary of Genetic Terms | NHGRI no date).

Enzymes are grouped by substances they are able to modify. In this study, 4 groups of enzymes are discussed: cell wall degrading enzymes (xylanases, cellulases, hemicellulases), proteases, phytases and amylases.

Enzymatic treatment

Enzymatic treatments are used in a wide range of applications, such as animal nutrition, cosmetics, textile development, fuel and energy generation. In food development and production, enzymes allow to improve the nutritional and functional properties of foodstuff without the negative effects such as alternation or deconstruction of amino acids which are associated with various chemical food processing. (Segura-Campos et al. 2012b.) They are extensively used to reduce the amount of antinutrients, improve digestibility, eliminate unpleasant flavours, etc. The most recognisable usage of enzymes in nutrition production are cheese curdling, beer brewing, and bread baking. (Britannica, no date c.)

2.3.1 Cell wall degrading enzymes

Cell wall degrading (CWD) enzymes are secreted by pathogenic fungi and bacteria to degrade wall and access cell's nutrients. In food production, these enzymes are used to transform insoluble DF to soluble DF in order to improve the physiochemical and functional properties of foodstuff. (Have et al. 2002).

Cellulases

Cellulases hydrolyse cellulose by degrading β-1,4-glycosidic linkages. There are three main types of enzymes which are combined together in different proportions to reach the desired activity: exoglucanase, β-glucosidase, endoglucanase. Endoglucanase randomly cleaves linkages of uncrystallised parts of cellulose, forming new chain ends which are later efficiently degraded by other enzymes. Exoglycanase breaks down chain's reducing and non-reducing ends of crystallized areas of cellulose, thereby forming glucose and cellobiose (two connected glucose units). β-glucosidase divides cellobiose from non-reducing ends of chain on glucose units. (Jayasekara and Ratnayake 2019.)

Hemicellulases

Hemicellulases is a broad group of enzymes which catalyse the hydrolysis of hemicellulose components into its monomers (Chadha et al. 2019). The commonest hemicellulases are D-xylanases, D-galactanases, D-mannanases, L-arabinases (Dekker and Richards 1976). The main component of hemicellulases is usually an endoxylanase which cleaves the pyranosyl linkages of xylan forming xylo-oligosachharides (Meena *et al.*, 2017).

2.3.2 Proteases

Proteases, also called a "peptidases" is a class of enzymes which catalyse the hydrolysis of proteins into oligopeptides, polypeptides and amino acids. The enzymes attack the peptide bonds of protein molecule chain which connect protein components. (Britannica 1988.) Proteases are used for food, textile

leather, detergent production as well as in waste recycling, water treatment, etc (Razzaq *et al.* 12 June 2019). Proteases can be categorised according to the chain sites which enzyme attacks. Enzymes which cleave terminal ends of chain are called exopeptidases, those which hydrolyse bonds within the protein are called endopeptidases. (Britannica, no date d.)

Proteolytic enzymes are classified into six groups depending on the residue they contain on the active site:

- serine peptidases, contains serine residue
- aspartic acid peptidases, contains aspartate carboxylic acid
- cysteine acid peptidases, contains cysteine thiol
- metallopeptidases, contains metal, in most of the cases, zinc
- threonine peptidases, contains threonine secondary alcohol
- glutamic acid peptidases, contains glutamate carboxylic acid

(Sino Biological no date.)

Proteases act differently than the rest of enzymes. Instead of focusing on the specific protein type, they focus the carboxylic group of the residue which forms the peptide bond. (Garcia-Carreon 1997.)

2.3.3 Phytases

Phytases is a group of enzymes which hydrolyse phytic acid or phytates to form free phosphorous available for animal and human consumption, therefore, widely used to increase the nutritional value of food and feed. In feed production, phytases decrease the demand in supplementary phosphorous and lower the phosphorous concentration in the cattle's manure, thereby decreasing farm's negative environmental impact on soil and water. (Guerrand 2018.)

Phytase assist protein solubility by releasing proteins from protein-phytate complexes, but not hydrolyses protein itself (Kies *et al.* 2006). It also liberates chelated molecules of minerals such as calcium, iron and zinc (Lei and Porres 2003). Most of the phytases are active in acidic condition with pH value 4-5.5. (BRENDA 2020).

Phytases release phosphorous groups by attacking specific site in inositol ring. They can be grouped to microbial and plant phytases. Microbial phytases degrade C_1 or C_3 (carbon) in inositol molecule ring, whereas plant phytases attack C_6 carbon. (Lei and Porres 2003.)

2.3.4 Amylases

Enzymes which hydrolyse starch into sugars, such as glucose, maltose or dextrins are called amylases. Amylases are capable of degrading the glyosidic bonds connecting sugar units in starch. (El-Fallal et al. 2012). Amylases are divided on two classes, alpha and beta amylases. They differ by the origin, alpha amylases are secreted by living organisms to digest nutrients, whereas beta amylases are produced by plants, bacteria, moulds and yeast. (Britannica 2020.) α-Amylases are frequently used in food production such as sugar, baking and brewing industries (Mehta and Satyanarayana 2016).

In biotechnology, it is a common practice to combine different enzymes to reach the desired properties of substrate. It is because, enzymes are able to create metabolic pathways, the consequence of enzymatic actions which lead to desired modification in the final product. (Blanco and Blanco 2017.)

2.4 Lactic acid bacteria fermentation

Fermentation is one of the oldest technique used to improve food properties. Traditional fermentation takes place through the naturally occurring microbes such as lactic acid bacteria (LAB) and yeast which originate in aerobic conditions and the presence of sugars. Nowadays, in food production, fermentation is enhanced by the specific strains of lactic acid bacteria that are inoculated from starter cultures. (Galle et al. 2012.)

During fermentation LAB metabolise sugars such as glucose, maltose, fructose etc forming lactic acid. The process of sugar hydrolysis is called glycolysis. Glycolysis is a metabolic pathway which breaks down sugars and produces energy used by bacteria. After glycolysis, pyruvate and lactate are formed.

Pyruvate acid reacts with the hydrogen, thereby producing ethyl in plants, microorganisms and lactic acid in animals, bacteria. (Kumari, 2018.)

In bread baking, it is used for preparing sourdough, which improves both functional and nutritional properties of bread, including texture, flavour, shelf-life and nutritional value. Sourdough increases the volume of bread depending on the level of lactic bacteria acidification. Acidification provokes the solubilisation of arabinoxylans, gluten and starch. LAB fermentation also increases the concentration of AAs through proteolysis activity. It improves the flavour of DF rich cereals, which means that, perhaps, it can be used for improving sorghum-based products sensory properties. LAB shows promising results in improving properties of gluten free flours. Most of the gluten-free flours are lacking health promoting properties and have unsatisfactory flavour and texture. LAB improves the taste, texture and volume of gluten-free bread. (Galle et al. 2012.)

Sorghum flour LAB fermentation

LAB fermentation improves the volume of sorghum based bread through the hydrolysis of sucrose to fructose and glucose. Fermentation provokes formation of exopolysaccharides which improves the functionality of sorghum flour. (International Journal of Food Microbiology, no date.) Schober et al. (2007) fermented sorghum flour using LAB. Authors reported, that solubilised proteins did not include kafirins, which are most desired to be hydrolysed. However, bread quality was significantly improved as hydrolysed proteins included proteins which cross-linked during baking, disrupting the starch network.

Cowpea flour LAB fermentation

The fermentation of cowpea reduces the amount of antinutritional components such as phytic acid, oxalate and increases the amount of crude protein (Ojokoh et al. 2013). Another study reported that fermented cowpea flour had higher viscosity, nitrogen solubility and water absorption rates (Lu and Sanni-Osomo 1988).

3 MATERIALS AND METHODS

The research described in this report can be divided into three parts: raw material characterization, bioprocessing, assessment of the effectiveness of bioprocessing techniques. Prevailing part of raw material characterisation and fermentation was done by other researchers working on NUTRIFOODS project.

3.1 Raw material characterization

Sorghum and cowpea samples used for this study are commercially available milled flours produced by African manufacturers from locally grown crops (figure 8, 9). Sorghum Bicolor flour is product of King Korn, Tiger Brands Limited (King Korn - Main no date). Cowpea of Bechuana white cultivar was supplied by Agricol (PTY) LTD (Agricol no date).

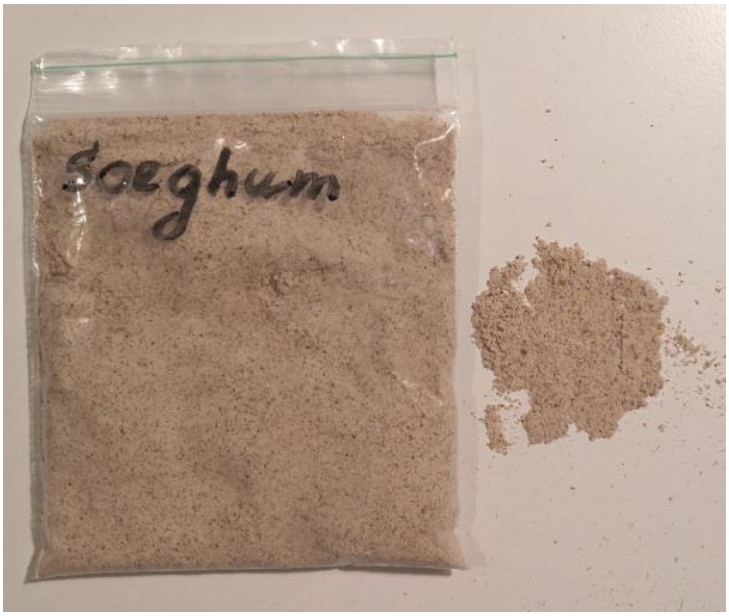

Figure 8. Milled sorghum used for the study

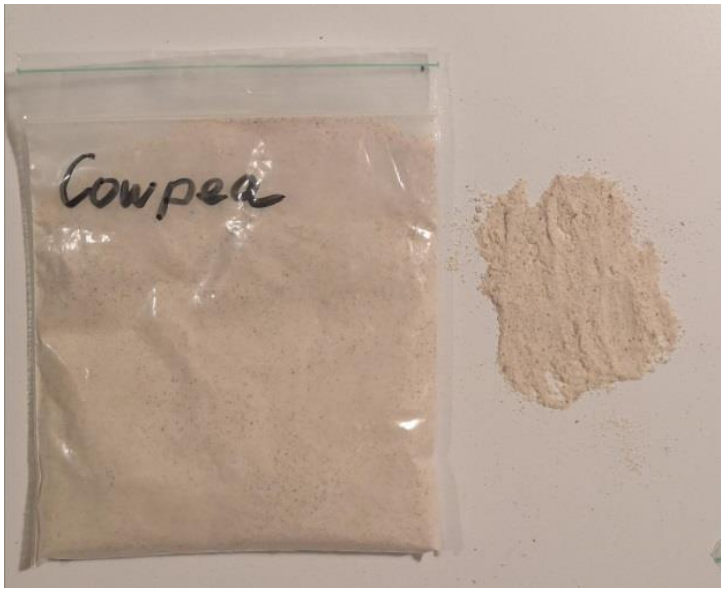

Figure 9. Milled cowpea used for the study

- **Moisture content.** All treatments and analyses conducted within this study took into account the moisture percentage of the flours which was measured using moisture analyser (MB120, OHAUS).
- **Protein concentration** was analysed with Kjeldahl autoanalyser (conversion factor = 6.25) according to the method 46-11A (AACC, 2003).
- **Dietary fibre** was analysed with the Method AACC 2011.25 using an ANKOM DF-equipment.
- **Starch** was quantified using Megazyme total starch assay kit according to the method 76-13.01 (AACC, 2003).

3.2 Enzymatic treatments

Sorghum and cowpea flours were enzymatically treated by various enzymes at different concentrations (tables 6 and 7). Volumes varied depending on the treatment, but components' proportions and the conditions of treatment were identical. Flours were weighted in Falcon test tubes or in borosilicate glass bottles. Enzymes were diluted with distilled water according to pre-calculated proportions. The concentration of enzyme was expressed in percentage in relation to dry flour weight.

Enzyme amount per each sample was calculated according to the Equation 1.

$$W = \frac{E * F}{100\%} \quad (1)$$

where	*W*	enzyme weight	[g]
	E	enzyme concentration	[%]
	F	DM flour weight	[g]

Flours were mixed with enzyme dilutions to reach the slurry concentration of 20% dry matter (figures 10 and 11). Along with each treatment, control sample containing no enzymes was prepared to assess the correctness of treatment performance. The contents of the tubes or bottles were shaken, and mixed using vortex mixer (if samples were in tubes) to eliminate the agglomerates of flour. Prepared samples were incubated in water bath for 2 - 4 hours at 50 °C. After incubation, samples treated by cell wall degrading enzymes were centrifuged (10.000 x G, 20 min, 4°C), supernatant collected and immediately frozen. Samples treated by proteases and their combinations with phytase, amylase and CWD enzymes were collected “as is” and immediately frozen. The impact of centrifugation before or after freezing is considered negligible.

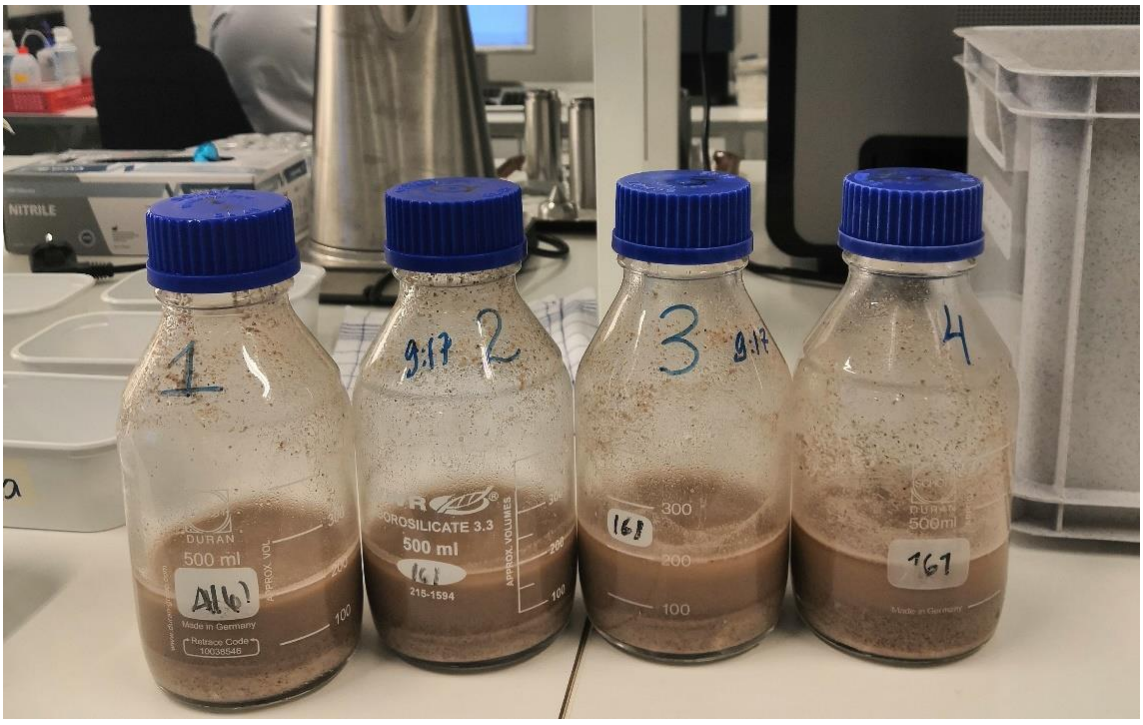

Figure 10. Example of sorghum samples

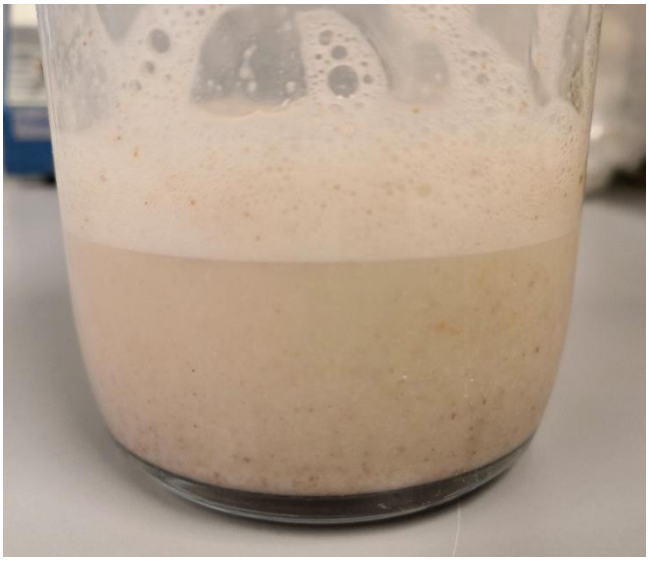

Figure 11. Example of cowpea flour sample

3.2.1 Enzymes used for sorghum and cowpea treatments

Flour samples were treated by commercially available cell wall degrading enzymes, proteases, phytase and amylase. Tables 6 and 7 list enzymes, their main activities and used concentration for sorghum and cowpea treatment.

Table 6. Enzymes, their main activities and dosages used for the treatment of sorghum

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	Enzyme (Producer)	Main activities	Dosages (%)	Time [h]	Source of activity info.
CWD enzymes	Depol 740 L (Biocatalysts)	Xylanase, endoglucanase, β-glucanase	0.015 0.03	[3h]	Arte 2019
	CelluclastBG (Novozymes)	Cellulase, Endoglucanase xylanase	0.1 / 1	[3h]	Gama et al. 2015
	Veron CP (ABenzymes)	β-glucanase, endoglucanase, endoxylanase	0.1 / 1	[4h]	Arte 2019
	Viscozyme L (Novozymes)	Cellulase, beta-glucanase, xylanase and arabanase,	0.1 / 1	[4h]	NCBE University of Reading 2018
	FiberCare R (Novozymes)	Endo-glucanase	0.1 / 1	[4h]	Bajpai 2015

Proteases	Brewers Clarex (DSM)	Protease(prolyl-endoprotease)	0.05 / 0.036 / 0.01	[2h]	DSM no date
	Corolase7089 (AB Enzymes)	Serimendoprotease and metalprotease	0.05 / 0.036 / 0.01	[2h]	AB Enzymes no date
	FlavourSEB (Advanced enzymes)	Leucine aminopeptidase	0.05 / 0.036 / 0.01 0.5	[2h] [4h]	Enzyme Innovation no date
Amylase	BAN 480L (Novozymes)	Alpha-amylase	0.2	[4h]	Novozymes 2014
Phytase	Phytase (Ultra Bio-Logics)	Phytase	Not used separately	[4h]	Ultra Bio-Logics Inc. no date

Enzyme combination	**Combined dosages (%, respectively)**
Celluclast BG + Depol 740L	0.1 + 0.03 / 0.1 + 0.015 / 1 + 0.03 / 1 + 0.015
Veron CP + Fibercare R	1 + 1
Viscozyme L + Veron CP	1 + 1
Viscozyme L + Fibercare R	1 + 1
FlavourSEB + Viscozyme	0.5 + 1
FlavourSEB + UltraBio	0.5 + 1
FlavourSEB + BAN 480L	0.5 + 0.2

Table 7 Enzymes, their main activities and dosages used for the treatment of cowpea.

	Enzyme (Producer)	**Main activities**	**Dosages (%)**	**Time [h]**	**Source of activity info.**
Proteases	Brewers Clarex (DSM)	Protease(prolyl-endoprotease)	0.05 / 0.036 / 0.01	[2h]	DSM no date.
	Corolase7089 (AB Enzymes)	Serine endoprotease an metalloprotease	0.05 / 0.036 / 0.01	[2h]	AB Enzymes no date.

	FlavourSEB (Advanced enzymes)	Leucine aminopeptidase	0.05 / 0.036 / 0.01 / 0.5	[2h] [4h]	Enzyme Innovation no date.
Amylase	BAN 480L (Novozymes)	Alpha-amylase	0.2	[4h]	Novozymes 2014.
Phytase	Phytase (Ultra Bio-Logics)	Phytase	0.1 / 1	[2, 4h]	Ultra Bio-Logics Inc. no date

Enzyme combination	**Combined dosages (%, respectively)**
FlavourSEB + Viscozyme	0.5 + 1
FlavourSEB + UltraBio	0.5 + 1
FlavourSEB + BAN 480L	0.5 + 0.2

3.3 Fermentation trials

Sorghum and cowpea were fermented with three strains of lactic acid bacteria (table 8). At the end of fermentation, pH was measured and samples were frozen and freeze-dried for further analysis (figure 12). A pH-control sample was also prepared in similar conditions, by adding lactic and acetic acid to the keep pH of the system at ~ 4.5. Fermentations were performed at 33 % dry matter content for 24 h at 30 °C in beakers without mixing. Fermentation trials were performed by other researchers working in the same project.

Table 8. Strains and conditions used for the fermentation of sorghum and cowpea.

Species	**Strain**	**Temperature, time**
Lactobacillus plantarum	VTT E-062634	30°C, 24h
Leuconostoc pseudomesenteroides	VTT E-981034	30°C, 24h
Pediococcus pentosaceus	VTT E-153483	30°C, 24h

Before the analysis, freeze dried samples were extracted. Ground lyophilised flour was mixed with distilled water at 1:10 ratio. Samples were mixed for 30 min at cold room 4◦C and centrifuged (10.000G, 10 min, 4◦C) to collect the supernatant for the further analysis.

Figure 12. Example of lyophilised sorghum flour sample.

3.4 Assessment of bioprocessing techniques effectiveness

Two types of analysis were used in this study, analysis of protein solubility and quantification of reducing sugars. Protein solubility analysis was used to assess the hydrolysis of protein, especially by the action of proteases. Reducing sugars (RS) quantification was done to assess the effectiveness of CWD enzymes.

Before the analyses 1-1.5ml frozen samples were thawed and centrifuged (10.000 x G, 10-15 min) to separate the supernatant for the following test.

3.4.1 Quantification of solubilized proteins using Lowry protein assay

To determine the concentration of solubilised proteins in treated samples, Lowry Protein Assay was conducted using commercial kit (DC Protein Assay, Bio-Rad, Hercules, CA, U.S.A). Lowry Protein assay is a colorimetric method based on the biuret reaction followed by the addition of Folin-Ciocalteu reagent which forms blue coloured complexes with protein's side chains. The characteristic blue colour is measurable at 650 - 750 nm. (Lowry et al. 1951.)

Measurements were conducted in 96x well microplates or in semi-micro cuvettes depending on the treatment. Bovine serum albumin was used as a standard protein.

For the standard assay in cuvettes, 25 µl of the pre-dilluted samples (2-3 dillutions) and standards were transferred into the cuvettes in duplicates. 125 µl of Reagent A from the testing kit was added to each cuvette. After, 1 ml of Reagent B was added into each cuvette and mixed. Resulting solutions were incubated for at least 15 minutes at room temperature. Absorbance was measured at 750 nm twice using spectrophotometer (UV-Vis Spectrophotometer UV-1800, Shimadzu Europa GmbH, Duisburg, Germany).

For the microplate assay, the proportions of assays's components were the same, but in lower volume. 5 µl of predilluted samples and standards were transferred into the wells in duplicates. 25 µl of Reagent A was added to each solution and mixed. 200 µl of Reagent B was added into each filled well and mixed again. Solutions were incubated for at least 15 minutes at room temperature. Absorbance was measured at 750 nm twice using microplate reader (Varioscan, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A).

The results of standard protein absorbance were used to draw standard curve. Taking into account the dilutions used, the protein concentration of supernatant was calculated using standard curve equation. The percentage of solubilised proteins was calculated using an Equation 2.

$$P = \frac{C_o}{C_s} \times 100\% \tag{2}$$

where	P	percentage of solubilized proteins	[%]
	C_o	concentration of protein in original sample	[mg/ml]
	C_s	concentration of protein in supernatant quantified by the assay	[mg/ml]

The concentration of the protein in the original samples was calculated on the basis of protein quantification analysis. Amount of protein in the flour (mg/g) was divided by the total volume of the sample (flour + water), which results in highest possible protein concentration, if all proteins in the flour are solubilised to supernatant.

For some samples, pH value were adjusted to 4 and 5 to find out the pH range at which protein hydrolysis is more effective. pH was adjusted by 0.1/1 M NaOH and 0.1/1M HCl. Additions of mentioned chemicals were taken into account while calculating protein solubility percentage.

3.4.2 Reducing sugars quantification using DNS method

In the scope of this study, quantification was done to assess the extent of cell wall degradation, i.e insoluble dietary fibre. Enzyme treatment degraded the glycosidic bonds of polysaccharides, thereby releasing monosaccharides, disaccharides and oligosaccharides. The analysis of unmodified supernatant determined the quantity of *Free* sugars (mono-, di- saccharides) hydrolysed during treatment. To quantify oligosaccharides released during the treatment, supernatant was hydrolysed with sulfuric acid (1 M, 1-2h, 100°C). The concertation of reducing sugars in acid hydrolysed sample displayed the total amount of mono-, di-, oligo- saccharides. *Oligo* sugars (oligosachharides) concentration was estimated by the difference in total and *Free* sugars values. In case if enzyme hydrolyse exclusively *Free* sugars, the difference total - free can be close to zero or even negative as analysis of *Free* and *Oligo* sugars is done separately and deviations of *Free* RS results apply also on *Oligo* RS values.

The concentration of reducing sugars was determined by DNS (3,5 Dinitrosalicylic Acid) method well described by Miller (1959). DNS reagent is an aromatic compound which interacts with reducing sugars forming 3-amino-5-nitrosalicylic acid (figure 13). The resulting acid has a characteristic colour which ranges from light yellow to dark red with the increase of reducing sugars

concentration. The colour is intensely adsorbed at 540 nm. Glucose dilutions (x - y mg/g) are used to draw a standard curve.

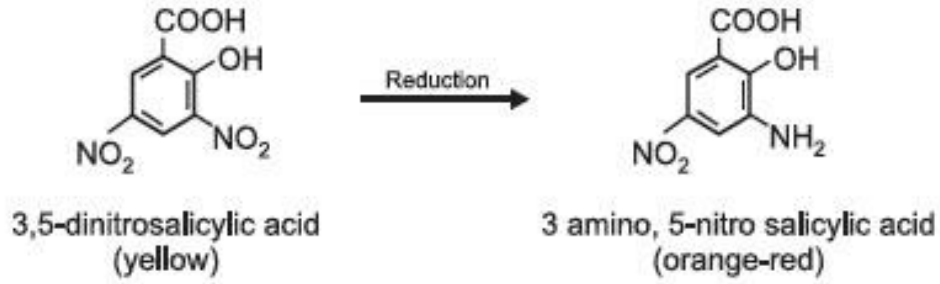

Figure 13. Principle of DNS analysis (Biocyclopedia no data).

RS quantification was done in 96x well microplates. Samples were diluted with distilled water to 2-3 different dilutions. 90 µl of each dilution and glucose standard was pipetted into the PCR plate in duplicates or triplicates. One blank sample of distilled water was transferred as well. 135 µl of DNS reagent was added to each solution and mixed. PCR plate was covered by aluminium foil sticker and boiled at 98 C° for exactly 5 minutes (Stuart Heating Block, Cole-Parmer, Vernon Hills, Illinois, U.S.A). Immediately after boiling, plate was placed to water bath to cool down to room temperature. 200 µl of each solution was transferred to microtiter plate. Absorbance at 540 nm was measured by microplate reader (Multiscan, Thermo Electron Limited, Altrincham, United Kingdom). Standards' absorbance values were used to draw a standard curve and calculate the standard curve equation.

Taking into account dilutions used, reducing sugars concentration in supernatant was calculated. Concentration of RS was presented in an amount of RS per gram of sample's dry matter using Equation 3.

$$C_{DM} = \frac{C_S \times V_L}{W_{DM}} \quad (3)$$

where C_{DM} concentration of RS in dry matter [mg/g]

C_S concentration of RS in supernatant [mg/ml]

V_L	volume of liquid fraction of sample	[ml]
W_{DM}	weight of sample's dry matter	[g]

4 RESULTS

4.1 Raw material characterization

The main chemical composition of the raw materials is presented in the table 9.

Table 9. Chemical composition of raw materials (% to DM)

Material	Dry matter content	Starch	Protein	Dietary fibre		
				Insoluble	Soluble	Total
Sorghum	90.18	73.1 ± 1.2	10.18 ± 0.03	9.25 ± 0.06	2.25 ± 0.02	11.5
Cowpea	90.87	40.7 ± 0.8	23.85 ±0.02	13.1 ± 0.21	7.54 ± 0.03	20.64

4.2 Enzymatic treatments

Results are presented in the order of treatments implementation. This study included 6 enzymatic treatments, of which 4 with sorghum and 2 with cowpea. The aim of CWD enzymes application was to solubilise the insoluble dietary fibre which is present in a form of polysaccharides, therefore, reducing sugars concentration assay is the main criteria for assessing the performance of CWD enzymes. The aim of protease treatment was to increase the protein solubility (PS). Lowry protein assay is used to assess proteases' effectiveness. However, both RS and PS analysis are conducted for samples treated by enzyme combinations to identify possible metabolic pathways. The plan was to find the most effective CWD and protease enzyme which later will be combined together and with phytase, amylase. In order to evaluate the effectiveness of the enzymatic treatments, the treated samples are compared to control sample which was treated on the same conditions, but without enzymes.

4.2.1 Cell wall degrading enzymes treatment of sorghum flour

The first set of CWD enzymes included Depol 740 L (Depol), Celluclast BG (Celluclast) and their combinations (figure 14).

The Control sample contained 15.1 and 2.8 mg/g of free and oligo RS, respectively. The highest RS content was found in samples treated with 1% Celluclast BG. The addition of 1% Celluclast BG alone increased Free and Oligo RS concentration in 1.4 and 1.9 times comparing to 3h control. Depol 740L did not have a significant effect on RS release. The results of enzyme combination indicate that RS release depended mainly on Celluclast concentration, whereas Depol addition had a minor effect. The highest concentration of Free RS in this treatment was found in Celluclast 1% + Depol 0.03%, 1.59 times higher than in control. Combination Celluclast 1% and Depol 0.015% was most effective in Oligo RS release. In comparison with the control, this sample contained 2.7 in times more Oligo RS. Celluclast BG was chosen for further analysis.

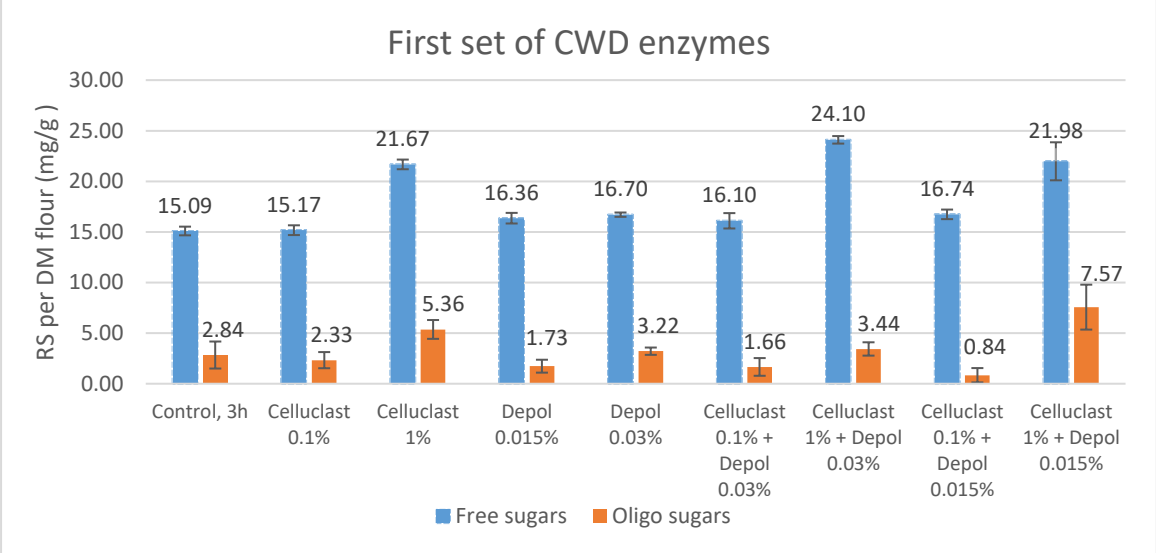

Figure 14. *Free* and *Oligo* sugars concentrations (mg/g) in sorghum samples treated by Depol 740L, Celluclast BG and their combinations for 3h, 50°C.

The second set of CWD enzymes included treatment with Veron CP (Veron), Viscozyme L (Visco), FiberCare R (FiberC) and their combinations. Samples were treated for 4 hours and compared with control (figure 15).

Among samples from the second set of enzymes, the highest amount of Free RS was released by Viscozyme L. 1% of Viscozyme L increased the Free RS content in 2.5 times compared to the Control. FiberCare R was the most effective in Oligosaccharides release. 1% of FiberCare R increased Oligo RS concentration 1.7 times. Viscozyme 1% did not have any effect on oligosugars hydrolysis. Among the combinations, FiberCare + Viscozyme sample released more Free RS than the rest, however, concentration did not differ a lot from RS content in sample containing Viscozyme alone. FiberCare + Veron combination was most effective in Oligo sugars hydrolysis.

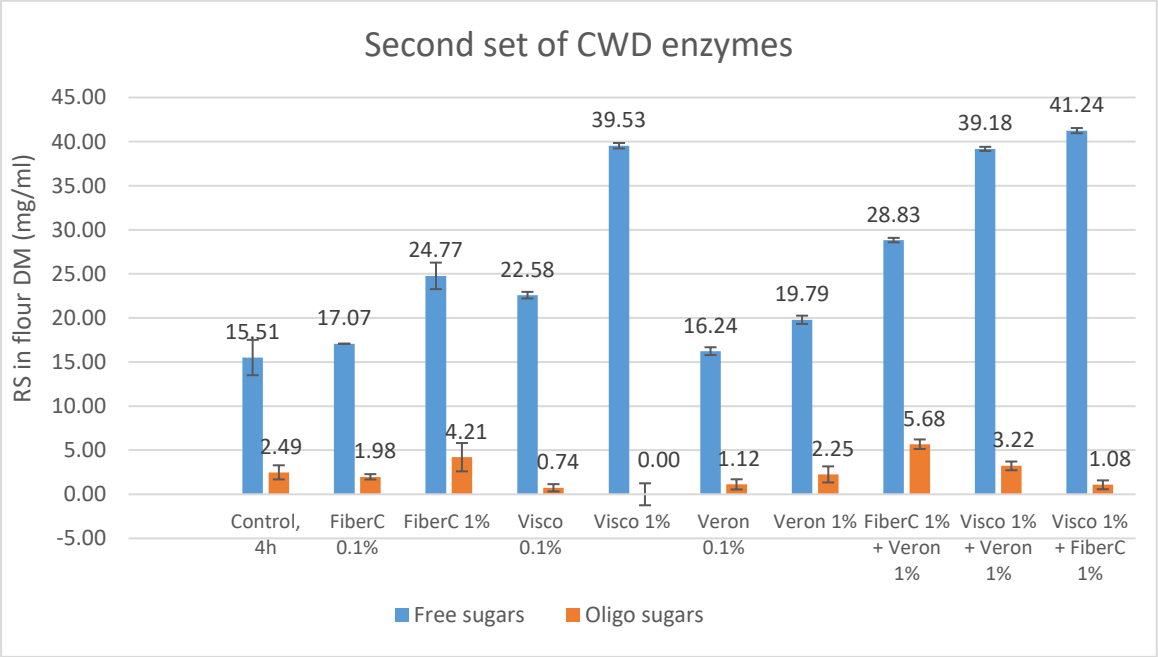

Figure 15. *Free* and *Oligo* sugars concentrations (mg/g) in sorghum samples treated by Veron CP, Viscozyme L, FiberCare R and their combinations for 4h, 50°C.

It was decided to continue trials only with Veron and Viscozyme. Even though FiberCare's overall activity was promising, FiberCare is not a food-grade enzyme, therefore, its addition to food production is not allowed except for the research purposes. (Bajpai, P. 2015.)

4.2.2 Impact of mixing on the PS and RS values

On a basis of results obtained from CWD enzyme treatments, bigger scale treatment was conducted in order to produce samples for further analysis. Treatment included Celluclast BG, Veron CP and Viscozyme L enzymes. Conditions of treatment were not changed except of two aspects: the volumes of components were proportionally increased, samples were not initially mixed using vortex mixer or magnetic stirrer, but were mixed manually. Use of magnetic stirrer was advised, but could not be performed because of the thick consistency of sample, thick glass bottom of the bottle and gap between the water bath and the magnetic stirrer. Samples in previous treatments were mixed every 30 minutes of incubation to enlarge the interactive surface of flour particles. Therefore, it was decided to conduct additional treatment to identify the impact of mixing on the PS and RS concentration. Four samples were prepared, two controls, one of which is mixed every 30 minutes for 4 hours at 50◦C and two samples of 1% Viscozyme (mixed, not mixed). The protein solubility and Free RS were measured.

Table 10. Impact of mixing on the PS and Free RS release.

Sample		PS, %	Free RS in aliquot, mg/ml
Control	not mixed	6.68 ± 0.01	4.26 ± 0.02
	mixed	5.76 ± 0.24	3.98 ± 0.11
Viscozyme 1%	not mixed	7.42 ± 0.05	10.21 ± 0.73
	mixed	6.45 ± 0.21	9.44 ± 0.61

It is important to take into account that mixed samples contained less DM than not mixed, because small amount of liquid fraction from mixed samples was collected for side analysis. Not mixed samples show a minor increase in Free RS concentration and PS (table 10). Most probably, it is due to the variations of DM fractions. After the discussion of results, it was agreed not to mix samples in following treatments.

4.2.3 Proteases treatment of sorghum

The following dosages were used for the treatment of sorghum with proteases: 0.01%, 0.036%, 0.05%. Proteases used included Brewers Clarex (BrewClar),

Corolase 7089 (Corolase), FlavourSEB (FlavourS) (2h, 50 °C). Samples were tested using Lowry protein assay to calculate the protein solubility. The protein amount in the original sample flour is 19.8mg/ml, this value was used to calculate the percentage of solubilised proteins in the sample flour.

In overall, enzymes' effect was insignificant. Highest dosages of all three enzymes solubilised almost equal amount of proteins compared to the Control sample (figure 16). At lower dosages (0.01%), FlavourSEB was more efficient in comparison with Corolase and Brewers Clarex. Sorghum control 4h was used to calculate the impact of longer incubation time. 4h control solubilised 1% more proteins than 2h control. Taking into account the low solubility of sorghum proteins and insignificant enzyme activity, 1% increase of PS is considered a significant effect. FlavourSEB was chosen for the next treatment. Also, it was decided to prolong the incubation time up to 4 hours.

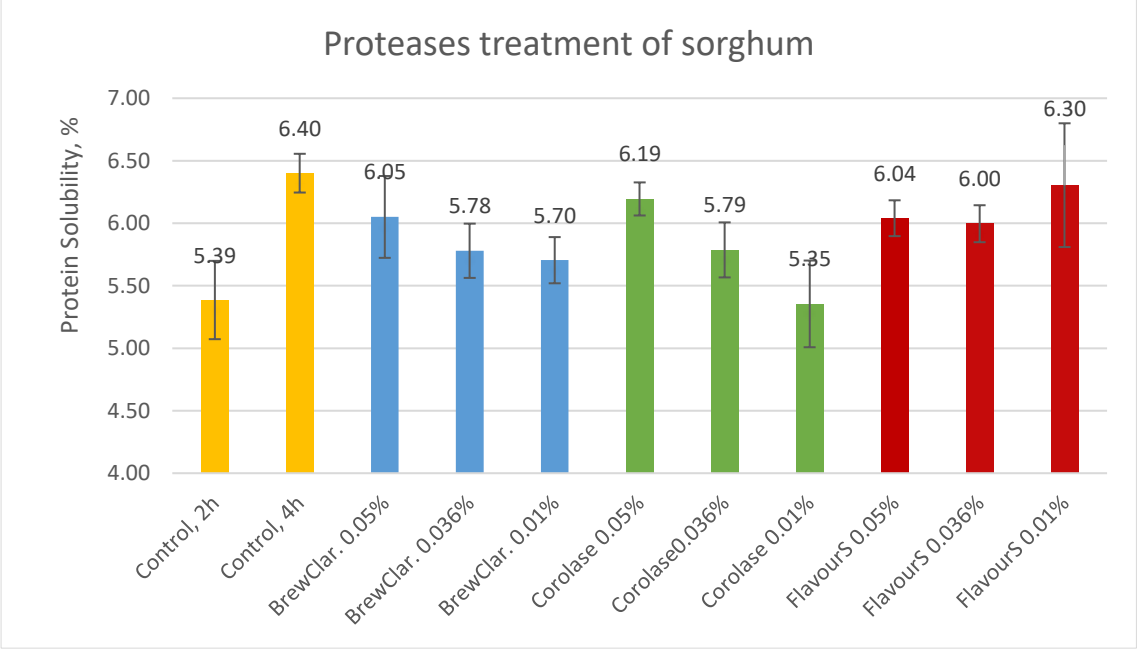

Figure 16. Protein solubility (%) of sorghum samples treated by Brewers Clarex (BrewClar), Corolase and FlavourSEB (FlavourS) (2h, 50°C).

4.2.4 Combination of CWD enzymes, proteases, amylase and phytase for sorghum treatment

Combination treatment included selected CWD enzyme Viscozyme L (Visco), protease FlavourSEB (FlavourS), combination of protease with amylase BAN

480L (BAN) and phytase UltraBio (UltraB). The concentration of FlavourSEB used in this treatment are significantly higher (0.5%) than in the previous proteases treatment (0.01-0.05%) because these low concentrations did not have a significant activity in previous treatment.

FlavourSEB at higher dosage (0.5%) and longer incubation time (4h) increased PS on 2.92% (figure 17). The addition of Viscozyme or UltraBio to FlavourSEB did not significantly increased the protein hydrolysis compared to FlavourSEB itself. FlavourSEB + BAN 480L sample showed the lowest PS value than the rest of combinations. BAN 480L itself was not effective in protein hydrolysis, as expected, because it is an amylase. Acidification negatively affected on the PS of control, protease and protease + phytase samples.

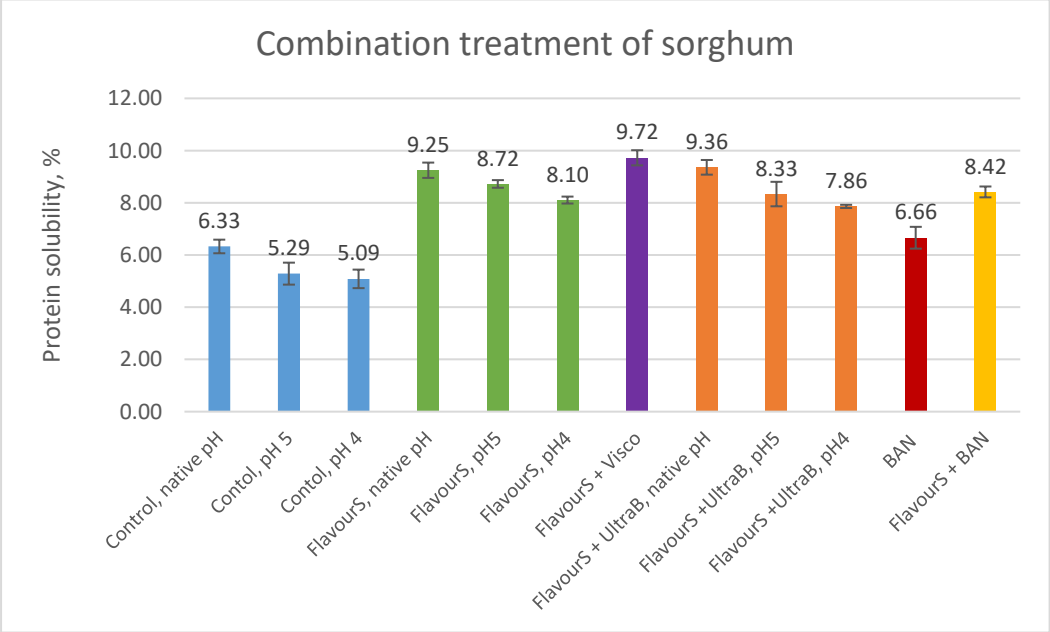

Figure 17. Protein solubility (%) of sorghum samples from combination treatment (4h, 50°C).

Samples from combination treatment were also analysed for Free RS content. Treatment with FlavourSEB itself released 2.4 times more RS than the Control (figure 18), indicating that FlavourSEB probably has carbohydrase activity besides protease. The combination of FlavourSEB with Viscozyme released 3.3 times more Free RS in comparison with the control sample. The combination of

FlavourSEB with BAN or UltraBio were almost equally effective as FlavourSEB alone. BAN alone increased the Free RS amount in 1.4 times.

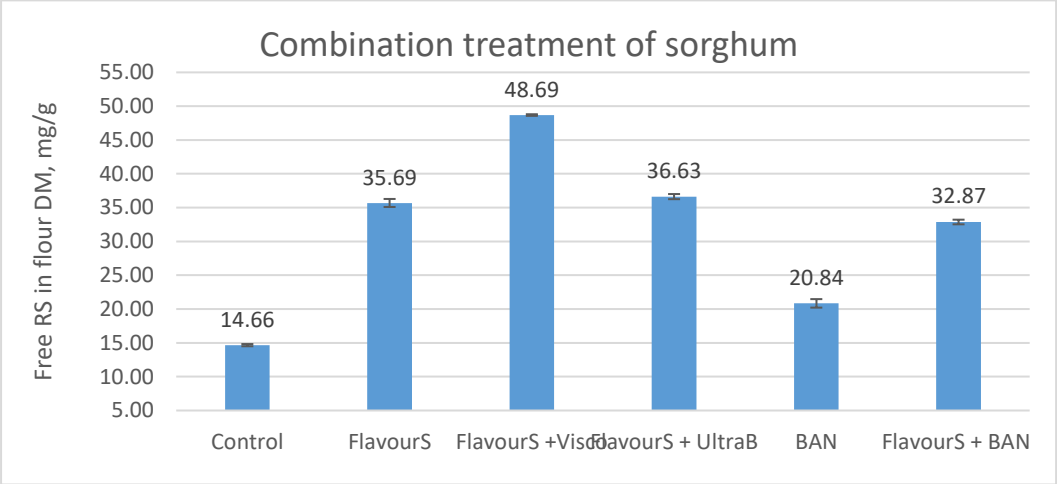

Figure 18. Free RS quantification (mg/g) of sorghum samples from combination treatment (4h, 50°C).

4.3 Protease treatment of cowpea

The protease treatment of cowpea included the application of Brewers Clarex, Corolase and FlavourSEB enzymes at the same concentration as in sorghum protease treatment. The concentration of protein in the control sample is 52.5mg/ml. This value was used to calculate the percentage of hydrolysed proteins.

In overall, FlavourSEB and Corolase showed similar results, whereas Brewers Clarex was slightly less effective. Highest PS value, 38.76%, was reached by FlavourSEB at 0.05% (figure 19). Brewers Clarex showed the lowest PS rates than the rest of enzymes. It was decided to continue research only with FlavourSEB.

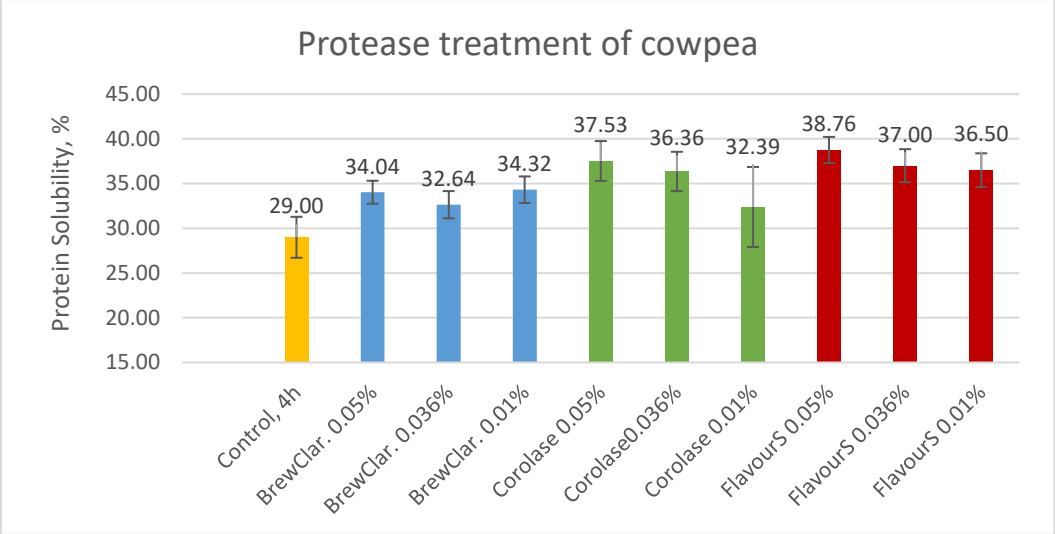

Figure 19. Protein solubility (%) of cowpea samples treated by Brewers Clarex (BrewClar), Corolase and FlavourSEB (FlavourS) (2h, 50°C).

4.4 Combination of protease with amylase, phytase and CWD enzymes for cowpea treatment

Enzymes and concentrations used for the treatments are the same as used for sorghum (described in item 7.3.5).

At native pH, the most effective enzyme combination was FlavourSEB + Viscozyme, which increased the protein solubility in 1.7 times in comparison with the control (figure 20). The PS of control samples at different pHs indicated that acidification decreased the protein release from 29% to 17.9%. Similar decrease was observed in FlavourSEB treated samples. However, the protein solubility rate of FlavourSEB+UltraBio sample at pH 4 is 1.5 times higher than at native pH and 3.1 times higher than pH 4 control sample. BAN 480L alone was not effective in PS, its combination with FlavourSEB increased PS on the same rate as FlavourSEB alone.

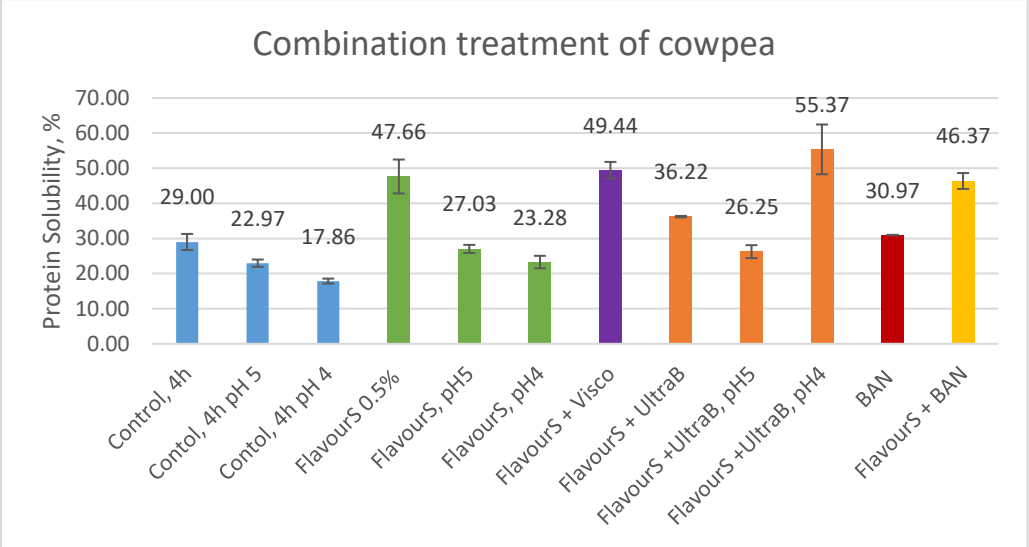

Figure 20. Protein solubility (%) of cowpea samples from combination treatment (4h, 50°C).

The most effective enzyme in Free RS release was BAN 480L which increased the concentration from 54.7 to 108.4 mg/g due to starch hydrolysis (figure 21). Among FlavourSEB combinations, the highest activity was observed in FlavourSEB+BAN 480L, but the amount of Free RS was slightly lower than in sample containing only BAN 480L. FlavourSEB alone had quite small impact on the release of free sugars.

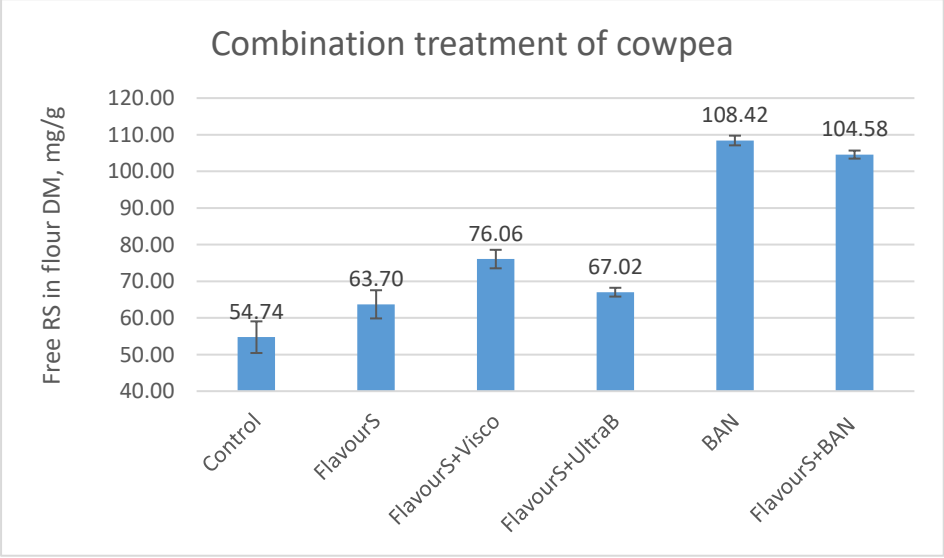

Figure 21. Free RS release (mg/g) in cowpea samples from combination treatment (4h, 50°C).

4.5 Lactic acid bacteria fermentation of sorghum and cowpea

Sorghum and cowpea were fermented with lactic acid bacteria and the impact on protein solubility was analysed. In cowpea trials, sample fermented with *Pediococcus pentosaceus* showed the highest protein solubility (28.3%) Among sorghum samples, most of solubilised proteins were found in acid control, in which 6% of the proteins were solubilised (figure 22).

pH values for sorghum were 3.9, 4.5 and 3.9 for *P. pentosaceus, L. pseudomesenteroides* and *L. plantarum,* respectively. In cowpea, pH values were 4.6 for *P. pentosaceus,* 4.0 for *L. pseudomesenteroides* and 4.1 for *L. plantarum.*

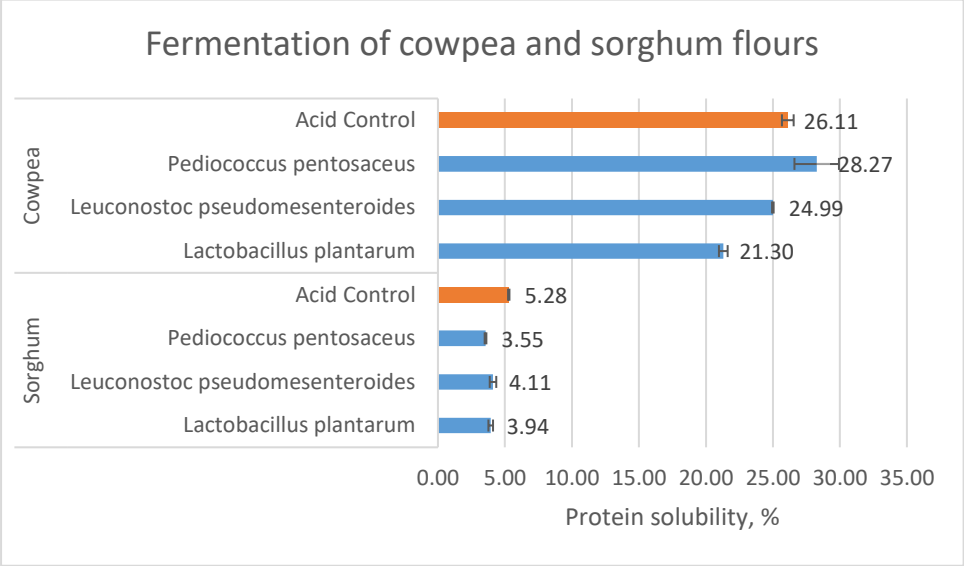

Figure 22. Protein solubility (%) of fermented cowpea and sorghum samples (24h, 30◦C)

5 DISCUSSION

To sum up the results derived during the research, several assumption can be made.

5.1 Impact of cell wall degrading enzymes on sorghum flour structure

Sorghum has a complex cell wall structure which affects the properties and the acceptability of sorghum-based products, including baked goods (Bader et al. 2019). 80% of DF in the untreated sorghum flour sample was insoluble. The treatment of sorghum flour by CWD enzymes solubilised significant part of insoluble dietary fibre. Among 5 applied CWD enzymes (Celluclast BG, Depol 740L, Viscozyme L, Veron CP, FiberCare R) Viscozyme L showed most promising results, followed by Celluclast BG and FiberCare R. The addition of 1% Viscozyme L increased the amount of solubilized cell wall from 15 (control) up to 40 mg/ml (4h, 50°C), meaning that 23% more DF was hydrolysed in comparison with control. Celluclast BG and FiberCare R were almost equally efficient. 1% of Celluclast increased the amount of hydrolysed cell wall components on 10% (3h, 50°C), while 1% FiberCare R on 12% (4h, 50°C). The combination of Viscozyme and FiberCare solubilised 27% more of DF than control (1%+1%, 4h, 50°C).

The effectiveness of Viscozyme could be due to many reasons. One of them could be due to the multi-activities reported in this enzyme, such as cellulase, β-glucanase, xylanase and arabinose (NCBE University of Reading, 2018). Viscozyme was the only enzyme reported to have an arabinase activity, which might had an impact as sorghum has high arabinose content. A better hydrolysis extension could be achieved with the combination of Viscozyme and a β-glucosidase (Gama et al. 2015). Higher amount of the released Free sugars could be explained by the enzyme cleave sites. Due to the complex cell wall structure, enzymes could not hydrolyse the arabinoxylans from the middle of the chain, but hydrolysed the ends of it, releasing free sugars.

5.2 Impact of proteases on the solubility of sorghum and cowpea proteins

The low protein solubility of sorghum compromises its nutritional potential. Sorghum proteins are extremely resistant to hydrolysis due to complex protein matrix, hydrophobic kafirin proteins and high AA proline content. Moreover, the increase of temperature provoke the formation of disulphide bonds

which decreases the solubility of proteins (Kanerva 2011; Taylor and Taylor 2018; Rom 1992). In this study, the application of proteases had a mild effect on the solubility of sorghum proteins. Three proteases were tested: Brewers Clarex, Corolase 7089, FlavourSEB. At equal concentrations (0.036%) and incubation time (2h, 50°C) Corolase 7089 and FlavourSEB samples were similar in PS values. The best result was reached by 0.5% FlavourSEB (4h, 50°C) which increased the protein solubility on 2.9% compared to the control. When the PS was measured in samples where the pH was adjusted to 4 or 5, the decrease of PS values was observed. Low protein solubility at pH 4 was also reported by Elkhalifa and Bernhardt, 2010. Probably, it could indicate that the isoelectric point of sorghum protein is around pH 4. The impact of FlavourSEB on the release of sugars worth attention. In comparison with the control sample, FlavourSEB at 0.5% concentration increased the RS content 2.4 times. There are several possible explanations. One of them is a carbohydrase side-activity of FlavourSEB, which has not been shown in previous studies. Another possible reason is polysaccharides and starch liberalisation from protein matrix which was hydrolysed by FlavourSEB, but a carbohydrate activity would be anyway needed to hydrolyse them into sugars.

In contrast with sorghum, the application of proteases on cowpea flour was more efficient in hydrolysing proteins. At equal concentrations (0.036%) Corolase 7089 and FlavourSEB showed almost identical values of the PS, increasing the PS on 8.5 and 9.8% respectively (2h, 50°C). The increase of incubation time and enzyme dosage significantly affected PS. 4h incubation with 0.5% of FlavourSEB increased the PS on 18.7% in comparison with 4h control sample.

When the pH of cowpea samples was reduced to 4 or 5, decrease in the PS rate was observed. This goes along with the findings of study conducted by Agustin et al. 2020, who observed the isoelectric point of cowpea protein isolates in the range pH 4.3 - 4.5. However, in sample prepared with 0.5% FlavourSEB and 1% phytase, PS at pH 4 was 19.2% higher than at native pH. Rosa-Sibakov et al. (2018) also observed a higher protein solubility at

pH < 5 in faba bean treated with phytase. This was explained by the fact that phytic acid forms a complex with proteins at acidic pH decreasing the protein solubility (Kumar et al., 2010). As cowpea also contains high amounts of phytic acid, the treatment with phytase might have had the same impact as observed for faba bean (Rosa-Sibakov et al. 2019). Sorghum treated with the same phytase did not show similar difference, probably because sorghum might have lower amount of phytic acid than cowpea, or the phytase treatment was not efficient in sorghum (Chitra et al. 1996; Olivera-Castillo et al. 2007).

Such a difference in the extent of protein hydrolysis in cowpea and sorghum possibly could be explained by their different structures and composition. The proteins of cowpea are not encapsulated by the cell wall and protein-starch matrix as in sorghum. Cowpea proteins are distributed along the cotyledon, allowing a better access to enzymes. Another explanation could be due to the difference in globulin amounts. In legume, most of insoluble proteins are reported to be globulins (Jayathilake 2018). If comparing globulins solubility to prolamins', the latter are more resistant to hydrolysis which results in significant difference between PS values of cowpea and sorghum (Agustin et al. 2020; Ejeta et al. 1987). Besides, cowpea flour was visibly finer milled than sorghum which could impact the interactive surface of flour particles and enzymes.

5.3 Effect of enzymatic cocktails

Both sorghum and cowpea flours were treated by the same set of enzyme combinations: FlavourSEB + Viscozyme L, FlavourSEB+Phytase, FlavourSEB+BAN 480L. Additional samples treated only by BAN 480L were prepared to identify the actual differences in results. Among sorghum samples, the combination of FlavourSEB and Viscozyme was the most effective, increasing the hydrolysis of proteins and insoluble DF. However, enzymes did not have a synergetic effect. The results of treatments where these enzymes were used separately show that DF was mostly hydrolysed by Viscozyme and proteins by FlavourSEB. The treatment of cowpea with the same enzymes show similar behaviour, except for increased PS value in FlavourSEV+Phytase sample at adjusted pH, as discussed

earlier in item 8.2. Such results, perhaps, could be explained by the structure of cell walls and proteins. Sorghum proteins are encapsulated in the protein matrix, therefore cell wall degradation did not affect its structure and two activities were independent (Ratnavathi and Komala 2016; Rooney 1996). In cowpea, the protein hydrolysis is not affected by fibre degradation because protein is not linked or encapsulated with fibres, therefore, hydrolysing the cell wall did not impact the PS.

5.4 Effect of LAB fermentation on cowpea and sorghum flours

Fermented samples were analysed for protein solubility. Pediococcus pentosaceus solubilised 2% more cowpea proteins in comparison with acid control (24h, 30°C). However, the rest of fermented cowpea samples showed lower PS than in acid control. In sorghum, acid control sample showed the highest PS. This could be due to the isoelectric point of sorghum and cowpea proteins. The same situation was observed in enzyme combination treatment, where PS decreased with the decrease of pH from native to 4. LAB acidified the samples, thereby decreasing the PS. Another explanation of the increased PS of acid control could be the presence of endogenous proteases, which are more active at lower pH than at native. (D'Silva et al.1998; Ng'andwe et al. 2008; Poutanen et al. 2009.) As acid control spent 24 h at the low pH its endogenous proteases had longer time to act than in fermented samples that were only gradually acidified.

It is still unclear what was the effect of LAB fermentation on functional or nutritional properties with PS assay. Application of LAB could increase the protein digestibility of sorghum and cowpea samples, but no assumptions can be done basing on the PS analysis. (Day et al 2018.) Further investigation of the degree of protein hydrolysis could be performed with other methods (e.g., SDS-page and OPA-method) in order to better evaluate the effect of lactic acid bacteria fermentation.

5.4.1 Limitation of the study and possible improvements

During the research, several limitations were revealed. However, for some of the limitations, there are methods which could improve the reliability of results and avoid problems arose during the study:

- The age of data about sorghum. Sorghum is rapidly adaptive crop. Prevailing part of researches about sorghum are conducted in 1970s-1980s. Such information as digestibility, the impact of processing on sorghum can be no longer valid due to changes in sorghum characteristics grain. Moreover, since then, assessment methods and equipment have improved.
- Lowry protein assay which was used for the quantification of protein solubilisation can give an indication that the treatment made proteins more accessible, but it does not reflect directly a better digestibility of proteins in vivo. Other methods such as SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis, which allows to categorise proteins based on their length, or the measurement of protein hydrolysis with OPA (orthophthalaldehyde) method, which measures the amount of free amino groups released during hydrolysis, could give a complementary information.
- Lack of information about enzymes activities does not allow to assess their effect on specific studied component. Therefore, the characterisation of enzyme activities is needed to understand why enzymes worked the way they did.
- Analysis of sugar profile is advised to identify which cell wall components were affected by treatment.

6 CONCLUSION

The impact of enzymatic treatments on the solubilisation of proteins and DF of sorghum was reasonable. Five different cell wall degrading enzymes (Depol, FiberCare, Veron, Viscozyme and Celluclast) were tested at various dosages. DF hydrolysis was significant. Viscozyme L, 1% and FiberCare 1% solubilised 27% more DF than control (4h, 50°C), but no significant improvement in protein solubility was observed in the tested conditions (enzyme dosage: 0.01-0.05%; 2h; 50°C). The increase of incubation time (4h) and protease dosage (0.5% FlavourSEB) increased the protein solubility from 6.3 (Control) up to 9.2%.

The impact of enzymatic treatments on the solubilisation of proteins and DF of cowpea was more effective in comparison with sorghum. At low dosages (0.01-0.05%), all proteases tested significantly increased the protein solubility from 29 (Control) up to 39.20%. When the dosage and time was increased (FlavourSEB 0.5%, 4h), the protein solubility increased up to 48%. The combination of protease (FlavourSEB) with phytase did not improve the protein solubility, when measured at native pH. However, an improvement was observed (PS: 55%) when the protein solubility was measured at pH 4. No significant metabolic pathways were detected in the combination of enzymes applied on both flours.

LAB fermentation did not have a significant impact on protein solubility. In sorghum, application of LAB mildly decreased the PS value. In cowpea treatment, 2 bacteria species decreased the value, while one increased.

Increasing the nutritional value and functionality of sorghum and cowpea flours can increase the acceptability of sorghum/cowpea based bakery goods. Replacing wheat flour in the bread baking can decrease the demand in wheat production in SSA region. Promoting the usage of locally grown climate adapted crops have a positive impact on economic, social and environmental sustainability in the region. The increased use of cowpea crops will replenish the soil deposits is phosphorous and nitrogen. Intercropping cowpea with cereal crops, such as sorghum can significantly increase the yields thanks to enriched soil composition. (Fatokun, C. et al. 2002.) Both cowpea and sorghum can withstand the negative effects of climate changes, such as longer heat waves and drought (Dadson et al. 2005).

The outcomes of the thesis will be used for the continuous research of NUTRIFOODS project. Selected enzymes and their combination will be combined with fermentation experiments and further used for bread baking trials. Flour samples prepared during this study are to be analysed for more comprehensive characterisation. Using the outcomes of this study can assist in decision making for other bioprocessing techniques applied on sorghum and cowpea flours.

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Appendix 1/1

Results of protein solubility and reducing sugars analyses

Table 1. Concentration of Free and Oligo RS in sorghum samples treated by Depol 740L, Celluclast BG and their combinations (3h, 50°C).

Samples	Concentrations, %	Free RS per flour DM, mg/g	S.D.	OligoRS per flour DM, mg/g	S.D.
Control, 3h	-	15.09	0.43	2.84	1.33
Control, 4h	-	15.51	2.01	2.49	0.80
Celluclast BG	0.1	15.17	0.48	2.33	0.80
	1	21.67	0.48	5.36	0.93
Depol 740L	0.015	16.36	0.53	1.73	0.64
	0.03	16.70	0.21	3.22	0.36
Celluclast BG + Depol 740L	0.1 + 0.03	16.10	0.76	1.66	0.87
	1 + 0.03	24.10	0.37	3.44	0.66
	0.1 + 0.015	16.74	0.47	0.84	0.71
	1 + 0.015	21.98	1.88	7.57	2.22

Table 2. Concentration of Free and Oligo RS in sorghum samples treated Veron CP, Viscozyme L, FiberCare R and their combinations (4h, 50°C)

Samples	Concentrations, %	Free RS per flour DM, mg/g	S.D.	OligoRS per flour DM, mg/g	S.D.
Control, 3h	-	15.09	0.43	2.84	1.33
Control, 4h	-	15.51	2.01	2.49	0.80
FiberCare R	0.1	17.07	0.03	1.98	0.30
	1	24.77	1.51	4.21	1.61
Viscozyme L	0.1	22.58	0.38	0.74	0.41
	1	39.53	0.31	-1.02	1.25
Veron CP	0.1	16.24	0.44	1.12	0.58
	1	19.79	0.47	2.25	0.91
FiberCare R + Veron CP	1+1	28.83	0.25	5.68	0.54
Viscozyme L + Veron CP	1+1	39.18	0.23	3.22	0.49
Viscozyme L + FiberCare R	1+1	41.24	0.30	1.08	0.50

Table 3. Protein solubility of sorghum samples treated by Brewers Clarex, Corolase 7089, FlavourSEB. (2h, 50°C)

Enzyme	Concentration, %	Soluble protein, %	S.D.
Control, 2h	-	5.39	0.31
Control, 4h	-	6.40	0.16
Brewers Clarex	0.050	6.05	0.33
	0.036	5.78	0.22
	0.010	5.70	0.18
Corolase 7089	0.050	6.19	0.13
	0.036	5.79	0.22
	0.010	5.35	0.35
FlavourSEB	0.050	6.04	0.14
	0.036	6.00	0.15
	0.010	6.30	0.50

Table 3. Protein solubility of sorghum samples treated by FlavourSEB, UltraBio, Viscozyme L, BAN 480L. (4h, 50°C)

Enzyme	Concentration, %	Soluble protein, %	S.D.
Control, native pH	-	6.33	0.26
Contol, pH 5	-	5.29	0.42
Contol, pH 4	-	5.09	0.36
FlavourSEB, native pH	0.5	9.25	0.29
FlavourSEB, pH5	0.5	8.72	0.15
FlavourSEB, pH4	0.5	8.10	0.13
FlavourSEB + Viscozyme L	0.5+1	9.72	0.29
FlavourSEB + UltraBio, native pH	0.5+1	9.36	0.28
FlavourSEB+UltraBio, pH5	0.5+1	8.33	0.47
FlavourSEB +UltraBio, pH4	0.5+1	7.86	0.06
BAN 480L	0.2	6.66	0.42
FlavourSEB + BAN 480L	0.5+0.2	8.42	0.21

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Table 4. Free RS content of sorghum samples treated by FlavourSEB, UltraBio, Viscozyme L, BAN 480L. (4h, 50°C)

Enzyme	Concentration, %	Free RS per flour DM, mg/g	S.D.
Control	-	14.66	0.16
FlavourSEB	0.5	35.69	0.59
FlavourSEB + Viscozyme L	0.5+1	48.69	0.12
FlavourSEB+UltraBio	0.5+1	36.63	0.38
BAN 480L	0.2	20.84	0.63
FlavourSEB+BAN 480L	0.5+0.2	32.87	0.34

Table 5. Protein solubility of cowpea samples treated by Brewers Clarex, Corolase 7089, FlavourSEB (2h, 50°C)

Enzyme	Concentration, %	Soluble protein, %	S.D.
Control, 4h	-	29.00	2.29
Brewers Clarex	0.050	34.04	1.29
	0.036	32.64	1.53
	0.010	34.32	1.48
Corolase 7089	0.050	37.53	2.23
	0.036	36.36	2.20
	0.010	32.39	4.48
FlavourSEB	0.050	38.76	1.46
	0.036	37.00	1.85
	0.010	36.50	1.89

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Table 6. Protein solubility of cowpea samples treated by FlavourSEB, UltraBio, Viscozyme L, BAN 480L. (4h, 50°C)

Enzyme	Concentration, %	Soluble protein, %	S.D.
Control 4h, native pH	-	29.00	2.29
Contol 4h, pH 5	-	22.97	1.03
Contol 4h, pH 4	-	17.86	0.71
FlavourSEB, native pH	0.5	47.66	4.83
FlavourSEB, pH5	0.5	27.03	1.15
FlavourSEB, pH4	0.5	23.28	1.77
FlavourSEB + Viscozyme L	0.5+1	49.44	2.37
FlavourSEB + UltraBio, native pH	0.5+1	36.22	0.19
FlavourSEB+UltraBio, pH5	0.5+1	26.25	1.84
FlavourSEB +UltraBio, pH4	0.5+1	55.37	7.09
BAN 480L	0.2	30.97	0.05
FlavourSEB + BAN 480L	0.5+0.2	46.37	2.26

Table 7. Free RS content of sorghum samples treated by FlavourSEB, UltraBio, Viscozyme L, BAN 480L. (4h, 50°C)

Enzyme	Concentration, %	Free RS per flour DM, mg/g	S.D.
Control	-	54.74	4.32
FlavourSEB	0.5	63.70	3.85
FlavourSEB + Viscozyme L	0.5+1	76.06	2.53
FlavourSEB+UltraBio	0.5+1	67.02	1.20
BAN 480L	0.2	108.42	1.32
FlavourSEB+BAN 480L	0.5+0.2	104.58	1.10