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Probiotic Beverage Production from a Mixture of Baobab (*Adansonia digitata* L.) Pulp and Lemongrass (*Cymbopogon citratus* L.) Extract using Lactic Acid Bacteria

A THESIS

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CERTIFICATION

We the undersigned, **Prof NSO Emmanuel JONG and Prof DESOBGO ZANGUE Steve Carly** supervisors of the thesis entittled "**Probiotic Beverage Production from a Mixture of Baobab (*Adansonia digitata* L.) Pulp and Lemongrass (*Cymbopogon citrates* L.) Extract using Lactic Acid Bacteria**" attest that it is the original work of Miss **WIYEH Claudette BAKISU MUALA** (Registration Number: **11I092EN**) submitted in the Department of Process Engineering, University of Ngaoundere in partial fulfillment of the requirements for the award of a Doctorate/PhD Degree in Process Engineering. This work was carried out in the Food Technology and Engineering Laboratory (LAGETA)", of the National School of Agro-Industrial Sciences (ENSAI), University of Ngaoundere (Cameroon) and in the Department of Microbiology and Fermentation Technology, CSIR-Central Food Technological Research Institute, Mysore, India. It has not been presented in any application for a degree or any academic pursuit. All borrowed ideas nationally and internationally have been acknowledged through citations.

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DEDICATION

This work is dedicated to my beloved late dad, Michael Wiyeh Tatah, my mum, Lukong RoseMary Kila, whose greatest wish is to offer me the best in life

and to my daughter Asherinyuy Ngangoum Penielia Sarah

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LIST OF ABBREVIATIONS

AAD: Absolute Average Deviation

ABTS: 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

A_f: Accuracy factor

AOAC: Association of Official Analytical Chemists

AUA: Anhydrouronic Acid Content

B_f: Bias factor

Cfu: Colony forming units

Conc: concentrated

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

DE: Degree of esterification

DP: Degree of Polymerisation

DH: Degree of Hydrolysis

DNS: Dinitro salicylic acid

DM: Degree of Methylation

ES: Enzyme Substrate complex

Ent: Enterococcus

FRAP: Ferric Reducing Ability of Plasma

GAE: Gallic acid equivalent

GalA: Galacturonic acid

GIT: Gastrointestinal Tract

GRAS: Generally Regarded as Safe

HPLC: High Performance Liquid Chromatography

Lb: Lactobacillus

LAB: Lactic Acid Bacteria

PMG: Polymethylgalacturonases

PMGL: Polymethylegalacturonate lyases

PGL: Polygalacturonate lyases

PG: Polygalacturonases

POS: Pectin oligosaccharides

TPC: Total Polyphenol Content

RSM: Response Surface Methodology

RSME: Root Mean Square Error

ROS: Reactive Oxygen Species

SEM: Scanning Electron Microscopy

TE: Trolox Equivalent

LIST OF SYMBOLS

Ø: Diameter

[S]: Pectin concentration

Vmax: maximum reaction rate

n (Hill equation): Degree of coperativity

μ_{max}: Maximum specific growth rate (h^{-1})

Xm: Maximum biomass concentration

Yx/s: Biomass yield coefficient (g biomass g^{-1} glucose)

m_s: Maintenance coefficient (g glucose h^{-1} g^{-1} biomass)

α: Growth associated constant (g lactic acid g^{-1} biomass)

β: Non growth associated constant (g lactic acid g $biomass^{-1}$ h^{-1})

K: Consistency index

n (Rheology): Flow behavior index

τ_0: Yield stress

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ABSTRACT

Beverages occupy a very important part in human diet. Consumers nowadays have special interest for functional beverages such as juice-based products containing probiotics and/or prebiotics with natural preservatives. This work aims to produce a probiotic beverage from baobab juice and lemongrass extract using autochthonous lactic acid bacteria (LAB) from baobab pulp. To achieve this, the pectinaceous matter in baobab pulp was hydrolysed at 50 °C using different enzyme concentrations of 0.03, 0.07, 0.1, and 0.2 (% v/v). The degree of hydrolysis (DH) and rate of enzymatic reaction were determined. The DH increased with time for each enzyme concentration and depectinisation kinetics revealed a sigmoidal curve trend and was modelled using the Hill Equation. The degree of cooperativity (n) decreased from 9 to 5 and Hill extrapolated Vmax increased from 0.336 to 2.378 g/L.min^{-1} with increase in pectinase concentration. Through a decoction extraction process, central composite design was investigated to recover optimal quantity of phenolic compounds from *C. citratus* leaves. The extraction variables were, lemongrass powder/water ratio (2-5 g /100 mL), temperature (85 -95 °C), and time (5-10 min). Antioxidant activity (DPPH), and total polyphenol content (TPC) were monitored as responses. The TPC and DPPH for lemongrass extracts were 71.98±0.33 mg GAE/100 mL extract and 80.63±0.49 mg TE/100 mL extract, respectively under optimal conditions (lemongrass powder/water ratio, 5g/100 mL; temperature, 93.8 ^{o}C and time 11.3 min). The extracted baobab juice and lemongrass obtained were mixed in different proportions, 50/50, 75/25 and 100/0 baobab juice and lemongrass extract then subjected to fermentation. Fermentation was done in batch mode using *lactobacillus fermentum* isolated from baobab and a fermentation kinetics followed. The cell growth rate, reducing sugar, and lactic acid production were evaluated throughout the fermentation process and the results fitted to Monod, Logistic, and Leudeking-Piret equations. At the end of fermentation, a stability study was done for 28 days. Growth was best in the 50/50 baobab/lemongrass probiotic beverage followed by the 75/25 baobab/lemongrass and then the 100/0 baobab. The Logistic model fitted best than the Monod model and the 50/50 baobab/lemongrass probiotic beverage presented best results with a maximum specific growth rate (μmax) and maximum biomass concentration (Xm) of 0.0731 h^{-1} and 13.9100 cfu/mL respectively. While for the substrate utilisation and product formation models, the 75/25 baobab/lemongrass probiotic beverage showed a better fitting than the other cases. The parameters biomass yield coefficient (Yx/s), maintenance energy (ms), growth associated constant (α) and non-growth associated constant (β) were respectively 2.9675 g/g, 0.0003 g/l.g^{-1}, 0.0118 and 0.0038. At the end of 28 days of storage, the viability of the *lactobacillus fermentum* in the 50/50, 75/25 and 100/0 baobab/lemongrass probiotic juice were 8.31, 7.65 and 4.79 respectively at 4 ^{o}C. Therefore, the mixture of baobab and lemongrass powders (50/50) constitutes a good substrate for the production of probiotic juices using *lactobacillus fermentum*.

Keywords: *Adansonia digitata* L., *Cymbopogon citratus*, hydrolysis, decoction, LAB fermentation

RESUME

Les boissons occupent une place très importante dans l'alimentation humaine. Les consommateurs de nos jours ont un intérêt particulier pour les boissons fonctionnelles, telles que les produits à base de jus contenant des probiotiques et/ou des prébiotiques avec des conservateurs naturels. Ce travail vise donc à produire une boisson probiotique à partir du jus de baobab et d'extrait de citronnelle utilisant les bactéries lactiques autochtones de baobab. Pour y parvenir, la matière pectinée de la pulpe de baobab a été hydrolysée à 50 °C avec des concentrations enzymatiques de 0,03, 0,065, 0,1 et 0,2 (% v/v). Le degré d'hydrolyse (DH) et la vitesse des réactions enzymatiques ont été déterminés. Le DH a augmenté avec le temps pour chaque concentration enzymatique et la cinétique de dépectinisation modélisée à l'aide de l'équation de Hill, a révélé une allure de courbe sigmoïde. Le degré de coopérativité (n) a diminué de 9 à 5 et la V_{max} extrapolée de l'équation de Hill a augmenté de 0,336 à 2,378 g/L.min^{-1} avec l'augmentation de la concentration enzymatique. D'autre part, une procédure d'extraction par décoction a été mise en œuvre pour extraire les composés phénoliques totaux (CPT) des feuilles de *C. citratus*. Dans le but d'optimiser le processus d'extraction, un plan composite centré dont les variables sont la concentration en poudre de citronnelle (2-5 g/100 mL), la température (85 -95 °C) et le temps (5-10 min) a été mis en œuvre. Les réponses suivies sont, l'activité antioxydante (DPPH) et la teneur en CPT. Dans les conditions optimales déterminées, concentration en poudre de citronnelle (5 g/100 mL), température (93.8 °C) et temps (11.3 min), les valeurs optimales des CPT et l'activité antioxydante d'extraits de la citronnelle étaient respectivement de 71,98 ± 0,33 mg GAE/100 mL d'extrait et de 80,63 ± 0,49 mg TE/100 mL d'extrait. Le jus de baobab et les extraits de citronnelle obtenus ont été mélangés à des différentes proportions (50/50, 75/25 et 100/0 baobab et citronelle) et soumis à une fermentation. Une fermentation discontinue a été effectuée en utilisant *lactobacillus fermentum* isolé du baobab, suivi d'une cinétique de fermentation. La concentration en biomasse, la production de sucre réducteur et d'acide lactique ont été évaluées tout au long du processus de fermentation et les résultats ajustés aux équations de Monod, Logistic et Leudeking-Piret. A la fin de la fermentation, l'étude de la stabilité a été réalisée. La croissance a été meilleure dans la boisson probiotique à 50% de baobab, suivie de 75% puis 100% de baobab. Le modèle logistique explique mieux le processus que le modèle Monod et la boisson probiotique à 50/50 de baobab a présenté les meilleurs résultats avec une vitesse maximale (μ_{max}) et une concentration maximale en biomasse (Xm) de 0,0731 h^{-1} et 13,9100 cfu/mL respectivement. Alors que pour les modèles d'utilisation de substrat et de formation de produit, la boisson probiotique à 75/25 de baobab s'est mieux illustrée comparée aux autres avec un Yx/s, ms, α et β respectivement de 2,9675 g/g, 0,0003 g/l.g^{-1}, 0,0118 et 0,0038. Après 28 jours de stockage, la viabilité du *lactobacillus fermentum* dans les jus probiotiques à 50/50, 75/25 et 100/0 de baobab et citronelle étaient respectivement de 8,31, 7,65 et 4,79 à 4 °C. De ce fait, le mélange de poudres de baobab et de citronnelle a un ratio de 50:50, est le substrat idéal pour la production de jus probiotique utilisant *lactobacillus fermentum*.

Mots clés : *Adansonia digitata L.*, *Cymbopogon citratus*, hydrolyse, décoction, fermentation lactique.

GENERAL INTRODUCTION

There is an increasing consumer interest in health-enhancing foods such as functional foods. These are products which are beneficial to one or more target functions in the body, beyond adequate nutrition, in a way that improves health and well-being or reduces the risk of diseases (Roberfroid, 2000a). Amongst functional foods, probiotics and prebiotics are the most prominent. Probiotics are defined as ‘‘live microorganisms, which when consumed in adequate amounts confer a health benefit on the host’’ (FAO/WHO, 2006). Microorganisms of the genera Lactobacillus and Bifidobacterium are the most employed as probiotic bacteria (Siro *et al*., 2008; Song *et al*., 2012). These probiotics exhibit several important properties such as anti-inflammatory, antiallergic, immunomodulatory, antimutagenic, anticarcinogenic, and antimicrobial properties (Saadat *et al*., 2019; Shah, 2001). Probiotics have been mostly applied in dairy products. There is however an increasing demand and a growing market for non-dairy probiotic products and an increase in consumer vegetarianism. Non-dairy products are rich sources of protein, vitamins, minerals, dietary fibres, bioactive compounds suitable of probiotic survival and stability (Min *et al*., 2018; Vasudha and Mishra, 2013). Fruits and vegetables have thereby been exploited in the production of non-dairy probiotic beverages.

Baobab (*Adansonia digitata* L.) is an iconic plant indigenous to Africa and known for its numerous importance in human nutrition and health (Namratha and Sahithi, 2015). It has a unique fruit type with a slight sour taste. It is considered a ‘super fruit’ due its rich nutritional content. The fruit pulp is an excellent source of vitamin C and its vitamin C content is ten times higher than that of oranges. It is also a good source of calcium, potassium, iron, pectin and carbohydrates (Adekanmi *et al*., 2013; Chabite *et al*., 2019; Coe *et al*., 2013). Pectin are complex polysaccharides present in the cell wall of higher plants. It is a methyl esterified polygalacturonic acid joined in chains by α-(1, 4)-glycosidic bonds (Sriamornsak, 2003).

It causes cloudiness and increase viscosity of juice which can present subsequent pumping and mixing difficulties during processing of fruits. Moreover, they are complex carbon sources for microbial metabolism during fermentation and therefore necessity of pre-treatment to hydrolyse into fermentable sugars (Abbasiliasi *et al*., 2017). Hydrolysis can either be by acid hydrolysis followed by ethanol precipitation or by enzymatic hydrolysis. Acid hydrolysis however, has drawbacks such as it is an analytical method and a low yield is obtained at the end when compared to enzymatic hydrolysis (Locatelli *et al*., 2019; Sandarani, 2017). On the other hand, enzymatic hydrolysis is specific, increases juice yield, and safe (Siewe *et al*., 2020). Pectic enzymes (pectinlyase, pectinmethylesterase, polygalacturonases) (Sharma *et al*., 2014) act by trans elimination or hydrolysis of pectins (Tapre and Jain, 2014) to release galacturonic acid and oligomers (POS) (Combo *et al*., 2012). Baobab also has its natural microbial flora made of LAB (Chadare et al., 2010; Cisse and Montet, 2012). This LAB can therefore be isolated from baobab pulp and used as autochthonous starter in the fermentation of baobab juice. Autochthonous starters are advantageous because they prolong shelf life, improve nutritional, and sensory properties of the fermented foods (Di Cagno *et al*., 2013; Terzic-Vidojevic *et al.*, 2015). The shelf life of fermented beverages can also be further extended by the use of natural preservatives. The search for products with natural preservatives is one of the key global market trends today (Caleja *et al*., 2016; Illupapalayam *et al*., 2014). Several herbal extracts have been employed in fermented foods amongst which are: green, white and black tea (Muniandy *et al*., 2016); green tea, roselle, white tea, and lemongrass aqueous extract (Granato *et al*., 2017). Lemongrass (*Cymbopogon citratus*) is a tropical perennial plant that belongs to the Poaceae family with a lemon scent (Asaolu *et al*., 2009; Boeira *et al*., 2018). It has a high crude protein content and contain nutrients such as fibre and minerals, and also several important bioactive compounds (Adeyemo *et al*., 2018; Nambiar

and Matela, 2012; Ojo, 2017). The bioactive compounds and essential oils act as natural antioxidants and anti-inflammatory agents(Godwin *et al*., 2014). Looking at the high demand of functional foods, the nutrient content and properties of baobab pulp and lemongrass, it is therefore of great interest to investigate the use of autochthonous starter culture from baobab pulp in the fermentation of baobab pulp-lemongrass extracts. Fermentation challenges however involve both upstream processes and the fermentation process. Both parameters however, have to be taken in to consideration during processing. The problem was therefore, the potential of LAB isolated from baobab pulp in the fermentation of baobab and lemongrass juice. This work therefore aims to produce a probiotic-fermented beverage from baobab - lemongrass extracts using autochthonous LAB from baobab pulp. Specifically, the goal was to:

1. Optimise hydrolysis conditions of baobab pulp pectin.
2. Determine optimum decoction conditions for the extraction of bioactive components of lemongrass.
3. Determine kinetic parameters of growth and substrate utilisation models of lactic acid fermentation of baobab-lemongrass juice.

The specific objectives are sustained by the following hypotheses:

1. Hydrolysis of baobab pectin depends on the pectinase concentration and hydrolysis time.
2. Extraction of bioactive components in lemongrass depends on lemongrass concentration and decoction temperature.
3. Increase in the concentration of lemongrass extract improves the kinetic parameters of growth and substrate utilisation models.

CHAPTER 1: LITERATURE REVIEW

1.1 Functional food concept

During the past two decades, the health/nutrition paradigm has changed significantly in which food is not merely considered to supply essential nutrients for proper growth and development, but as a route to optimal health (Granato *et al*., 2010). This spell out the concept of functional foods. This term was first coined out in Japan in 1984, and was defined as food products fortified with special constituents that possess advantageous physiological effects (Martirosyan and Singh, 2015). Japan termed functional foods as Foods for Specific Health Uses (FOSHU) (Roberfroid, 2000a). So far, there is no unanimous definition for this group of food *(Siro´ et al*., 2008) but the functional food centre (FFC) defined functional food as Natural or processed foods that contains known or unknown biologically-active compounds; which in defined amounts provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease (Martirosyan and Singh, 2015).

All foods are therefore functional at some physiological level, because they provide nutrients or other substances that furnish energy, sustain growth, or maintain/repair vital processes (Granato *et al*., 2010). Changes in world food economy and lifestyle has resulted in a shift in dietary patterns which has led to an increase in cardiovascular diseases, hypertension, obesity, osteoporosis and periodontal diseases (Ares *et al*., 2014; WHO, 2003). Consumers nowadays however, believe that foods contribute directly to their health (Mollet and Rowland, 2002). Functional foods therefore, play an outstanding role, due to their increasing demand (Bigliardi and Galati, 2013; Roberfroid, 2000b). Accordingly, there is the development of a variety of new functional food products by the food industry, which has increased the supply for such products in the marketplace (Granato *et al*., 2010). Functional foods includes designer foods, medicinal foods, nutraceuticals, pharmafoods, vitafoods, and probiotics (Arihara, 2014; Shah, 2001). Among the functional components,

probiotics, prebiotics, plant antioxidants, vitamins and calcium are the most important and the most frequently demanded (Grajek *et al*., 2005).

1.2 PROBIOTICS

Probiotics are “life microorganisms which when administered in adequate amounts confer a health benefit on the host’’ (FAO/WHO, 2006). While probiotic food products are fermented foods containing sufficient amount of viable and active microorganisms enough to reach the intestine and exert an equilibrating action on the intestinal microflora (FAO/WHO, 2002).

1.2.1 Probiotic microorganisms

Most probiotics are generally; gram-positive, catalase-negative, rods with rounded ends, and occur in pairs, short, or long chains. They are non-flagellated, non-motile and non-spore-forming, and are intolerant to salt (Song *et al.*, 2012). Lactic acid bacteria (LAB) are a large group of closely related bacteria that have similar properties such as lactic acid production, which is an end product of fermentation. LAB include Lactobacillus, Lactococus, Streptococcus and Leuconostoc species (Chelule *et al*., 2010). Table 1 represents the different probiotic microorganisms. The optimum growth temperature for most probiotics is 37 ^{o}C but some strains such as *Lb. casei* prefer 30 ^{o}C and the optimum pH for initial growth is 6.5-7.0. *Lb. acidophilus* is microaerophilic with anaerobic referencing and capability of aerobic growth. Bifidobacterium are anaerobic but some species are aero-tolerant (Song *et al*., 2012).

Table 1: Microorganisms considered as probiotics

Lactobacillus species	Bifidobacterium species	Other LAB	Non-LAB
Lb. acidopphilus	*B. adolescentis*	*Ent. faecalis*	*Bacillus cereus*
Lb. amylovorus	*B. animalis*	*Ent. faecium*	*Escherichia coli*
Lb. casei	*B. bifidum*	*Sporolactobacillus inulinus*	*Propionibacterium freudenreichii*
Lb. crispatus	*B. breve*		*Saccharomyces cerevisiae*
Lb. delbrueckii subsp. bulgaricus	*B. infantis*		
Lb. gallinarum	*B. lactis*		
Lb. gasseri	*B. longum*		
Lb. johnsonii			
Lb. paracasei			
Lb. plantarum			
Lb. reuteri			
L. rhamnosus			

1.2.2 Selection of appropriate strains

Bifidobacteria prevalent in the faeces of breast-fed infants could be a major reason for its selection for use as probiotics. There are a number of favourable factors that determine the use of Lactobacillus strains: their association with traditional fermented foods; their association with the human gastrointestinal tract (GIT) and beneficial interactions in the gut ecosystem; the adaptation of many lactobacilli to milk and other food substrates (Holzapfel, 2006). The food industry is faced with a major challenge of selecting new strains. The primary objective is to select microbial strains with one or more proven functional properties. Though probiotic microorganisms are suggested to

promote health and well-being, the challenge as to defining particular end points or biomarkers by which such strains can be characterized, remains to be elucidated. Desirable technical features and factors related to health promotion or sustaining health serve as important criteria for strain selection (Holzapfel, 2006). There are therefore different characteristics for strain selection.

- **Characteristics of probiotics**

Characteristics of probiotics determines their ability to survive the upper digestive tract and to colonize the intestinal lumen and colon for an indefinite time period (Song *et al*., 2012). For microorganisms to be considered as probiotic strains, they must fulfil a number of criteria linked to safety, functional effects and technological properties (FAO/WHO, 2002).

For safety, most probiotic strains are considered commensal microorganisms with no pathogenic potential (Salminen *et al*., 1999). They are considered safe when they possess: their intrinsic properties (antibiotic resistance, excessive degradation of mucus); pharmacokinetic properties; commensal interaction between the strain and the hosts, haemolytic activity, metabolic activities (D-lactate, bile salt de-conjugation); (Awaisheh, 2012; Barbés, 2008).

To be recognized as functional food components, the strains must be able to survive and grow under the physiological conditions of the desired ecological unit (Barbés, 2008). They should therefore have the following properties: acid- and bile-stability, resistance to digestive enzymes, adhesion to intestinal surface. They should also have, antagonistic activity against human pathogens, anti-carcinogenic and anti-mutagenic activity, cholesterol-lowering effects. Stimulation of the immune system without inflammatory effects, maintenance of mucosal integrity, improvement of bioavailability of food compounds and production of vitamins and enzymes (Sharma *et al*., 2012) are also important functional properties.

The technological properties of bacteria play a very significant role in the production of probiotics (Saarela *et al*., 2000). During product manufacture and storage, strain viability and the maintenance of desirable characteristics are also a prerequisite for probiotic strains. Strain survival depends on factors such as the final product pH, the presence of other microorganisms, the storage temperature, and the presence or absence of microbial inhibitors in the substrate. Proper growth and pleasant aroma and flavour profiles are of importance when developing probiotic functional foods (Barbés, 2008).

Several probiotic strains have been isolated with respect to the characteristics of probiotics from different sources. To continually satisfy the increasing demand probiotics with more active cultures and better probiotic characteristics in the market, studies on the microbial diversity of unexplored sources have led to the isolation of an endless number of novel bacterial species (Di Cagno *et al*., 2009). To this effect, fruits and vegetables have served as a good matrix for the isolation of LAB. Table 2 summarises some sources which LAB has been isolated from and amongst them, to the best of our knowledge, LAB has not yet been isolated from baobab.

Table 2: Different sources of isolation of Lactic Acid Bacteria

Source	**Strain**	**Reference**
Turmeric (*Curcuma longa* Linn.)	*Enterococcus faecium, Lactococcus lactis Lactobacillus plantarum*	(Pianpumepong and Noomhorm, 2010)
Sweet cherry (*Prunus avium* L.)	*Lactobacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus and Leuconostoc mesenteroides*	(Di Cagno *et al*., 2011b)
Fermented table olives	*Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus paraplantarum*	(Bautista-Gallego *et al*., 2013)
Wine	*Pediococcus pentosaceus, Lactobacillus casei Lactobacillus plantarum*	(García-Ruiz *et al*., 2014)
Fermented mustard	*Lactobacillus plantarum*	(Wang *et al*., 2014)
Palm tree	*Lactobacillus pentosus Lactobacillus plantarum Lactobacillus brevis*	(Fossi *et al*., 2015)
Fermented foods and beverage	*Lactobacillus brevis Lactobacillus plantarum*	(Angmo *et al*., 2016)
kimchi	*L. mesenteroides, L. plantarum*	(Lee *et al*., 2016)
Banana	*Lactobacillus musae sp.*	(Chen *et al*., 2017)
Kahudi (traditional rapeseed fermented food)	*E. durans, L.plantarum, L. fermentum, L. casei*	(Goswami *et al*., 2017)

1.2.3 Growth requirements of probiotics

LAB are fastidious organisms that require complex nutrition for growth (De Vos *et al*., 2009). This is because they require both carbon and nitrogen sources for growth and product formation (Abbasiliasi *et al*., 2017). Glucose is the main carbon source for microbial growth and bacteriocin production. In general, LAB cannot ferment complex carbohydrates unless they are

pre-treated and enzymatically hydrolysed (Abbasiliasi *et al*., 2017; Lubeck and Lubeck, 2019). LAB obtain their energy from the metabolism of simple sugars, and in addition depends on exogenous sources for amino acids, vitamins, purines and pyrimidines (Lubeck and Lubeck, 2019). Pantothenic acid and nicotinic acid are required by all species except some few strains while thiamine is only necessary for the growth of the heterofermentative lactobacilli. Folic acid, riboflavin, pyridoxal phosphate, and *p*-aminobenzoic acid are also necessary for growth of various species with riboflavin being the most frequently required compound (De Vos *et al.*, 2009; Snell, 1945). Snell and Strong (1939) tested the requirement of riboflavin for growth of LAB and out of eleven organisms tested, three proved unable to grow in its absence. Those which grew in the absence of riboflavin synthesized it.

Amino acids such as adenine, guanine, uracil, thymine have been shown as growth factors for several LAB (Snell, 1945). A survey of the literature on amino acid requirements of lactic acid bacteria revealed that glutamate and valine are required by most species of Lactobacillus, Streptococcus, Leuconostoc, and Pediococcus that have so far been examined (Morishita *et al*., 1981).

1.2.4 Health benefits of probiotics

Health benefits of probiotic bacteria are very strain specific; thus, there is no strain that would provide all benefits (Song *et al*., 2012). LAB confer preservative and detoxifying effects on foods and fermented LAB foods boost the immune system and strengthen the body in the fight against pathogenic bacterial infections. They play an important role in immunological, digestive and respiratory functions in humans (Chelule *et al*., 2010). Probiotic beverages are thereby gaining importance because of their numerous benefits. LAB exhibit several health benefits such as: antimicrobial activity, improvement in lactose metabolism, reduction in serum cholesterol,

immune system stimulation, antimutagenic properties, anti-carcinogenic properties, anti-diarrheal properties, improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection by addition of selected strains to food products (Shah, 2001; Shinde *et al*., 2012). To confer these health benefits, they have several mechanisms of actions.

1.2.5 Mechanism of action of probiotics

The mechanisms by which probiotics beneficially affect human health are either by direct antagonism to pathogens, barrier function and prevention of adhesion, competition for nutrients and Immunity stimulation or modulation.

- **Direct antagonism**

This may be mediated by antibacterial agents produced and secreted by the probiotic organisms (Fuller and Gibson, 1997). Probiotic organisms produce organic acids at the end of fermentation that lowers the pH of the medium thereby interfering with the maintenance of the membrane potential of bacterial cytoplasmic membrane and inhibits active transport (Blandino *et al*., 2003). They also produce antimicrobial compounds such as hydrogen peroxide, bacteriocins or bacteriocin-like substances, biosurfactants. Biosurfactants are surface-active compounds produced by microorganisms that are able to reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures. It has also been suggested that biosurfactants could be involved in microbial adhesion (Barbés, 2008). Probiotic bacteria produce deconjugated bile acids which are derivatives of bile salts. Deconjugated bile acids show a stronger antimicrobial activity compared to the bile salts synthesized by the host organism (Oelschlaeger, 2010).

- **Barrier function and prevention of adhesion**

Adhesion to intestinal mucosa is one of the major selection attributes for probiotics as it is required for intestinal colonization, and is also important for modulation of the immune system and antagonism against pathogens. LAB display various surface determinants that are involved in their interaction with intestinal epithelial cells and mucus which help competitive exclusion of pathogens from the mucus. Several Lactobacillus proteins (along with saccharide moieties and lipoteichoic acids) have been shown to promote mucous adhesion and bacteria display surface adhesions that mediate attachment to the mucous layer. Bacterial adhesins (mucus-binding protein) have been reported from *Lb. reuteri*. When lactobacilli are ingested, they compete for binding sites, leaving fewer binding sites open for pathogens. Pathogens pass through the gut and leave the body sooner when no binding site is available (Bajaj *et al*., 2015). Not only adhesion to but also invasion of epithelial cells is an important property for full pathogenicity of many gut pathogens (Oelschlaeger, 2010).

- **Competition for nutrients**

The gut is so rich in nutrients but the limitation of just one essential nutrient may cause inhibition. The presence of health promoting bacteria in the gut, utilize more nutrients, leaving fewer nutrients for pathogenic bacteria, which may suffer starvation, and not survive. The competitive exclusion thus prevents the proliferation and growth of pathogens in the gut environment (Bajaj *et al*., 2015). Iron is one of the essential nutrients for most bacteria with the exception of lactobacilli but is often available in limited amounts. This might be a crucial advantage in competition with other microorganisms which depend on iron. Nevertheless, some probiotics such as *Lb. acidophilus* and *Lb. delbrueckii* bind ferric hydroxide at their cell surface, and make it unavailable to pathogenic microorganisms. Thus, probiotic bacteria alter the physical

environment in such a way that the pathogenic bacteria cannot survive (Bajaj *et al*., 2015; Oelschlaeger, 2010).

- **Immunity stimulation or modulation**

Probiotics can influence the immune system by products like metabolites, cell wall components and DNA. Immune modulatory effects might be achieved with dead probiotic bacteria or just probiotics-derived components like peptidoglycan fragments or DNA. LAB products exert immunomodulatory activity via inhibition of inflammatory responses, regulation of the expression of Toll like Receptors, activation of Dendritic cells and Natural Killer cells in innate immunity; proliferation of lymphocytes, balancing T-helper (Th1/Th2) cells responses, secretion of specific IgA, among several other ways. Probiotic bacteria exert its beneficial effects and modulate the immune system of the host against potentially harmful antigens via activation of lymphocytes and production of antibodies. The colonization of healthy microbes leads to maturation of the humoral immune mechanisms, particularly circulation of the IgA and IgM secreting cells (Bajaj *et al*., 2015).

- **Degradation of toxin receptors**

Probiotics modify toxin receptor through an enzymatic mechanism, because of the degradation of toxin receptor on the intestinal mucosa. Other mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence. Probiotics can also modify toxin receptors and block toxin-mediated pathology. Probiotics may also promote nonspecific stimulation of the host immune system, including immune cell proliferation, enhanced phagocytic activity of macrophages, and increased production of secretory immunoglobulin IgA and IgM (Bajaj *et al*., 2015).

1.2.6 Incorporating probiotics to food

For decades, probiotics have been used in fermented dairy products such as yogurts and fermented milks, where the techniques and technologies to incorporate the probiotics are now relatively advanced. Food companies are searching for ways to incorporate these ingredients into a broader range of foods and beverages while keeping them alive throughout shelf life (Crittenden, 2009). Food regulatory bodies demands that at the time of consumption of probiotic beverages, they should have a LAB load of 10^6–10^8 cfu/g. The viable count of probiotic organisms generally declines during product storage but an acceptable viable count can sometimes be achieved by introducing higher numbers of probiotics during manufacture (overage). The consumption of probiotic organisms at high doses is safe and appears not to pose a health risk. There may also be organoleptic limitations to the amount of probiotics that can be acceptably added to foods. Therefore, it is vital to maintain the viability of probiotics in foods during production and storage (Crittenden, 2009).

When incorporating probiotics into foods, there are five main points to be addressed:

1. Select a compatible probiotic strain/food type combination.
2. Use food-processing conditions that are compatible with probiotic survival.
3. If fermentation is required, ensure that the food matrix will support probiotic growth.
4. Select a product matrix, packaging, and environmental conditions to ensure adequate probiotic survival over the product's supply chain and during shelf storage.
5. Ensure that addition of the probiotic does not adversely impact on the taste and texture of the product.

Considering all the above points in order to select a compatible strain for fermentation of baobab juice, this work was therefore based to isolate LAB from baobab and use it in the fermentation of baobab and lemongrass juice.

1.3 Baobab

1.3.1 Generalities on Baobab

African baobab is a member of the Bombacaceae family with the binomial *Adansonia digitata* given by Linnaeus, the generic name honouring Michel Adanson who had been to Senegal in the eighteenth century and described Baobab. African baobab is a very long-lived tree with some trees over 1000 years old (Sidibe *et al*., 2002). Baobab (*Adansonia digitata* L.) is widely grown throughout sub-Saharan Africa and has been successfully introduced out of Africa. It is related to 7 other species: *A. digitata L., A. grandidieri Baillon, A. suarezansis H., A. gibbosa* (*A. Cunn*.), *A. rubrostipa* Jum. & H. Perrier, *A. madagascarensis* Baill, *A. za* Baill., *A. perrieri* Capuron (Baum, 1995; Sidibe *et al*., 2002).

Several names are used to describe the baobab depending on its geographical location. It is also known as the dead-rat tree (from the appearance of the fruits), monkey-bread tree (the dry fruit is a source of food for monkeys), upside-down tree (as the bare branches resemble roots) and cream of tartar tree (due to the acidic taste of the fruits) (Kamatou *et al*., 2011; Sidibe *et al*., 2002). The species name digitata (hand-like) is in reference to the shape of the leaves. The African baobab tree is typified by its massive size, reaching to a height of 18-25 m, producing rounded crowns and showing a stiff branching habit. The trunk is swollen and stout, up to 10 m in diameter (Sidibe *et al*., 2002). The baobab tree is the most drought resistant tree. During the rains, the tree absorbs water and thus swells greatly storing thousands of litres of water in their trunk for later use (Sundarambal *et al*., 2015). Branches are large and distributed irregularly, the bark is smooth,

reddish brown to grey, soft and fibrous (Sidibe *et al*., 2002). It takes about eight and twenty-three years before the baobab produces seeds and the mature plant (over 60 years) can produce more than 160–250 fruits per year. It grows on a wide range of well-drained soils, from clays to sands, but not on deep unconsolidated sands, where it is unable to obtain sufficient moisture (Kamatou *et al*., 2011).

Figure 1: African baobab (*Adansonia digitata* L.) tree
Source: (Chadare *et al*., 2010)

1.3.2 Baobab fruit

The baobab fruit (figure 2) is composed of an outer shell (epicarp) (45%), fruit pulp (15%) and seeds (40%). The woody epicarp or pod contains the internal fruit pulp (endocarp) which is split in small floury, dehydrated and powdery slides that enclose multiple seeds and filaments, and

the red fibres, subdivide the pulp in segments as in figure 2 (De Caluwé *et al*., 2010; Namratha and Sahithi, 2015).

Figure 2: Baobab fruit pulp

Source: (Chadare *et al*., 2010)

- **Fruit pulp**

The baobab fruit pulp is one of the most important part of the tree. It is dry, acidulous and mealy, and rich in mucilage, pectins, tartarate and free tartaric acids. The presence of the tartarate gives rise to the name 'cream of tartar tree' (Sidibe *et al*., 2002). The pulp is rich in several nutrients.

1.3.3 Chemical Composition of baobab pulp

A. digitata fruit is known as super fruit because of its rich nutrient profile (Gruenwald, 2009; Namratha and Sahithi, 2015). The chemical composition of baobab pulp is presented in table 3 and differs according to different literature sources (Aluko *et al*., 2016; Bamalli *et al*., 2014a;

Ibrahima *et al*., 2013; Osman, 2004). The fruit pulp contains a high amount of carbohydrate, low protein, and extremely low fat (Osman, 2004). Murray *et al.* (2001) reported simple sugars in baobab pulp account for about 35.6% of the total carbohydrate content which explains the noticeable sweet taste of the pulp. However, the sweetness may vary for different types of pulp (Chadare *et al*., 2009). Nour *et al*. (1980) reported the absence of starch in the pulp and it also has high dietary fibre, much higher than other dehydrated fruits containing 50.57 g/100g compared with only 1.88 g/100g in dried banana and 3.96 g/100g in dried apricots (Leatherhead, 2009). The baobab fruit pulp is acidic, due to the presence of organic acids: citric, tartaric, malic, succinic and ascorbic (Bamalli *et al*., 2014a; Nour *et al*., 1980). Baobab pulp is rich in pectin ranging from 23.4 to 30.00 g/100g (Leatherhead, 2009). The pectin is mainly water soluble and has a low degree of esterification and a low intrinsic viscosity. This implies it will yield a low quality jell of high solids content, because it tends to precipitate rapidly in acid media to form irregular gels (Bamalli *et al*., 2014a).

The baobab pulp is an excellent source of potassium, calcium, and magnesium, but poor sources of iron, zinc, and copper (Osman, 2004). The pulp has an exceptionally high calcium content (Osman, 2004) which is rare in fruits and vegetables (Leatherhead, 2009). Baobab contains 4 times the amount of calcium found in dehydrated apricots and 13 times that of dehydrated apples (Leatherhead, 2009). The high calcium contents of the fruit pulp make the baobab fruit attractive as a natural source of calcium supplementation for pregnant and lactating women, as well as for children and the elderly (Osman, 2004).

Vitamin C and some of the B vitamins are the main vitamins found in baobab fruit pulp. The vitamin C content in baobab dried fruit pulp is tenfold higher than that in dried peaches and apricot. In addition, this value is twice the amount of ascorbic acid commonly detected in fresh oranges

(46 mg/100g) or fresh strawberries (61 mg/100g) (Leatherhead, 2009). The vitamin C content contributes to its overall antioxidant capacity and is a good source of polyphenols in baobab (Bamalli *et al*., 2014a).

Table 3: Nutritional composition of baobab fruit pulp

Macronutrients (g/100g)	
Protein	2.04-3.24
Fat	0.4-0.70
Total dietary fibre	45.8-53.90
Total carbohydrate	78.3-78.90
Total sugars (as glucose)	16.9-25.30
Ash	5.5-6.60
Pectin	23.4-33.80
Micronutrients (mg/100g)	
Sodium	7.00-31.10
Potassium	2010.00 - 2390.00
Calcium	257.00 - 370.00
Magnesium	126.00 - 179.00
Phosphorus	56.10 - 73.30
Iron	3.95 - 9.13
Copper	0.53 - 0.75
Zinc	0.70 - 1.02
Manganese	0.65 - 1.30
Vitamins (mg/100g)	
Vitamin C	74.00 - 163.00
Thiamine	0.01 - 0.09
Riboflavin	0.01 - 0.03

Source:(Leatherhead, 2009)

- **Amino Acid and fatty acid Profile**

Most essential amino acids are present in baobab fruit pulp as shown in table 4. They have a high content of tyrosine, glutamic acid, aspartic acid, arginine, and glycine and low amounts of sulphur containing amino acids, namely cystein and methionine (Chadare *et al*., 2009; Osman, 2004). Most fatty acids in the pulp do not reach detectable levels with oleic acid being the highest reported value among all fatty acids (25 mg/g dw).

1.3.4 Baobab flora

Afolabi and Popoola (2005) evaluated the effect of baobab pulp powder on the micro flora involved in tempe fermentation (a nutritious fermented food obtained by the fermentation of soybeans using the fungus *Rhizopus oligosporus*) and discovered an increase population in LAB when baobab pulp concentrations were increased in tempe fermentation. The LAB dominating in the fermented tempe were; *Lb. plantarum, Lb. fermentum, Lb acidophilus* and *Rhizopus* sp. It was speculated that the source of LAB could be due to its presence in baobab pulp. Cisse *et al.* (2009) and Cisse and Montet (2012) evaluated the microbial load of baobab pulp before pasteurization for the production of baobab nectar and they noticed the presence of LAB and other microorganisms. Also, Makawi *et al.* (2019) fermented sorghum flour using different concentrations of baobab pulp as starter. They noticed higher concentrations of starter enhanced protein, fibre, ash, and major mineral contents of the fermented sorghum. In addition, increased in the concentration of starter also led to an increase in the microbial load, particularly LAB, of the fermented samples. LAB has also been identified in Maari, a fermented food condiment obtained by spontaneous fermentation of seeds from the baobab tree (*Adansonia digitata*) (Kaboré *et al*., 2012). Due to these works, there is therefore the presence of LAB in baobab fruit pulp which can serve as a good matrix for LAB isolation.

Table 4: Amino acid and fatty acid profile of baobab fruit pulp

Amino acids (g/100g proteins)	**Range**
Alanine	3.3- 8.2
Arginine	4.4 – 8.4
Aspartic acid	5.2 – 11.0
Cysteic acid	1.0 – 1.7
Glutamic acid	4.1 – 14.6
Glycine	2.9 – 11.4
Histidine	1.2 – 3.4
Isoleucine	2.2 – 5.1
Leucine	4.1 – 7.6
Lysine	1.7 – 6.0
Methionine	0.2 – 4.9
Phenylalanine	2.1 – 4.4
Prolamine	2.2 – 5.1
Proline	5.4 - 8.7
Serine	2.2 – 4.4
Threonine	2.4 – 2.8
Tryptophan	0.7 – 6.4
Tyrosine	0.9 – 20.6
Valine	3.8 – 6.0
Fatty acids (mg/g dw)	
C14:0 (Mystiric)	0.2 – 0.2
C16:0 (Palmitic)	0.2 – 27.0
C18:0 (Stearic)	3.3 – 3.3
C18:1 (Oleic)	25 – 25
C18:2 (Linoleic)	0.0 - 27.0
C18:3 (Linolenic)	0.2 – 0.9
C20:0 (Arachidic)	0.7 – 0.7
C20:1 (Gadoleic)	0.04 – 0.04

1.3.5 Medicinal uses of baobab

In the Sahel (Africa), the tree is called "Mother of the Sahel" by some people because more than thirty different uses of the tree are known. The pharmacological active substances in baobab are responsible for its beneficial effects. The various parts of the plant (leaves, bark, seeds) are used as a panacea, for the treatment of malaria, tuberculosis, fever, microbial infections, diarrhoea, anaemia, dysentery, toothache, etc. The fruit is used for the management of malaria, febrifuge, small pox, measles, dysentery, wound disinfection, eye lotion and general fatigue of children (Kamatou *et al*., 2011). They possess several pharmacological properties. They have antioxidant, anti-inflammatory, antipyretic, analgesic and antimicrobial properties (Kaboré *et al*., 2011; Kamatou *et al*., 2011; Kumar *et al*., 2016; Vertuani *et al*., 2002).

1.3.6 Applications of baobab pulp

Adansonia digitata, a Non-Timber Forest Product (NTFPs) is a multipurpose tree as every part of the plant are consumed mostly by rural population in Africa, often the main ingredient in sauces, pastes, porridges and beverages (De Caluwé *et al.*, 2010). The pulp and leaves have high antioxidant content there by can be considered as functional foods.

The fruit pulp is the most important part of the tree. Since 2008, the fruit pulp has been authorized as a novel food in Europe where as in the United States of America, the fruit pulp was approved as a food ingredient in 2009 (Sokeng *et al*., 2019).The dried pulp is commonly used to prepare fruit juice or mixed with porridge or gruels in parts of East Africa to benefit from generally higher levels of vitamin C than oranges and calcium than cow's milk. Dissolved pulp is added to cooled gruels after cooking to preserve the vitamin C content of the pulp (Bamalli *et al*., 2014). The dissolved pulp is also mixed with porridge of maize, (roasted) sorghum or millet, sorghum paste or the dry pulp can be mixed with sugar and milk or water for beverages. Dissolving the powder

together with sugar in water forms a solute, which has a syrup-like texture and when frozen in small plastic bags, gives ice lollipop. A less sophisticated sweet snack consists of directly sucking the baobab fruit pulp (De Caluwe *et al.*, 2009). In Tanzania, it is added to aid fermentation of sugar cane for beer making and is also a substitute for cream of tartar in baking (Bamalli *et al*., 2014) and also employed as ingredients in ice products, juice and jams (De Caluwé *et al*., 2010). The local process for transformation of baobab pulp to baobab juice is as shown in figure 3.

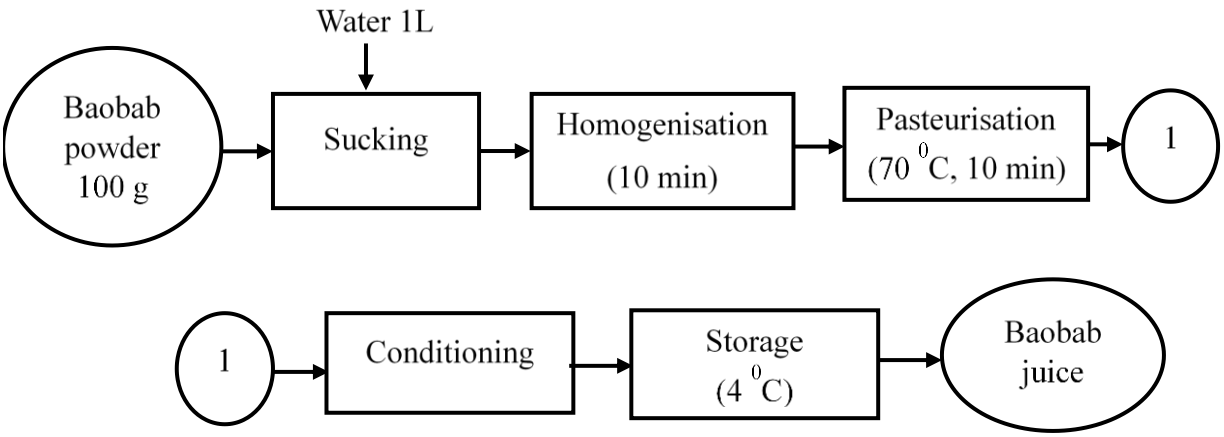


Figure 3: Processing of baobab powder to baobab juice

The baobab fruit pulp can either be sucked directly or first transformed to powder before sucking. Baobab fruits are cracked, and the pulp milled in a mortar and pestle to separate the pulp powder from the fibres and seeds. The pulp powder is sieved with a 1 mm test sieve shaker to obtain the fine powder. Tap water heated and held at boiling point (100 ^{o}C, 10 min) to inactive potential spoilage microorganisms and cooled to 45 ^{o}C is used to suck the baobab powder (100 g/L of water). The mixture is homogenized for 10 min and pasteurized at 70 ^{o}C for 10 min. The juice is immediately cooled in running tap water, conditioned in bottles and stored at 4 ^{o}C for consumption (Cisse and Montet, 2012; Tembo *et al*., 2017).

Aluko *et al.* (2017), enriched probiotic yoghurt with baobab pulp and there was an increase in the nutritional content of the baobab enriched probiotic yoghurt. This is advantageous nutritionally, as it contributes to the health benefit to the human body.

The processing of fruit and vegetable juices requires methods for extraction, clarification and stabilization. To this effect, suitable pectinases, cellulases and hemicellulases from food-grade microorganisms (*Aspergillus niger* and *Trichoderma sp*.), are used to help overcome these difficulties (Bhat, 2000). However, to the best of our knowledge from literature, authors didn't use maceration enzymes to hydrolyse the pectin present in baobab in the process of production of baobab nectar/juice, of which baobab contains 23.4-33.80 g/100 g DW pectin.

1.3.7 Pectin hydrolysis

Pectin and other pectic substances are complex plant polysaccharides, which are part of primary cell wall and middle lamella component of plants (Naidu and Panda, 1998). Pectic substances are complex polysaccharides, with galacturonic acid residues at the backbone linked by α-1,4-glycosidic linkages (Kashyap *et al*., 2001) (figure 4). The carbonyl side groups are 60-90% esterified with methanol (Gummadi and Panda, 2003; Naidu and Panda, 1998) and the side chains consist of L-rhamnose, arabinose, galactose and xylose (Kashyap *et al*., 2001).

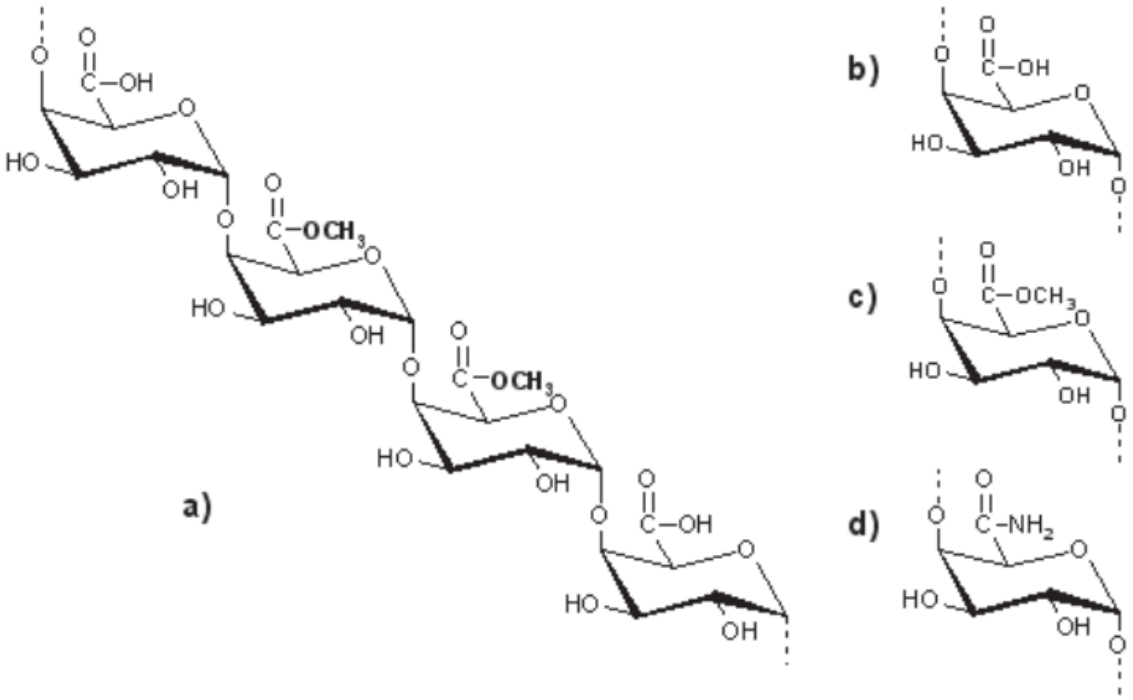


Figure 4: Pectin main component. (a) A repeating segment of pectin molecule and functional groups: (b) carboxyl; (c) ester; (d) amide in pectin chain (Sriamornsak, 2003).

Pectic substances are classified into four main types based on the type of modifications of the backbone chains. They are classified into protopectin, pectic acid, pectinic acid and pectin (Kashyap *et al.*, 2001; Jayani, Saxena and Gupta, 2005).

-Protopectin: is the water insoluble pectic substance present in plant tissues. Protopectin on restricted hydrolysis yields pectin or pectic acids.

-Pectic acid: is the soluble polymer of galacturonans that contains negligible amount of methoxyl groups. Normal or acid salts of pectic acid are called pectates.

- Pectinic acids: is the polygalacturonan chain that contains 0 - 75% methylated galacturonate units. Normal or acid salts of pectinic acid are referred to as pectinates.

-Pectin (Polymethyl galacturonate): is the polymeric material with widely differing compositions containing pectinic acid as the major component in which, at least, 75% of the carboxyl groups of

the galacturonate units are esterified with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall.

The percentage of galacturonic acid (GalA) that is methoxylated is defined as the degree of methoxylation (DM), the main parameter determining pectin functionality. In the presence of sugars, pectin with high DM (>50% esterified) can form a gel in acidic media (Fraeye *et al*., 2010). High methyl ester pectins gel at up to pH 3.4 (rapid set pectin) or 3.2 (slow set pectin) (Endreß and Christensen, 2009). On the other hand, pectin with low DM (< 50% esterified) contains a higher amount of free carboxylic acid groups, which can interact with divalent ions such as Ca^{2+}, resulting in the formation of a continuous network (Fraeye *et al.*, 2010). They form gels with or without only a small amount of sugars in the presence of Ca^{2+} and at low pH (<3.5) (Endreß and Christensen, 2009). Because of its gel-forming ability, low DM pectin is often used in the food industry (Fraeye *et al*., 2010).

Due to various forms of pectic substances present in plant cells, pectinases exist in various forms used in the hydrolysis of pectin. Pectinases were one of the first enzymes to be used in the production of wines and fruit juices of which their commercial applications were first observed in 1930. Today, they are one of the upcoming enzymes in the commercial sector (Kashyap *et al*., 2001). Pectinases are extracted and purified from various sources of microorganisms (bacteria, yeast and fungi). Those from fungi (*Aspergillus niger*) are extensively used in industries because they possess GRAS (Generally Regarded as Safe) status (Gummadi and Panda, 2003). This fungal strain produces various pectinases that act in different forms on their substrates, they are divided into three main types:

- Protopectinases: degrade the insoluble protopectin and yield highly polymerized soluble pectin.

- Pectinesterases: It is also known as pectinmethyl hydrolase. It catalyses the de-esterification of pectin by the removal of methoxy esters forming pectic acid. Its action is preferential on a methyl ester group of galacturonate unit next to a non-esterified galacturonate unit
- Depolymerases: These are enzymes which have different hydrolysing mechanisms.

1. They hydrolyse glycosidic linkages and they include:

- Polymethylgalacturonases (PMG) which can be Endo-PMG that causes random cleavage of α-1,4-glycosidic linkages of pectin, preferentially highly esterified pectin and Exo-PMG that causes sequential cleavage of α-1,4-glycosidic linkage of pectin from the non-reducing end of the pectin chain.
- Polygalacturonases (PG) catalyse hydrolysis of α-1,4-glycosidic linkages in pectic acid (polygalacturonic acid). They are also of two types:

-End-PG: also known as poly (1,4-α-D-galacturonide) glycanohydrolase, catalyses random hydrolysis of α-1,4-glycosidic linkages in pectic acid.

-Exo-PG: also known as poly (1,4-α-D-galacturonide) galacturonohydrolase, catalyses sequential hydrolysis of α-1,4-glycosidic linkages on pectic acid.

2. Cleaving: Cleaving α-1,4-glycosidic linkages by trans-elimination, which results in galacturonide with an unsaturated bond between C4 and C5 at the non-reducing end of the galacturonic acid formed. These include:

- Polymethylegalacturonate lyases (PMGL). They act by trans-eliminative cleavage of pectin. They are: Endo-PMGL (also known as poly (methoxygalacturonide) lyase) catalyses random cleavage of α-1,4-glycosidic linkages in pectin and Exo-PMGL, catalyses stepwise breakdown of pectin by trans-eliminative cleavage.

- Polygalacturonate lyases (PGL). Catalyse cleavage of α-1,4-glycosidic linkage in pectic acid by trans-elimination. They are also of two types: Endo-PGL (also known as poly (1,4-a-D-galacturonide) lyase) and Exo-PGL (also known as poly(1,4-a-D-galacturonide) exolyase), that catalyses random and sequential cleavage respectively of α-1,4-glycosidic linkages in pectic acid.

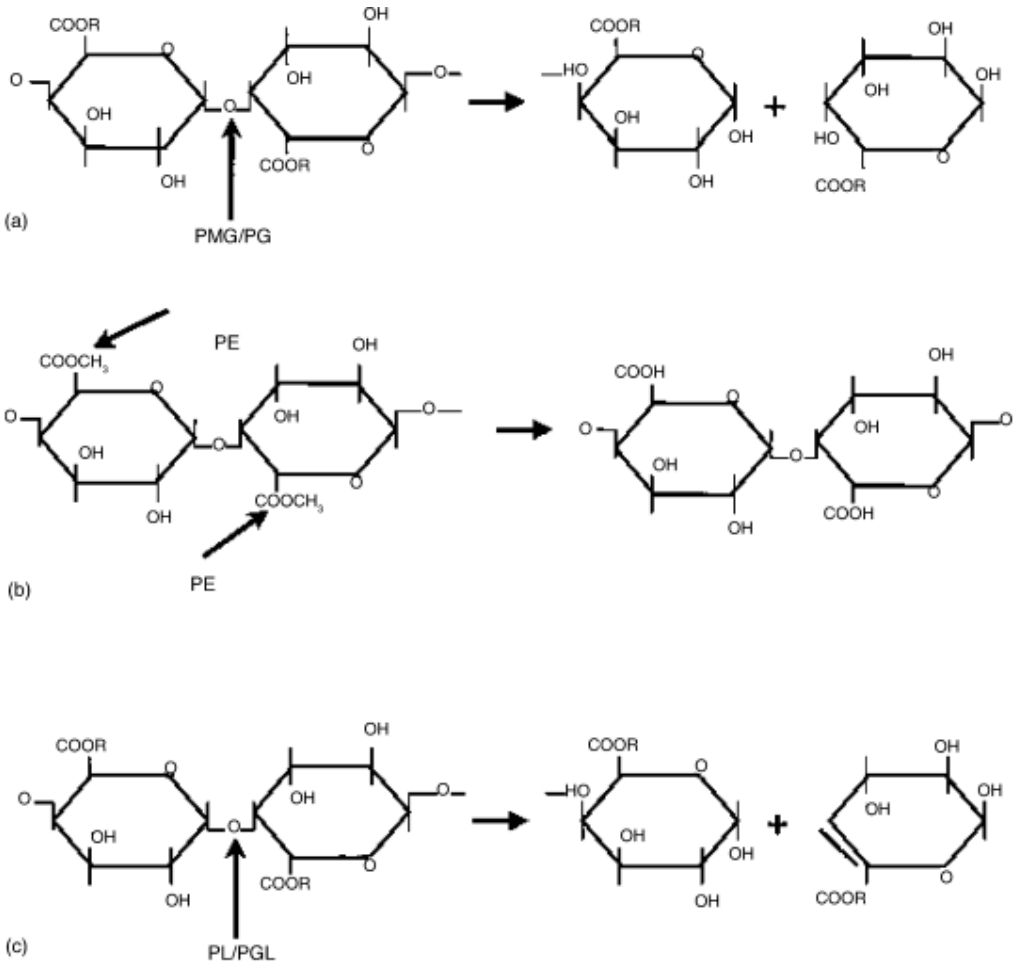


Figure 5:: Different pectinases and their mode of action: (a) R = H for PG and CH3 for PMG; (b) PE; and (c) R = H for PGL and CH3 for PL. The arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases; PG, polygalacturonases (EC 3.2.1.15); PE, pectinesterase (EC 3.1.1.11); PL, pectin lyase (EC-4.2.2.10) (Jayani et al., 2005)

Pectinases are largely applied in the industrial extraction and clarification of fruit juice. Several works have been done on the hydrolysis of pectin in different fruits and enzymatic degradation depends upon the type of enzyme, incubation time, incubation temperature, enzyme concentration, agitation, pH and use of different enzyme combinations (Sharma *et al.*, 2014). Cerreti *et al.* (2017) optimized the pectinase and protease clarification treatment of pomegranate juice using RSM. They realized an optimum enzymatic treatment conditions of temperature 25-30 ^{o}C, time 100-110 min, protease-pectinase complex enzyme amount (the ratio of protease: pectinase was 1:2) 0.22-0.25 g/100 g of pomegranate juice. Surajbhan *et al.* (2012) optimized the enzymatic clarification of guava juice by response surface methodology. The optimal conditions for clarification were: 0.11% enzyme concentration, incubation temperature and time of 41.30 ^{o}C and 115.98 min respectively. Kaur *et al.* (2009) optimized the clarification of guava juice and obtained a compromised optimum condition of incubation temperature 45.35 ^{o}C, incubation time 7.23 h and enzyme concentration 0.70 mg/100 g guava pulp. Liew Abdullah *et al.* (2007) obtained optimum clarification condition of carambola juice at 0.10% enzyme concentration at 30 ^{o}C for 20 min using response surface methodology. Sin *et al.* (2006) worked on the optimisation of enzymatic clarification of sapodilla juice using response surface methodology. Enzyme concentration was the most important factor affecting the characteristics of the juice and the recommended enzyme clarification condition was 0.1% enzyme concentration at 40 ^{o}C for 120 min. Rai *et al.* (2004) and Kaur *et al.* (2009) also optimized the clarification of mosambi and guava juice respectively by response surface methodology. Most of the research on depectinisation of fruit juice was studied using RSM, a mathematical method of fitting a polynomial equation to the experimental data, without providing any insight on fundamental understanding of depectinisation kinetics (Ninga *et al*., 2018). In order to better predict the rate of enzyme kinetics, some authors studied kinetics of

depectinisation in fruits, an adequate method of predicting enzyme kinetic parameters. Ninga *et al.* (2018) studied kinetics of enzymatic hydrolysis of pectinaceous matter in guava juice. While, Sagu *et al.* (2014) optimized the extraction of banana juice by RSM and at the end carried out depectinisation kinetics at the optimal conditions of incubation temperature 33 ^{0}C, time 108 min and concentration of the enzyme 0.03% v/w.

1.3.8 Enzyme kinetics

An enzymatic reaction is a two-step process, where a substrate (S) binds to an enzyme (E) and there is the formation of an enzyme–substrate (ES) complex, followed by an irreversible breakdown of the enzyme–substrate complex to free enzyme and product (P) (Marangoni, 2003).

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

The Michaelis–Menten model is used to describe the enzyme-substrate saturation curve expressed mathematically (equation 1) as:

$$v = \frac{V_{max}[S]}{K_s + [S]} \qquad (1)$$

Where, v is the initial velocity, V_{max} is the maximum velocity when the enzyme is fully saturated with substrate, and K_m is a measure of the affinity of enzyme for substrate and corresponds to substrate concentration at $\frac{1}{2}$ V_{max}. Equation 1 describes the velocity versus substrate concentration curve (Fig. 5).

The Michaelis–Menten model are based on several assumptions (Parkin, 1993; Marangoni, 2003):

1. The substrate-binding step and formation of the ES complex are fast relative to the breakdown rate. This leads to the approximation that the substrate binding reaction is at equilibrium.

2. The concentration of substrate remains essentially constant during the time course of the reaction ([S0] ≈ [St]). This is due partly to the fact that initial velocities are used and that [S0] ≫ [ET].

3. The conversion of product back to substrate is negligible, since very little product has had time to accumulate during the time course of the reaction.

These assumptions are based on the following conditions:

1. The enzyme is stable during the time course of the measurements used to determine the reaction velocities.

2. Initial rates are used as reaction velocities.

3. The reaction velocity is directly proportional to the total enzyme concentration.

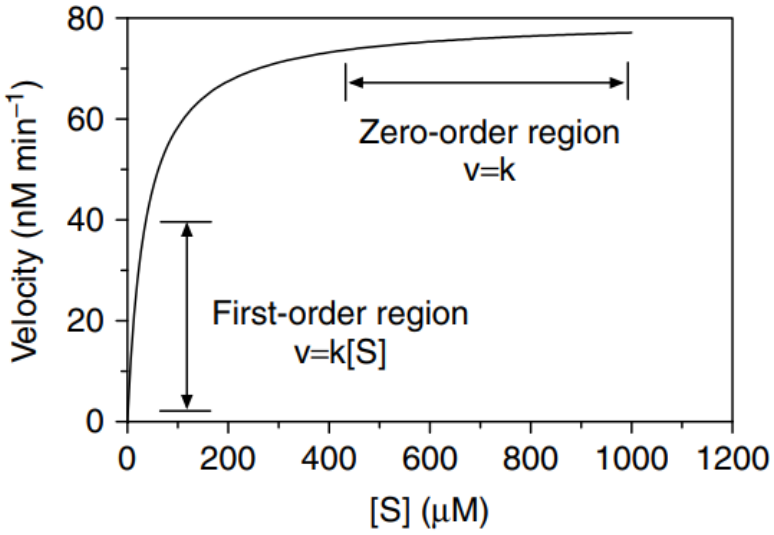


Figure 6: Initial velocity versus substrate concentration plot for an enzyme-catalyzed reaction. Notice the first- and zero-order regions of the curve, where the reaction velocity is, respectively, linearly dependent and independent of substrate concentration.

Langmuir (1918) showed independently that the same hyperbolic curve is found for adsorption of gases onto metal surfaces, soluble solutes onto charcoal, etc.

Many enzymes applied in food processing, do not follow the Michaelis-Menten kinetics (Parkin, 1993; Whitaker, 2003). The kinetics are sigmoidal or allosteric in nature and the equation (equation 2) is expressed as

$$v = \frac{V_{max}[S]^n}{K_s + [S]^n} \qquad (2)$$

Sigmoidal kinetics are sometimes found when more than one substrate molecule binds to the enzyme molecule. Cooperative enzymes show sigmoidal behaviour. Cooperativity occurs when the binding of one substrate molecule induces structural and/or electronic changes that result in altered substrate binding affinities in the remaining vacant sites. Cooperativity can either be positive, when enzyme's substrate binding affinity increase or negative when there is a decrease (Marangoni, 2003; Whitaker, 2003). Allosteric enzymes also display sigmoidal curve. This is due to the presence of multiple binding or active sites that can display dissimilar affinities for substrates, modulators, or inhibitors. Allosteric enzymes are not usually exploited in food applications (Parkin, 1993). Cooperative and allosteric enzymes therefore do not respect the Michaelis–Menten model. The sequential interaction and concerted transition models are used to describe the behaviour of cooperative enzymes (Marangoni, 2003).

In the sequential interaction (SI) model, significant changes in enzyme conformation take place upon substrate binding, which result in altered substrate binding affinities in the remaining active sites (Figure 7).

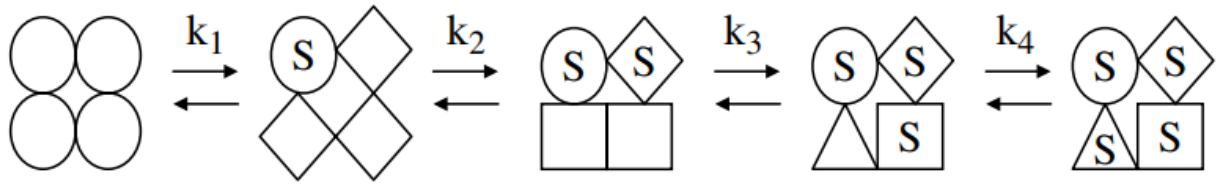


Figure 7: Diagrammatic representation of the sequential interaction of substrate with a four-site cooperative enzyme. Binding of one substrate molecule alters the substrate affinity of other sites. The constants k depicts microscopic dissociation constants for the first, second, third, and fourth sites, respectively

The Hill equation (equation 2) is used to describe sequential interaction. The Hill parameters are used to characterize the catalytic properties of cooperative enzymes. The Hill constant, K, is not the enzyme–substrate dissociation constant but the affinity of the enzyme for the substrate. The Hill coefficient, n, is an index of the cooperativity in the substrate binding process. Cooperativity increases with increase in value of n. The Hill equation reduces to the Michaelis–Menten model when n = 1 (no cooperativity). If the cooperativity of the sites is low, n will not correspond to the number of substrate-binding sites, but the minimum number of effective substrate-binding sites. Regardless of this limitation, the Hill equation can still be used to characterize the kinetic behaviour of a cooperative enzyme. In this case, n becomes merely an index of cooperativity, which can have non integer values (Marangoni, 2003).

The concerted transition (CT) or symmetry model, accounts for allosterism but could not explain anticooperativity and it is based on several postulates (Marangoni, 2003).

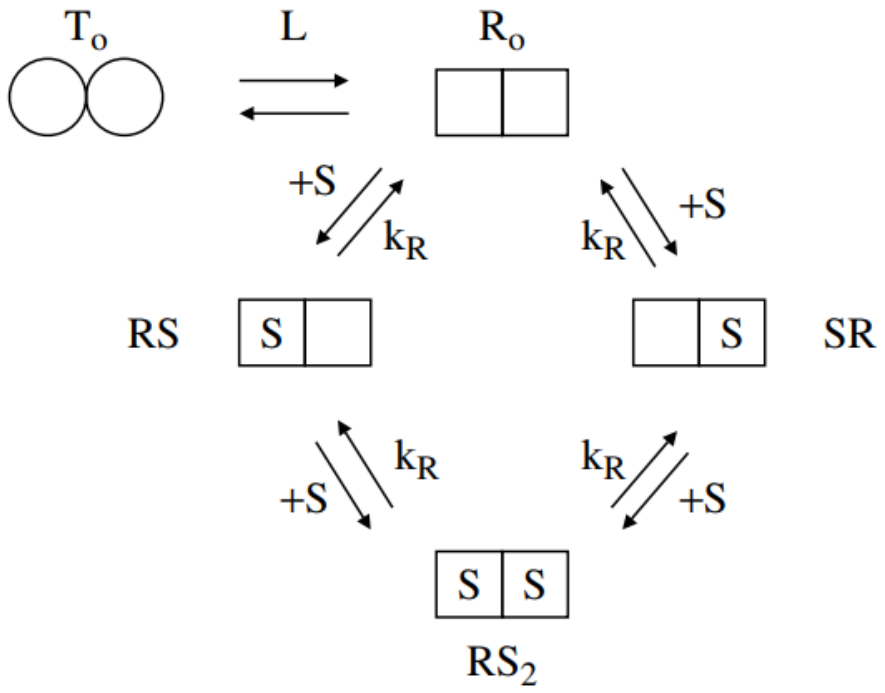


Figure 8*:* Simplified version of the concerted transition model for a two-site cooperative enzyme. In this case the T state of the enzyme is assumed not to bind substrate.

1.4 Lemongrass (*Cymbopogon citratus* Stapf)

1.4.1 Generalities on Cymbopogon citratus Stapf.

Lemongrass is a tall perennial grass with lemon scented leaves. Lemon grass is native to Asia (Pakistan, India and Sri Lanka) but grows in the tropical and sub-tropical South East Asia and Africa (Wifek *et al*., 2016). It belongs to the family Poacea and has as scientific name; *Cymbopogon citratus, C. flexuosus, C. giganteus, C. bombycinus, C. ambiguus, C. obtectus, C. refractus, C. nardus, C. proximus, C. schoenanthus, C. citriodora*. The common names are: West Indian lemon grass or lemon grass (English), hierba limon or zacate de limón (Spanish), citronelle or verveine des indes (French) and xiang mao (Chinese) (Lawal *et al*., 2017).

Lemongrass is about 1 m in height and has a width of 5–10 mm. It has a bulbous stem that increase the clump size as the plant grows and the leaves are long, glabrous, glaucus green, linear, tapering upward and along the margins, with very short ligule and tightly clasp sheaths at the base, narrow and separating at distal end (Lawal *et al.*, 2017). Lemongrass can grow under all types of soils, from over medium fertile soils to moderate irrigation soils. But actually, does well on drained sandy loam soils. It sprouts under warm and humid climate with sufficient sunshine and 250-330 cm rainfall per annum, evenly distributed over most part of the year. A temperature ranging from 20-30 ^{o}C and good sunshine throughout the year is favourable to high crop yield (Nambiar and Matela, 2012).

1.4.2 Chemical composition of lemongrass

A large number of studies have been conducted on the chemical composition of *C. citratus* and it is discovered that the chemical composition varies according to the geographical origin, genetic differences, part of the plant used, method of extraction, age/stage of maturity, and season of harvest (Ekpenyong *et al*., 2014). Ojo (2017) carried out proximate analyses of *Cymbopogon citratus* from Ilorin, Nigeria and the results obtained are as presented in Table 5. The ash content is an indication that *Cymbopogon citratus* has a reasonable amount of inorganic nutrients (Asaolu *et al*., 2009). The protein content is moderate and is an important part of animal structure and metabolism as they are a principal part of the body structure. The crude fibre content (4.49%) shows it's a good source of crude fibre than other conventional leaves (Ojo, 2017). In addition, its carbohydrate content is a good source of energy.

The mineral composition was also determined of which iron had the highest value (268.5±0.81), followed by manganese (61.56±0.15), phosphorus (30.15±0.00), zinc (25.91±0.06), copper (5.73±0.00), potassium (1.59±0.01%), calcium (0.62±0.00%), magnesium (0.44±0.00%) and

sodium (0.28±0.01) parts per million (ppm) in descending order. However, Asaolu, Oyeyemi and Olanlokun (2009) also determined the mineral composition of lemongrass in Nigeria and phosphorus (1245) was the highest followed by sodium (323), potassium (298), magnesium (226), calcium (242), iron (43), manganese (25) and zinc (16) ppm in descending order. Potassium is essential in the maintenance of cellular water balance, pH regulation in the body and it is also associated with protein and carbohydrate metabolism. The potassium content could be exploited for the management of hypertension and other cardiovascular conditions. The low level of sodium is desirable because high dietary sodium has been associated with essential hypertension. Zinc boosts the immune system and act as antioxidant. Presence of zinc in the leaves contributes to the antioxidant activity demonstrated by the plant (Oloyede, 2009).

Table 5: Proximate composition of *Cymbopogon citratus*

Nutrients analysed	**Mean composition (% DW)**
Dry matter	92.95±0.00
Ash	8.02±0.04
Crude protein	8.51±0.06
Crude fibre	4.49±0.19
Carbohydrates	76.84±0.01
Mineral elements	**Mean composition**
Potassium (%)	1.59±0.01
Calcium (%)	0.62±0.00
Magnesium (%)	0.44±0.00
Sodium (ppm)	0.28±0.01
Phosphorus (ppm)	30.15±0.00
Iron (ppm)	268.5±0.81
Zinc (ppm)	21.91±0.81
Copper (ppm)	5.73±0.00
Manganese (ppm)	61.56±0.15

C. citratus is rich in phytonutrients and its major phytonutrients are essential oils that contain citral α, citral β, nerol geraniol, citronellal, terpinolene, geranyl acetate, myrecene and terpinol Methylheptenone (figure 9) (Nambiar and Matela, 2012). They contribute to the aroma of the plant and citral, geranial and neral form nearly 75% of the aldehydes present in these oils (Promila and Madan, 2018). Other compounds found are tannins, saponins, flavonoids, alkaloids, phenols and steroids (Ekpenyong *et al*., 2014). Phenolics such as chlorogenic, caffeic, and *p*-coumaric acids, elemicin, catechol, and hydroquinone have been isolated from *C. citratus* essential oils (Lawal *et al*., 2017).

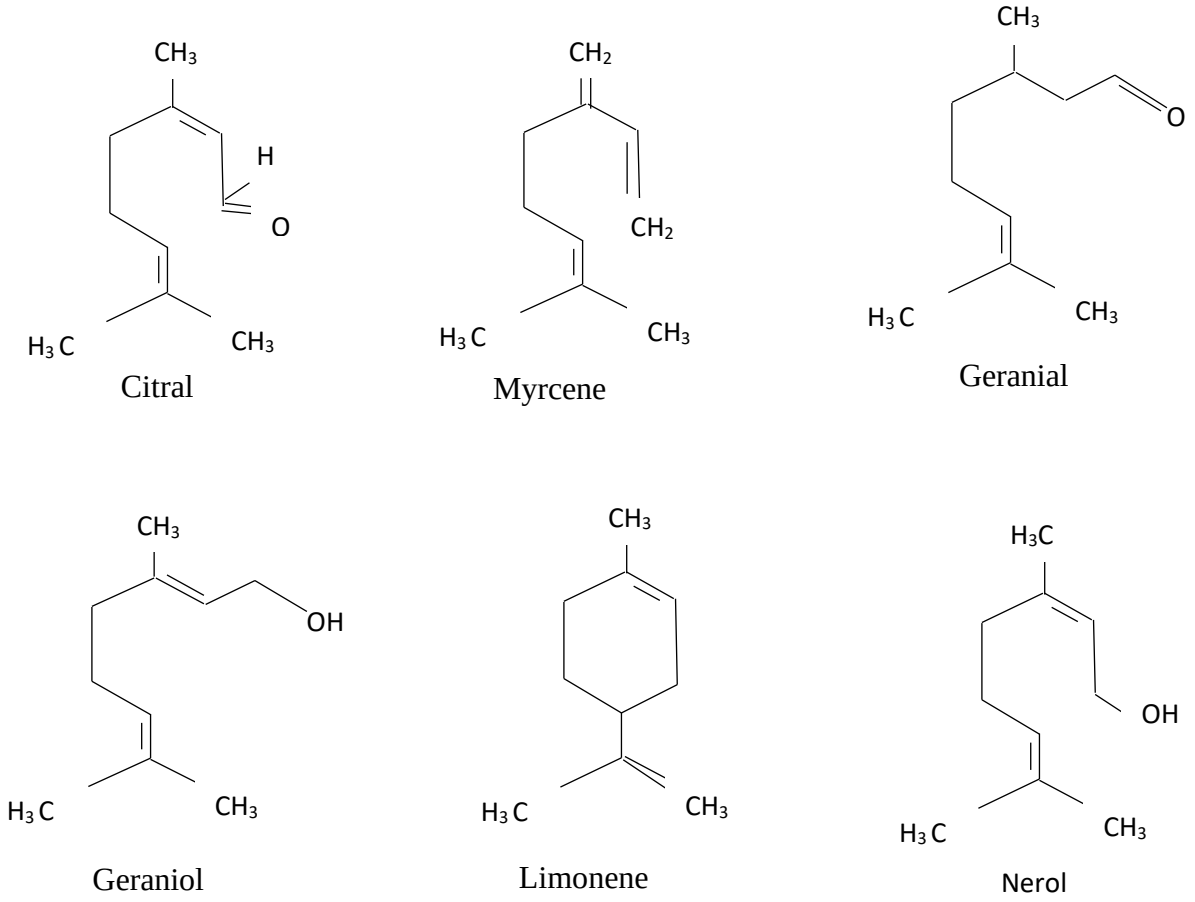


Figure 9: Chemical structures of different components in lemongrass (Ekpenyong *et al*., 2014)

1.4.3 Pharmacological properties of lemongrass

The bioactive components present in *C. citratus* confer its pharmacological properties. It possesses antioxidant, anti-inflammatory, antimicrobial, antimutagenicity and carcinogenicity activities (Olorunnisola et al., 2014; Promila and Madan, 2018). It has diverse uses and in folk remedy, it is used; for coughs, elephantiasis, influenza, headache, arthritis, leprosy, malaria, and inflammatory disorders (Lawal *et al*., 2017).

1.4.4 Applications of lemongrass

After harvesting of lemongrass, the final treatment depends on its application. The first pre-treatment involves washing to remove extraneous materials. It can thereby be processed by drying, extraction of bioactive components with different solvents, distillation to obtain essential oils of different grades (Chime and Onyishi, 2016). After processing, it can be used as, in cosmetics, folk medicines and as food ingredient.

- **Cosmetics and industrial uses**

Essential oils of lemongrass are rich in citral which impart its aroma. Due to its aroma, it is therefore incorporated in products like soaps, perfumes, candles, and mosquito and other insect repellent. It is also employed in aromatherapy, and it improves circulation and muscle tone (Chime and Onyishi, 2016; Nambiar and Matela, 2012).

- **Pharmaceutical and therapeutic uses**

The infusion or decoction of lemongrass is widely used in folk medicine. It is considered a diuretic, tonic, antiseptic and stimulant. It promotes good digestion, and a preparation of lemongrass with pepper is used for relief of menstrual troubles and nausea. It is used to treat diarrhoea, stomach-ache, headaches, fevers, and flu. It is helpful in treating muscular pain, poor

circulation, and muscle tone and slack tissue. The antiseptic oil treats athlete's foot and acne (Directorate plant production, 2009; Nambiar and Matela, 2012).

- **Food ingredient**

Because of the lemon flavour of the leaves, the stem and fresh leaves of lemongrass are used in culinary preparations in the Eastern world and other regions. The dried grass is used as tea either pure or blended with teas and used as herbal drink. It is also often used as an ingredient in curries, marinades and seafood dishes and soup, salads, etc (Directorate plant production, 2009; Chime and Onyishi, 2016).

C. citratus leaves have been used in beverages like yogurt (Fattah et al., 2010), orange, lime and cantaloupe juice (Assous *et al.*, 2012), soy ice cream (Natisri *et al*., 2014), ice cream (Chanmchan *et al*., 2016), curd yogurt (Kim and Kang, 2017), guava cheese (Sinha and Mishra, 2017). To the best of our knowledge, works have not been done in fermenting baobab and lemongrass juice.

1.5 Fermentation

Fermentation is the assimilation of glucose either through the homofermentative or heterofermentative pathway. By the homofermentative pathway, one mole of glucose is converted into two moles of lactate via glycolysis (Embden–Meyerhof–Parnas pathway) while heterofermentative, one mole of glucose is converted into one mole of lactate and one mole of ethanol or acetic acid (Axelsson, 2004). Microorganisms need both intrinsic and extrinsic factors for growth. The intrinsic factors include; nutrients, growth factors, and inhibitors (or antimicrobials), water activity, pH, and redox potential. Extrinsic factors include; the environmental conditions in which it is stored. These are temperature, relative humidity, and

gaseous environment (Ray, 2004). Fermentation can be carried out as batch, fed-batch or continuous process. The mode of operation used depends on the end product.

- **Batch growth**

Batch growth is a closed system where culturing cells are put in a vessel with an initial charge of medium, that are not altered by further nutrient addition or product removal (Shuler and Kargi, 2002). The fermentation is terminated when one or more of the following has been reached: (i) microbial growth has stopped due to the depletion of the nutrients or the build of toxic compounds; (ii) after a fixed predetermined period of time; (iii) the concentration of desired product has been achieved (Macauley-Patrick and Finn, 2008). When cells grow in batch culture, they undergo a number of phases; the lag, exponential, stationary, deceleration and death phases. This form of cultivation is simple and widely used both in the laboratory and industrially (Shuler and Kargi, 2002). The batch culture is advantageous because of its simplicity of use, operability and reliability, production of secondary metabolites that are not growth-related, and fewer possibilities of contamination. It has disadvantages such as culture ageing, and more importantly differentiation, can be a specific problem. There can be the build-up of toxic metabolites that restrict cell growth and product formation, initial substrate concentrations may have to be limited due to problems with inhibition, and also batch-to-batch variability (Macauley-Patrick and Finn, 2008).

- **Fed-batch culture**

Fed-batch culture is similar to batch culture, but does not operate in closed systems. Nutrients, and/or inducers are introduced into the bioreactor at a given point in time. Fed-batch cultures can run either at a fixed volume where at a certain time point, a portion of the fermenter content (consisting of spent medium, cells, product, and unused nutrients) is drawn off and

replaced with an equal volume of fresh medium and nutrients. It can operate at a variable volume where nothing is removed from the bioreactor during the time course of the process, with the cells and product remaining within the vessel until the end of the fermentation period, and the addition of fresh medium and nutrients having the effect of increasing the culture volume. This feeding strategy allows the organism to grow at the desired specific growth rate, minimising the production of unwanted by-products, and allowing the achievement of high cell densities and product concentrations (Macauley-Patrick and Finn, 2008).

- **Continuous culture**

In the continuous culture, the organisms are fed with fresh nutrients, and spent medium and cells are removed from the system at the same rate. This ensures that several factors remain constant throughout the fermentation, such as, culture volume, biomass or cell number, product and substrate concentrations, as well as the physical parameters of the system such as pH, temperature and dissolved oxygen. Continuous culture are widely used in industrial scale where these techniques are used for such processes as vinegar production, waste water treatment, ethanol production and single cell protein production (Macauley-Patrick and Finn, 2008).

1.5.1 Growth kinetics

Different models are used in describing growth kinetics. Models may be structured and segregated, structured and nonsegregated, unstructured and segregated, and unstructured and nonsegregated. Models containing both structure and segregation are the most realistic, but they are also computationally complex. The choice of model therefore depends on the system.

- **Unstructured nonsegregated model**

The growth assumes the form of a saturation kinetics and assumptions are made that, the single chemical species, S, is growth-rate limiting (i.e., an increase in S influences growth rate,

while changes in other nutrient concentrations have no effect). These kinetics are similar to the Langmuir–Hinshelwood kinetics in traditional chemical kinetics or Michaelis–Menten kinetics for enzyme reactions. When applied to cell growth, this kinetics can be described by the Monod equation (equation 3):

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{3}$$

where μ_{max} is the maximum specific growth rate when S >> Ks. The constant Ks is the saturation constant and is equal to the concentration of the rate-limiting substrate when the specific rate of growth is equal to one-half of the maximum. That is, Ks = S when $\mu = \frac{1}{2}\mu_{max}$.

The Monod equation describes substrate-limited growth only when growth is slow and population density is low. Other equations have been proposed to describe the substrate-limited growth phase. Depending on the shape of μ–S curve, one of these equations (equations 4 – 7) may be more reliable than the others. The following equations are alternatives to the Monod equation:

Blackman equation:

$$\mu = \mu_{max} \quad \text{iff } S \geq 2K_s$$
$$\mu = \frac{\mu_m}{2K_s} S \quad \text{iff } S < 2K_s \tag{4}$$

Tessier equation:

$$\mu = \mu_m (1 - e^{-KS}) \tag{5}$$

Moser equation:

$$\mu = \frac{\mu_m S^n}{K_s + S^n} = \mu_m (1 + K_s S^{-n})^{-1} \tag{6}$$

Contois equation:

$$\mu = \frac{\mu_m S}{K_{sx} X + S} \tag{7}$$

The Blackman equation often fits the data better than the Monod equation, but there is discontinuity in the equation. The Moser equation is the most general form of these equations, and

it is equivalent to the Monod equation when n = 1. The Contois equation has a saturation constant proportional to cell concentration that describes substrate-limited growth at high cell densities.

Growth models with inhibitors

Growth becomes inhibited in the presence of high substrate or product concentrations and in the presence of inhibitory substances in the medium (Shuler and Kargi, 2002). The inhibition models are as follows

Non-competitive substrate inhibition: $$\mu = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)\left(1+\frac{S}{K_1}\right)} \quad (8)$$

Competitive substrate inhibition: $$\mu = \frac{\mu_m S}{K_s\left(1+\frac{S}{K_1}\right)+S} \quad (9)$$

Competitive product inhibition: $$\mu = \frac{\mu_m S}{K_s\left(1+\frac{P}{K_p}\right)+S} \quad (10)$$

Non-competitive product inhibition: $$\mu = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)\left(1+\frac{P}{K_p}\right)} \quad (11)$$

Competitive toxic inhibition: $$\mu = \frac{\mu_m S}{K_s\left(1+\frac{1}{K_1}\right)+S} \quad (12)$$

Non-competitive toxic inhibition: $$\mu = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)\left(1+\frac{1}{K_1}\right)} \quad (13)$$

The Monod type models cannot fit processes of fermentation well in many cases, although there are many modified types. Recently the logistic model (a sigmoidal shaped model), has been a most popular one due to its “goodness of fit” and has been widely used in describing the growth of

microorganisms (Wang *et al*., 2004). For cell concentration, X, the logistic model was derived as follows:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right) \quad (14)$$

By integration of equation 14, the kinetic model can be formulated. The biomass production rate yields the following equation (equation 15):

$$X = \frac{X_0 X_m e^{\mu_m t}}{X_m - X_0 + X_0 e^{\mu_m t}} \quad (15)$$

This equation shows the relationship of biomass and the fermentation time, which is used to fit the experimental data of biomass concentration.

The relationship between cell growth and product formation were identified by Leudeking-Piret kinetics (Gordeeva et al., 2019). Leudeking-Piret model (equation 16) was used for kinetic analysis of cell production.

$$\frac{dp}{dt} = \alpha\frac{dx}{dt} + \beta x \quad (16)$$

Where α and β are the growth associated and non-growth associated factors respectively. x and p show the concentration of dry cell weight and product concentration.

The relationship for substrate consumption is given as (Gordeeva et al., 2019):

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\frac{dX}{dt} + m_s X \quad (17)$$

Where ms is the maintenance coefficient that takes into account the consumption of the substrate for maintaining the vital activity of microorganisms, h^{-1}.

CHAPTER 2: HYDROLYSES OF PECTINACEOUS MATTER IN BAOBAB PULP

Abstract

Baobab (*Adansonia digitata* L.) pulp is a superfruit because of its rich nutrient content. Its high pectin content can be exploited by hydrolysing it in the production of baobab juice. This therefore gave the objective of this section of the work, to determine optimal pectinase hydrolysing conditions to hydrolyse baobab pectin releasing fermentable sugars and pectin oligosaccharides that could favour fermentation of the juice. Hydrolysis of baobab pectin was realized at 50 ^{o}C with enzyme concentrations; 0.03, 0.065, 0.1 and 0.2% (v/v) and the degree of hydrolysis and rate of enzymatic reaction determined. In addition, the rheology, reducing sugar content, pH, TPC, antioxidant activity, and organic acid profile of the juice were determined and the results compared to the control which was the non-hydrolysed sample. The depectinisation kinetics revealed a sigmoidal curve trend and to elucidate the sigmoidicity of the curve, the Hill Equation was used to fit the data. The degree of cooperativity (n) decreased from 9 to 5 and the Vmax ranged from 0.336 to 2.378 g/L.min^{-1} with increase in enzyme concentration. The optimum hydrolysis conditions were enzyme concentration, 0.2% (v/v) and time 120 min. The hydrolysed juice obtained with the different pectin concentrations showed a non-Newtonian flow behaviour. The data fitted accurately to the Herschel-Bulkley model and the flow type was shear thickening. The pH of the juice decreased with increase in enzyme concentration and the organic acid profile showed a high concentration of galacturonic acid after hydrolysis. On the other hand, the reducing sugar content, TSS, TPC and antioxidant activity of the juice increased with enzyme concentrations. At the optimal condition of pectin hydrolysis, the pH was 3.62±0.01, reducing sugar content (25.23±0.27 g/L), TPC (184.20±0.20 mg GAE/100 mL), DPPH, FRAP and ABTS were respectively, 164.49±3.80, 219.95±0.56, 235.44±0.67 mg TE/100 mL. In comparison to the control with characteristics, 4.2±0.00, 11.84±0.69 (g/L), 167.70±0.36 mg GAE/100 mL, 143.62±3.00 mg TE/100 mL, 205.67±0.41, 185.86±2.40 respectively, the juice obtained have a rich nutrient profile which can serve as substrate for fermentation by autochthonous LAB.

Keywords: *Adansonia digitata* L., pectin, enzymatic hydrolysis

2.1 Introduction

Baobab fruit pulp is rich in pectin (23.4-33.80%) (Leatherhead, 2009) and Alba *et al.* (2020) identified xylogalacturonans (~69 mol% uronic acids and ~13 mol% xylose) as the main polysaccharide of baobab pulp. Pectin are complex polysaccharides present in the cell wall of higher plants (Sriamornsak, 2003). They are complex carbon sources for microbial metabolism during fermentation and therefore necessity of pre-treatment to hydrolyse to fermentable sugars (Abbasiliasi *et al*., 2017). Enzymatic hydrolysis of pectin using pectinases yields a juice with a much lower pectin content, lower viscosity (Rai *et al*., 2004), POS (Combo *et al*., 2012) and increase in the sugar content which is advantageous for fermentation. Hydrolysis of the pectin in the actual baobab juice, is complicated due to the presence of other polysaccharides which can inhibit the enzyme action. Therefore, the study of kinetics of the enzyme hydrolysis of the baobab pulp pectin is of interest to find the optimal operation points. The Michaelis-Menten model has been shown to fit hydrolysis kinetics of extracted and pure pectin. Deviation from the Michaelis-Menten model could however, be experienced in real life scenario in the depectinization of actual juice (Ninga *et al*., 2018). The two parameters, the Michaelis-Menten constant, K_m and the maximal velocity V_{max}, are important enzyme kinetic data. They describe the nature of the relationship between the initial reaction rate and the substrate concentration (Vasic-Racki *et al*., 2003). To the best of our knowledge, no works have been done on the hydrolysis of pectinaceous matter of baobab pulp using pectinase. Therefore, the objective of this section is to study the kinetics of hydrolysis of baobab pectin.

2.2 Materials and methods

2.2.1 Materials

Dry baobab pulp (photo 1) was purchased in the month of April, 2018 from Kaele (10° 6’ 33” North 14° 27’ 3” East), Far North region of Cameroon. Sampling was done in Kaele because consumers in the septentrional part of Cameroon generally accept that baobab obtained from Kaele is less acidic (Anonymous). This is of great interest since there is the need of baobab with lower acidic content (i.e. higher sugar content) destined for fermentation.

Photo 1: Baobab fruit pulp with seeds and fibres

Once in the laboratory, baobab fruit pulp was processed following the steps presented in Figure 10. Seeds were separated from the pulp by gentle milling using a mortar and pestle. A sieve of 400 µm was used to obtain a fine powder. Smaller particle size enhances enzyme hydrolysis due to increased enzyme accessibility (Yeh *et al*., 2010). The powder was immediately packaged in polyethylene bags and stored at -40 °C until analysis.

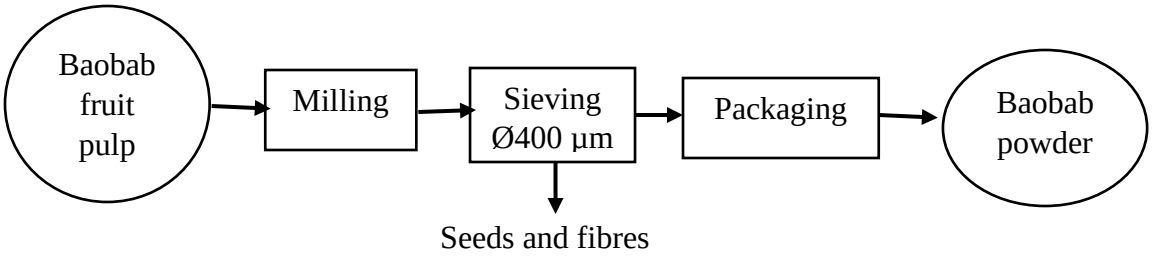


Figure 10: Block diagram of obtaining baobab powder

2.2.2 Methods

2.2.2.1 Determination of proximate and bioactive composition of baobab pulp

The raw materials before using were characterised by carrying out some physico-chemical analysis. Moisture, ash, crude fibre, fat, and protein contents were determined by procedures provided by the Association of Official Analytical Chemists (AOAC, 2000). The bioactive component of baobab pulp was extracted in 80% methanol and TPC, DPPH, FRAP and ABTS analysed.

2.2.2.1.1 Determination of moisture content

Moisture content was determined according to AOAC (2000) method. Baobab (5g) was dried in an oven (ADITYA, Vijay enterprises, India) at 105 ^{o}C for 7h. The dried samples were put in a desiccator to cool and later weighed. The moisture content was expressed following the formula:

$$Moisture\ content\ (\%) = \frac{M_1 - M_2}{M_1} \times 100 \qquad (18)$$

Where: M_1 is the mass in grams of the sample before drying and M_2 is the mass in grams of the sample after drying.

2.2.2.1.2 Determination of total ash content

Clean crucibles were dried in a muffle furnace (DTC PR, India) at 550 ^{o}C for 30 min, cooled in a desiccator and the weights taken. Baobab (5 g) was put in crucibles and kept in the furnace at 550 ^{o}C for 6 h until white ash was obtained (AOAC, 2000). The crucible was cooled, weighed and the percentage ash calculated as:

$$Ash\ (\%) = \frac{M_2 - M_0}{M_1} \times 100 \qquad (19)$$

Where, M_0 is the mass of the empty crucible, M_1 is the mass of the sample and M_2 is the mass of the sample and crucible after ashing. The results are a mean of three trials.

2.2.2.1.3 Determination of crude protein

Protein was determined by Kjeldahl method. Briefly, 500 mg of sample was introduced in a digestion flask. Ten millilitres of concentrated H_2SO_4 and 1 g of well ground digestion mixture of K_2SO_4:$CuSO_4$: SeO_2 (40:10:1) was added. The flask was swirled in order to mix the contents thoroughly and digested according to the program set until a clear solution was obtained. The digest was cooled and transferred to 100 mL volumetric flask and the volume completed with Milli-Q water. 5 mL of the digest was introduced in distillation tubes and 5 mL of 40% NaOH solution added gradually. Distillation was done in the distillation unit and NH_3 produced was collected as NH_4OH in a conical flask containing 10 mL of 2% boric acid solution. A drop of methyl red indicator was added and the mixture titrated against standard N/140 HCL solution till a pink colour was observed (AOAC, 2000). A standard solution of ammonia was run and blank solution of Milli-Q water titrated and the results is mean of three trials. The protein content was calculated using the formula:

$$Protein\ (\%) = \frac{(A-B)}{V} \times \frac{V_t}{M} \times \frac{100}{1000} \times \frac{1}{(C-B)} \times 6.25 \qquad (20)$$

Where A is the titre of sample, B is the titre of blank, C is the titre of standard, V is the volume of sample taken, V_t is the total volume of sample, and M is the weight of sample.

2.2.2.1.4 Determination of fat content

Soxhlet flasks were dried in oven, cooled in a desiccator and the weights taken. Sample (5g) free of moisture was folded in tissue and carefully fit in thimbles. It was connected to the Soxhlet system and about 50 mL of petroleum ether (60-80 ^{o}C) poured in. Extraction was carried out at 40 ^{o}C, the solvent retrieved and the flask dried in the oven for 2 h at 102 ^{o}C to completely evaporate the solvent. It was cooled in a desiccator and the weight of the flask and oil taken (AOAC, 2000). The percentage fat present in the sample was calculated as:

$$Fat\ (\%) = \frac{(M_2 - M_0)}{M_1} \times 100 \qquad (21)$$

Where M_0 is the mass of the empty Soxhlet flask, M_1 is the mass of the sample and M_2 is the mass of the flask and oil after extraction.

2.2.2.1.5 Determination of crude fibre

Fat free samples were weighed and put in conical flasks where, 200 mL of 1.25% H_2SO_4 was added. The whole mixture was boiled for 30 min and filtered on a muslin cloth. The residue was washed several times with hot distilled water to completely remove the acid. The residue was then digested with 1.25% NaOH for 30 min, rinsed with hot water to completely remove the base and later rinsed with acetone. The residue was put in fibre crucibles, the excess water drained out and then dried in an oven at 100 ^{o}C until constant weight. The dried crucible was removed, cooled

and weighed. Then, difference in weight was calculated and expressed in percentage crude fibre (AOAC, 2000).

$$Crude\ fibre\ (\%) = \frac{M_1 - M_2}{M_0} \times 100 \qquad (22)$$

Where M_0 is the mass of the original sample, M_1 is the mass of the sample before incineration and M_2 is the mass of the sample after incineration.

2.2.2.1.6 Determination of carbohydrate content

The total carbohydrate content was calculated by subtracting the sum of moisture, protein, fat, ash and crude fibre from 100 (AOAC, 2000).

2.2.2.2 Determination of pectin content

The pectin content was determined by colorimetric method described by Ranganna (1986) with some modifications. The colorimetric method is based on the reaction of galacturonic acid (GA), the basic structural unit of pectin molecule, with carbazole in the presence of H_2SO_4 and measurement of the colour change at 520 nm.

Briefly, 100 mg of baobab sample was mixed with 100 mL of 0.05 N NaOH, allowed to stand for 30 min to deesterify the pectin. Furthermore, 2 mL of the solution was mixed with 4 mL of 98% H_2SO_4 and heated for 10 min at 95 ^oC. The whole was then immediately cooled in ice water bath to ambient temperature. A volume (0.5 mL) of 0.15% carbazole (prepared in pure 95% ethanol) was added to the cooled solution. It was then homogenized and allowed to stand for 15 min at ambient temperature for the colour to develop. The colour changed from brown to pink and the transmittance was read at 520 nm. The blank was prepared as in sample preparation using pure

ethanol in place of sample and used to set the UV spectrophotometer (Shimadzu, UV-1800, serial No A114550, Kyoto, Japan) to 100% transmittance.

Standard curve: The standard curve for GA was prepared same as for sample by varying concentration of GA (0-1 mg/mL) in Milli-Q water. While the standard curve of pectin was prepared by mixing 1 mg of pure pectin in 0.05 N NaOH. The concentration was varied (0-1 mg/mL) in 0.05 N NaOH. 2 mL of each concentration was then mixed with 0.5 mL carbazole, allowed for 15 min and the transmittance read.

LOG of % transmittance was plotted against different GA concentrations and the GA content of the commercial pectin determined. The GA content of commercial pectin was then plotted against pectin concentration.

2.2.2.3 Determination of reducing and total sugars

Reducing sugars was extracted and analysed according to the method described by Fischer and Stein (1961).

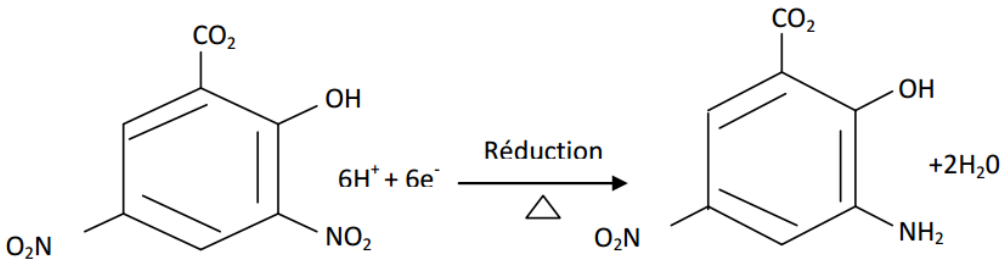
2.2.2.3.1 Extraction of reducing sugars

Baobab (5 g) was introduced in 50 mL Milli-Q water in a 250 mL conical flask and put in a water bath at 100 ^{o}C for 15 min. The mixture was filtered using Whatmann No. 1 filter paper and the residue rinsed twice with 2*10ml Milli-Q water. One millilitre of zinc acetate (2 g/100 ml) and 1 mL of potassium ferrocyanide (10.6 g/100 ml) was added to the filtrate and filtered in a 100 mL volumetric flask. The volume of the filtrate was then completed to 100 mL with Milli-Q water.

Principle:

In a hot alkaline medium, DNS reacts with soluble sugars to transform it from its yellow oxidised form to its reduced orange form that has a maximum absorption at 530 nm. The colour

intensity is proportional to the wavelength and permits to quantify the reducing sugar in solution (figure 11).



3,5- Dinitrosalicylic acid

Yellow component

3- Amino 5- nitrosalicylic acid

Orange component

Figure 11: Reduction reaction equation for DNS during measurement of reducing sugars

Procedure

The DNS solution was prepared by dissolving 1 g of DNS in 20 mL of hot 10% NaOH, in a 100 mL volumetric flask. Furthermore, 30 g of sodium and potassium tartrate was dissolved in a 50 mL volumetric flask using Milli-Q water till the mark. The two solutions were mixed and the volume completed to 100 mL with distilled water.

A standard glucose solution, S1 was prepared at a concentration of 4 mg/mL and the concentration varied in order to obtain the standard curve. The procedure to quantify the reducing sugar is as in appendix 1. The reducing sugar content was calculated from the formula of the standard curve and the results expressed as mg Glucose equivalent/100 g DW of sample.

2.2.2.3.2 Extraction of total sugars

The total sugar was extracted and determined by the method described by Dubois *et al.* (1956). Five millilitres of 1.5 N H_2SO_4 was introduced in 0.2 g of sample in a 50 mL conical flask, heated in water bath at 95 ^oC for 5 min. It was immediately cooled by putting the conical flask in ice water bath to attain ambient temperature. Ten millilitres of 70% ethanol, 1 mL zinc acetate (2

g/100 mL) and 1 mL potassium ferrocyanide (10.6 g/100 mL) were added for defecation. The mixture was filtered through Whatmann paper N°. 1 in a 50 mL volumetric -flask and the volume of the filtrate completed to 50 mL with Milli-Q water.

Principle:

In hot acid, pentoses (C5) and hexoses (C6) undergo cyclization to yield furfural and hydroxymethyl furfural respectively. These compounds can react with phenol to produce a yellow orange coloured complex (figure 12) which has a maximal absorption at 490 nm. The coloured complex formed permits measurement of the sugar concentration and their derivatives by spectrophotometry.

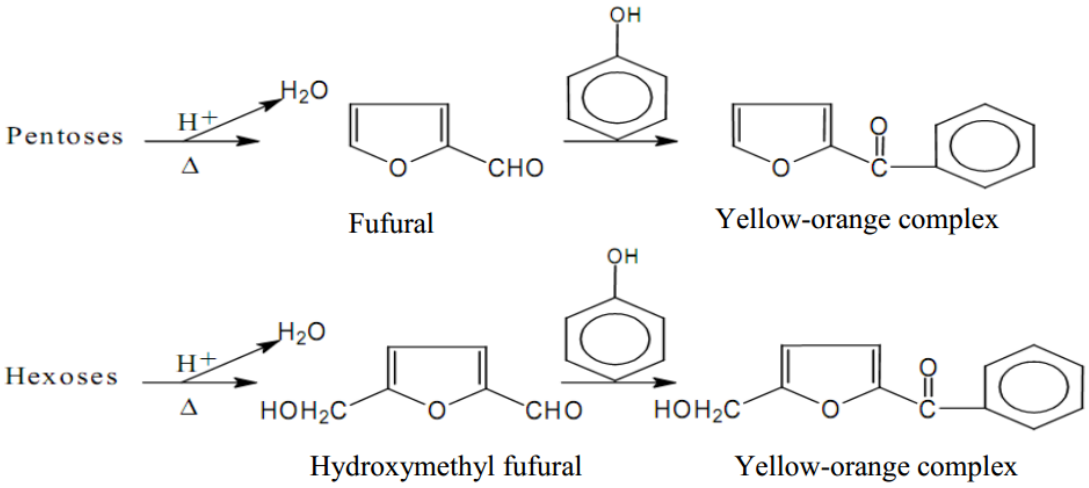


Figure 12: Reaction of oses with phenol in hot acidic medium

Procedure:

Total sugar was determined as in Appendix 2 and the standard curve varied from 0 to 1 mg/mL glucose. The quantity of the total sugar was calculated using the regression equation and the results expressed as mg Gluc equivalent/ 100 g DW of sample.

2.2.2.4 Determination of mineral content

A modified method of wet digestion as described by Ismail *et al.* (2011) was used to determine the mineral content of the sample. One gram of dried sample was pre-digested using 5 mL conc HNO_3 on hot plate for 1 h until semi dried. Conc HNO_3 and 2 mL H_2O_2 were added and digested until a clear semi dried solution was obtained. The semi dried digest was allowed to cool and filtered through a Whatman N° 1 filter paper and the volume of the residue made up to 25 mL with 2 N HNO_3. The mineral composition was analysed on Microwave plasma atomic emission spectrometry (MP-AES) (Agilent technologies, 4210, Stevens Creek, United States) against a mixture of mineral standards.

2.2.2.5 Extraction of bioactive components of baobab

In order to determine the total polyphenol content (TPC) and antioxidant activity, baobab pulp powder was extracted in 80% methanol. Briefly, 25 mL of 80% methanol was added to 2.5 g of baobab, agitated in a magnetic shaker at 25 ^oC for 30 min. It was centrifuged (Sigma 3-18KS, Germany) at 5000 *g* for 15 min at 4 ^oC and the supernatant filtered through Whatmann No. 1 paper. The volume was then completed to 25 mL.

2.2.2.5.1 Determination of total polyphenol content (TPC)

A modified method of TPC described by Marigo, (1973) was used. Twenty microliters of the extract were mixed with 680 µL of water and 100 µL of 1 N folin-ciocalteu reagent (FCR). The mixture was kept at ambient temperature for 5 min, and 200 µL of 20% Na_2CO_3 was added in the test tube. The whole mixture was agitated and incubated at 40 ^oC for 20 min in the dark. After the reaction, the absorbance of the blue complex was read at 725 nm using TECAN microplate reader (Infinite M200 PRO, Tecan Austria). A calibration curve was established using

standard gallic acid (0.001-0.008 g/L), and the results were expressed as mg of gallic acid equivalent (GAE)/100 mL extract. All samples were analysed in triplicate and an average taken.

2.2.2.5.2 Determination of antioxidant activity

- **DPPH Radical Scavenging Activity**

A modified method of DPPH radical scavenging activity was performed as mentioned by Shimada *et al.* (1992). Briefly, 30 µL of baobab extract was added to 150 µL of 0.1 mM DPPH in 96-well microplate. The mixture was homogenized and incubated at 37 ^{o}C for 30 min. The absorbance of the resulting mix was then measured at 517 nm and a standard curve was established using standard Trolox in the range of 0-50 µg/mL. The activity was expressed as mg Trolox equivalents (mg TE)/ 100 mL of extract.

- **Ferric Reducing Antioxidant Power (FRAP)**

The FRAP assay was carried out according to the method of Benzie and Strain (1996). FRAP solution was prepared fresh daily by mixing 0.3 M acetate buffer (pH 3.6), 0.01 M TPTZ in 40 mM HCl, and $FeCl_3$ (20 mM) in the ratio 10:1:1 respectively. Then, 30 µL of the extract were added to 200 µL of freshly prepared FRAP solution. The mixture was homogenized properly and incubated at 37 ^{o}C in the dark for 30 min. The ferrous tripyridyltriazine complex formed was measured at 595 nm against a blank prepared in the same manner using Milli-Q water. A standard curve was prepared using Trolox in the range of 0–50 µg/ mL, and the activity was expressed as mg TE/100 mL extract.

- **ABTS radical scavenging activity**

A modified method of ABTS radical-scavenging activity as described by Re *et al.* (1999) was used. The ABTS solution was prepared by mixing 5 mL of 7 mM ABTS and 88 µL of 140 mM potassium persulfate. The mixture was kept in the dark at room temperature for 16 h to react. Two millilitres of ABTS solution were diluted with methanol, in order to obtain absorbance of 0.80 ± 0.03 at 734 nm. In a 96-well microplate, 30 µL of baobab extract was mixed with 200 µL of ABTS solution (freshly prepared). The mixture was homogenized and incubated at 30 ^{o}C in the dark for 30 min. The absorbance was read at 734 nm using a Microplate Spectrophotometer. The blank was prepared in the same manner using Milli-Q water. A standard curve was prepared with Trolox in the range of 0–50 µM. The activity was expressed as mg TE/ g baobab sample.

2.2.2.6 Determination of amino acid profile

The amino acid profile of baobab pulp was determined with respect to the method described by Bidlingmeyer *et al.* (1987). Baobab powder, 100 mg were thoroughly mixed with 20 mL trichloroacetic acid solution (5%), stirred for 1 h at room temperature (28 ± 1 ^{o}C), then centrifuged (7780; Kubota, Bunkyo-ku, Tokyo, Japan) at 10,000 *g* for 15 min at 4 ^{o}C. The supernatant obtained was filtered using 0.22 µm PTFE membrane filters. Amino acid standards (essential amino acids (E): phenylalanine, leucine, methionine, threonine, valine, histidine, tryptophan, lysine, isoleucine and non-essential amino acids (NE): glutamic acid, glycine, proline, aspartic acid, tyrosine, hydroxyproline, alanine, serine, asparagine, cysteine, arginine, cysteine, glutamine) were prepared in milli-Q water (0.01 - 1 µg/ml) and standardized. The LC-MS/MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) - HPLC (Nexera LC-30AD) equipped with an autosampler (SIL-30AC), temperature-controlled column oven (CTO-20AC) and prominence diode array detector (SPD-M20A) coupled to triple quadrupole mass

spectrometer (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) was used to standardize the free amino acids. Shimadzu Shim-pack GISS C18 column (150 X 2.1 mm i.d, 1.9 µm) was used to quantify the amino acids. The mobile phase was water / formic acid (100/0.1) for solvent A and 100% methanol for solvent B. Amino acids were eluted with a linear gradient system as follows: 0.5 – 4.9 min 5% of solvent B, 5.0 – 13 min 85% of solvent B, and 13.1 – 15 min 5% of solvent B, a flow rate of 0.3 ml/min and oven temperature of 40 °C. The LC-MS/MS with electrospray ionization (ESI) was operated in multiple reaction-monitoring (MRM) mode, both positive and negative. 10 µl samples was injected, and the ion spray voltage was 4 kV. The collision-induced dissociation (CID) gas was 230 kPa. Analyses were done in triplicate and the free amino acids of the sample were identified and quantified by comparing with the authentic standard amino acid mixture.

2.2.2.7 Extraction and characterization of baobab pulp pectin

Before hydrolysing baobab pulp pectin, it was necessary to extract and characterise the type of pectin present in baobab pulp in order to know the type of pectin we are working on. Pectin was extracted and characterized by the method described by Azad *et al.* (2014) with some modifications. One part of baobab powder was mixed with 30 parts of distilled water and the pH adjusted to 2 with 1 N H_2SO_4. It was heated at 90 ^oC in a water bath (Julabo TW8, Germany) for 1 h while stirring every after 15 min with a stirring rod. The extractant was rapidly filtered on a muslin cloth, the filtrate collected and 2 parts of absolute ethanol corresponding to 1 part of filtrate was added for precipitation. The mixture was gently stirred to break up the precipitates and later kept at 27 ^oC overnight (12 h). The precipitated pectin was centrifuged at 10 000 *g*, 15 min at 10 ^oC and the pectin washed thrice with 75, 85 and 95% absolute ethanol. The resulting pectin was

dried at 55 ^{o}C in an oven for 24 h. The pectin was then ground in a mortar and pestle and stored at -40 ^{o}C for further analysis. The pectin yield was calculated as in equation (23)

$$Pectin\ (\%) = \frac{A}{B} \times 100 \qquad (23)$$

Where; A is the weight (g) of the dry pectin and B is the weight (g) of dried baobab powder taken for extraction.

2.2.2.7.1 Determination of equivalent weight of baobab pectin

The extracted baobab pectin (0.5 g) was mixed with 5 mL of ethanol, 1 g of NaCl, 100 mL distilled water and 6 drops of phenol red in a 250 mL conical flask. The mixture was titrated against 0.1 N NaOH until a purple colour was obtained. This neutralized solution was kept for determination of methoxyl content.

Equivalent weight was calculated by equation (24)

$$Equivalent\ weight = \frac{(A \times 1000)}{(V - N)} \qquad (24)$$

Where; A is the weight of sample, V (mL) is the volume of alkali and N is the normality of alkali.

2.2.2.7.2 Determination of methoxyl content (MeO) of baobab pectin

To the neutral solution, 25 mL of 0.25 N NaOH was added, homogenized and kept at 27 oC for 30 min. 25 mL of 0.25 N HCL was further added and titrated against 0.1 NaOH. The methoxyl content was calculated following equation 8.

$$Methoxyl\ content\ (\%) = \frac{V \times N \times 3.1}{A} \qquad (25)$$

2.2.2.7.3 Determination of Total Anhydrouronic Acid Content (AUA)

Total AUA of pectin was obtained by equation 26.

$$AUA\ (\%) = \frac{(176 \times 0.1z \times 100)}{(w \times 1000)} + \frac{(176 \times 0.1y \times 100)}{(w \times 1000)} \qquad (26)$$

Molecular unit of AUA (1 unit) = 176 g

Where z is the titre (mL) of NaOH from equivalent weight determination, y is the titre (mL) of NaOH from methoxyl content determination, and w is the weight of sample.

2.2.2.7.4 Determination of Degree of Esterification (DE)

The DE of pectin was calculated by equation 27.

$$DE\ (\%) = \frac{(176 \times \%MeO}{(31 \times \%AUA)} \times 100 \qquad (27)$$

Where 176 and 31 are the molar masses (g/mol) of anhydrogalacturonic acid and methoxy (–OCH_3) groups, respectively.

2.2.2.7.5 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the baobab pectin was determined using Vertex 70 FTIR spectrophotometer (Tensor II, Bruker, Billerica, USA). The surface of the diamond crystal was cleaned with isopropanol and the sample deposited. The flap of the FTIR was closed and the spectra recorded within the wavenumber range 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.2.2.8 Pectin hydrolysis

Due to the high pectin content in baobab, the pectin was hydrolysed with different concentrations of pectinase (0.03, 0.065, 0.1 and 0.2%) at 50 ^{o}C from 0 to 2 h, optimal condition for action of pectin lyase (Jayani *et al*., 2005; Yadav *et al*., 2009). The concentration of baobab powder for hydrolysis was chosen with respect to preliminary works which 8 % showed a better hydrolysis. In the formulation of baobab juice, 10 % baobab concentration has been used (Tembo, 2016). However, it forms a paste which during preliminary works led to a decrease in the degree of hydrolysis probably due to difficulties in diffusion of enzyme at higher substrate concentration (Ouyang *et al*., 2009). Eight grams of baobab powder was therefore mixed with 100 mL of Milli-Q water in a beaker and different concentrations (0.03-0.2 % v/v) of pectinex Ultra AFP (Novozymes, Bagsvaerd Denmark) with activity 10000 PECTU/ml added. The mixture was put in a shaking water bath (BS-11, Lab Companion, Korea) at 50 ^{o}C with an agitation speed set at 100 rpm. Agitation was essential to homogenise the system and ensure proper contact of enzymes to substrate and also, avoid accumulation of products in one area which could lead to possible inhibition of enzyme action (Jørgensen *et al*., 2006). Agitation avoids mass transfer problems and the speed is also important to prevent shear stress on the enzyme (Abd Rahim *et al*., 2015). Samples were then taken at 15 min time interval from 0 to 2 h and the pectinase denatured by heating at 95 ^{o}C for 5 min and immediately cooled in ice water. The mixture was centrifuged at 8000 *g* for 15 min at 4 ^{o}C. The supernatant was filtered through Whatman N° 1 filter paper and the volume of the filtrate completed to 100 mL and stored at -40 ^{o}C for further analyses.

2.2.2.8.1 Pectin concentration

Pectin was extracted from baobab juice as described by Ninga *et al.* (2018). Pectin was precipitated using 96% v/v ethanol and the precipitate incubated at 4 ^{o}C for 30 min. The pectin

gels were centrifuged at 8000 g for 15 min and the supernatant collected. The pectinaceous matter was dissolved in 0.05N NaOH and the volume made up to 100 mL. The samples were then allowed to stand for 30 min to de-esterify the pectin. In a test tube, 6 mL H_2SO_4 were introduced and cooled in an ice water bath till ambient temperature, whereafter 1 mL of sample was added and the tubes closed with caps. The tubes were heated for 10 min in a boiling-water bath, and then allowed to cool at room temperature (27 ^oC ± 2) for about 10 min. After that, 0.5 mL of 0.15% w/v carbazole reagent was added in each tube. The mixture was allowed to stand for 25 ± 5 min, and the transmittance was measured at 520 nm using a spectrophotometer (Shimadzu, UV-1800, serial No A114550, Kyoto, Japan). Pectin concentration was calculated according to the calibration curve obtained using galacturonic acid monohydrate standard. The degree of hydrolysis (DH) of pectinaceous matters was determined using equation 28:

$$Degree\ of\ hydrolysis\ (\%) = \frac{[S]_{t=0} - [S]_t}{[S]_{t=0}} \times 100 \qquad (28)$$

Where [S] is the pectin concentration and t is the time.

The rate of reaction was also determined using the following equation 29:

$$Rate\ of\ reaction, v = \frac{[S]_i - [S]_{i-1}}{t_i - t_{i-1}} \qquad (29)$$

The rate of reaction (v) was plotted against pectin concentration, and the model was fitted in Hill Equation (equation13) (Marangoni, 2003). The use of Hill equation is due to the fact that the plot of reaction rate with pectin concentration describes a sigmoidal pattern, instead of the typical Michaelis-Menten's hyperbola.

The Hill equation is given by

$$v = \frac{V_{max}[S]^n}{K + [S]^n} \quad (30)$$

Where Vmax and n refer to the maximum reaction rate and Hill coefficient, respectively. K is expressed as $K = [S_{50}]^n$, where $[S_{50}]$ is the substrate concentration at which the reaction rate is half of its maximum value, i.e., v=Vmax/2. For each enzyme concentration, the values of the Vmax and n were obtained by minimizing the sum of squares at each experimental data, using MATLAB 2014b.

2.2.2.8.2 Morphology of hydrolysed pulp

To evaluate the effect of hydrolysis with different enzyme concentrations (0.03 – 0.2% v/v), SEM analysis were carried out on the hydrolysed pulp. The method described by Elzeini *et al.*, (2017) was adapted for baobab pulp. The pulp was suspended in 4% glutaraldehyde in phosphate buffer (0.1 M, pH 7) and incubated at 4 ^{o}C for 3 h. It was further washed with phosphate buffer and dehydrated in graded series of absolute ethanol (30, 50, 70, 80, 90, 95%). The dehydrated pulp was suspended in absolute ethanol and subjected to SEM analysis. The dehydrated pulp was mounted on a specimen holder, coated with gold (3 nm) in a sputter coater (POLARON E5100, Watford, England) and transferred to the microscope (LEO 435 VP, Scanning Electron Microscopy Limited, UK) where it was observed at 10 kV under high vacuum.

2.2.2.9 Measurement of rheological properties of hydrolysed juice

The rheological properties of the baobab juice hydrolysed at different enzyme concentrations and the non-hydrolysed juice were evaluated according to the method described by Salinas *et al.* (2019). A rheometer HAAKE RheoStress 6000 (Haake, Karlsruhe, Germany) was

used with the measuring cup Z41 DIN 53018. In order to evaluate the rheological parameters of the fluid, the shear stress was varied from 0.01 to 300 s^{-1} at temperature, 25 ^{o}C, and the shear rate and apparent viscosity recorded.

2.2.2.9.1 Analysis and modelling

The experimental data were fitted to the power law (equation 31) and Herschel-Bulkley (equation 32) models.

$$\tau = K\dot{y}^{n} \quad (31)$$

$$\tau = \tau_0 + K\dot{y}^{n} \quad (32)$$

Where τ is the shear stress (Pa), τ_0 is the yield stress, k is the consistency index, n is the flow behaviour index that reflects closeness to Newtonian fluid and $\dot{y}$ is the shear rate (s^{-1}). For Newtonian fluids, n = 1, the consistency index K is equal to the viscosity of the fluid, η. When n < 1, the fluid is shear-thinning (pseudoplastic) and when n > 1, the fluid is shear-thickening (dilatant).

2.2.2.10 Microbial analyses of baobab juice

Microbial analyses of the baobab pulp powder and the juice obtained after hydrolysis was done by evaluating the total aerobic mesophile flora, yeast, fungi, Staphylococcus and coliforms.

- **Total aerobic mesophile flora**

It is a general indicator of the sanitary quality of food indicating the adequacy of temperature and sanitation control during processing, transport, and storage, and revealing sources of contamination during manufacture (Herrera, 2001).

Plate count agar was prepared according to manufacturer instruction and aliquot of serial dilutions of baobab pulp and juice was inoculated by pour plate technique under aseptic conditions. The petri dishes were labelled and incubated at 30 ^{o}C for 48 h. Furthermore, the number of colonies was counted and the results were expressed in colony forming units/ml (cfu/mL) by using the equation 33:

$$X = \frac{A \times V}{f} \qquad (33)$$

Where: A is number of colonies counted (between 30 and 300), V is the dilution reciprocal inoculated, and f is the volume of inoculum (in mL).

- **Yeast and mould**

Potatoes dextrose agar (PDA) was used to determine the presence of yeasts and moulds in the samples. The molten PDA was prepared with respect to manufacturer instructions and acidified with sterile 10% tartaric acid to pH 3.5 ± 0.1 in order to favour the growth of only yeast and mould. The medium is not reheated after addition of acid and pour plate technique was used to inoculate aliquot of the samples. The plates were then incubated 25 ^{o}C for 3-5 days, the colonies counted on plates containing 30–300 colonies, and the number computed as yeast and moulds per millilitre of food.

- **Total coliforms**

These include total coliforms, faecal coliforms and faecal streptococci and are the main representatives of faecal contamination. They include members of the family that are capable of fermenting lactose with the production of acid and gas within 48 h at 35 ^{o}C (Herrera, 2001).

Total coliform was determined using Violet Red Bile agar (VRBA). It was prepared according to manufacturer instructions and the samples inoculated by pour plating. The plates were incubated at 37 ^{o}C for 48 h and the number of colonies counted.

- **Staphylococcus**

Staphylococcus aureus is one of the leading causes of food poisoning resulting from the consumption of contaminated food with staphylococcal enterotoxins. Baird-Parker (BP) medium was used to determine the presence of Staphylococcus in baobab samples. The media was prepared and appropriate dilutions of the baobab samples inoculated by pour plating. They were incubated at 35 ^{o}C for 48 h and the colony types were counted differentially, based on egg yolk clearing and morphology of the colonies on the BP plates.

2.2.2.10 Colour analyses of baobab juice

The colour of baobab extract was measured with a Minolta colorimeter (CM-5, Konica Minolta, Tokyo, Japan) against distilled water as blank. The parameters determined were L* (lightness), a* (greenness), and b* (yellowness) based on the CIELAB colour scale. Measurements were carried out in triplicate, and the average and standard deviation reported. The colour of the baobab powder was also measured.

2.2.2.11 Polyphenol profile of baobab

Free and bound polyphenols of baobab pulp was extracted and analysed with respect to the method described by Pang *et al.*, (2018).

2.2.2.11.1 Extraction of free polyphenols

Baobab pulp powder (1 g) was mixed with 80% methanol (20 mL) and extracted twice on a mechanical shaker at 150 rpm for 30 min at room temperature. The mixture was centrifuged at 4000 *g* for 15 min at 20 ^{o}C. The supernatant of the extracts was combined and the pH adjusted to 2 with 6 M HCL. The supernatant was further concentrated to dry on a rotary evaporator (IKA RV 10, Staufen, Germany) at 45 ^{o}C. The free polyphenols were extracted three times from the concentrated fraction using ethyl acetate at a ratio of 1:1 v/v. The organic phase was separated from the aqueous phase and concentrated by evaporating on a rotary evaporator. The concentrate was dissolved in 5 mL HPLC grade methanol (50%), filtered through 0.45 µm syringe filter and analysed for crude free polyphenol by HPLC.

2.2.2.11.2 Extraction of bound polyphenols

The solid residue obtained after extraction of the free polyphenols was washed with Milli-Q water and digested with 20 mL of 4 M NaOH at room temperature on a mechanical shaker for 2 h. The pH of the mixture was adjusted to 2 with concentrated HCL. The bound polyphenol was then extracted thrice with ethyl acetate (1:1 v/v). The fractions were evaporated to dryness at 45 ^{o}C and the concentrate dissolved in 5 mL HPLC grade methanol and analysed as crude bound polyphenol. The baobab juice obtained after pectin hydrolyses was filtered using a 0.45 µm syringe filter and then used for polyphenol analyses.

2.2.2.11.3 Analysing polyphenols by HPLC

HPLC analyses of baobab extract was performed on LC-10AS (Shimadzu, Kyoto, Japan) system equipped with Shimadzu LC-10AT pumps, and CBM-20A communication bus module. Chromatography was carried out in a gradient system using 250 × 4.60 mm, 5µm C18 column

(Gemini, Phenomenex, California, USA). A flow rate of 1 mL/min and the injection volume of 10 µL was employed. The mobile phases consisted of A (99.9% acetonitrile and 0.1% acetic acid) and B (0.1% acetic acid in Milli-Q water). The gradient elution was 0 - 15 min (8% A and 92% B), 30 min (22% A and 78% B), 45 min (78% A and 22% B), 55 min (8% A and 92% B), and 60 min (8% A and 92% B). A UV-Visible DAD detector was used, and the wavelengths detected at 280 and 320nm. Polyphenols were identified by comparing their retention times with those of pure standards (gallic acid, dihydroxybenzoic acid, catechin, vanillic acid, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid, trans ferulic acid, quercitin, and trans cinnamic acid).

2.2.2.12 Organic acid profile

Organic acid of the baobab pulp powder was extracted by ultra-sonication following a modified method described by Wang *et al.* (2018). Baobab powder (4 g) was thoroughly mixed with 50 mL of Milli-Q water and sonicated at 70 Hz for 30 min (pulse 3 sec on and off). The sonicated mixture was centrifuged (8000 *g*, 15 min at 4 ^{o}C), the supernatant was filtered through 0.45 µm filter and analysed by HPLC for organic acids.

The analysis of organic acids was performed according to the method of Sharma and Devi (2018). Baobab extract was filtered using 0.45 µm syringe filter. Filtrates were used for the determination of organic acids using LC-8A HPLC (Shimadzu, Kyoto, Japan) equipped with SPD-M10A VP diode array detector. A C18 (250 × 4.60 mm, 5µm) column (Gemini, Phenomenex, California, USA) was used and the mobile phase was 8 mM H_2SO_4. The extract (10 µL) was injected and the separation of organic acids carried out at a flow rate of 1 mL/min for 30 min, at a wavelength of 210 nm. Standard organic acids (D-galacturonic acid, oxalic, tartaric, malic, iso-citric, ascorbic, citric, succinic, propionic and glutaric acids) were used for identification and quantification of individual organic acids. Organic acid content was expressed as mg/100 mL extract.

2.2.2.13 Determination of Vitamin C

Vitamin C content of baobab juice was determined by HPLC method described by Gundogdu *et al.* (2014) with some slight modifications. Baobab juice (1 mL) was mixed with 4 mL of 3% metaphosphoric acid, centrifuged at 8000 rpm for 10 min at 4 ^{o}C. The supernatant was collected and filtered through 0.45 µm membrane filter.

For the baobab powder, 1 g was mixed with 10 mL of 3% metaphosphoric acid, agitated for 30 min in a mechanical shaker at 100 rpm. It was centrifuged and the volume of the supernatant completed to 20 mL with m-phosphoric acid and then filtered through 0.45µm membrane filter. Quantification of ascorbic acid was done by using standard ascorbic acid prepared and the concentrations varied in 3% metaphosphoric acid. Samples were separated using C18 (250 × 4.60 mm, 5 µm) column (Gemini, Phenomenex, California, USA). The mobile phase was 8 mM H_2SO_4 and 10 µL of extract was injected and the flow rate was 1 mL/min. Ascorbic acid peaks were detected at a wavelength of 245 nm.

2.2.3 Statistical analysis

ANOVA test was used to assess the statistical significance of the regression coefficient on the level of significance declared at $p \leq 0.05$ using Statgraphics (Statgraphics Centurion, 16.1.18, StatPoint Technologies, Inc). Differences between mean were analysed by Duncan test at the significance level of $p \leq 0.05$. The results were expressed as a mean ± standard deviation.

2.3 Results and Discussion

2.3.1 Physicochemical analysis of baobab powder

The proximate composition of baobab fruit pulp is presented in table 6. The moisture content of baobab fruit powder, 7.43±0.08% was lower than 10.4 ± 0.4% reported by Osman (2004) and 9.16±1.15 to 10.30±1.28 of three different baobab locations reported by Aluko *et al.* (2016). Moisture content of ≤12% is desired for shelf life stability of food on long storage (Ibrahim *et al.*, 2016). This therefore implies the baobab powder can be conserved for long before use.

Table 6: Proximate and mineral composition of baobab pulp

Macronutrients (g/100g DW)	
Moisture content	7.43±0.08
Ash	6.32±0.05
Fat	0.18±0.05
Protein	2.89±0.08
Crude fibre	10.31±0.03
Reducing sugar	16.46±0.20
Total sugar (as glucose)	41.52±0.21
Pectin	28.34±1.07
Micronutrients (mg/100g DW)	
Calcium	180.67±1.89
Iron	22.96±0.81
Potassium	1181.81±0.54
Magnesium	85.88±1.08
Sodium	18.85±0.59
Vitamin C (mg/100mL)	
Vit C	137.44±1.46

Ash content was 6.32±0.05% and falls within the range 5.5 – 6.6% reported by Leatherhead (2009). Ash is a representative of the inorganic material present in samples (Ibrahim *et al*., 2016) and therefore, baobab is rich in minerals. The fat content (0.18±0.05%) is much lower than that of literature, 0.4 - 0.70% (Leatherhead, 2009); 0.46±0.13 - 1.98±0.69% (Aluko *et al*., 2016); 0.5 – 2.1% (Ibrahima *et al*., 2013). The crude protein (2.89±0.08%) is in the range of 2.04 - 3.24% analysed by Leatherhead (2009) and 2.5 – 6.3% by Ibrahima *et al.* (2013). Baobab fruit pulp is therefore not a good source of protein due to its low protein content. The low protein content of baobab is not favourable for the growth of LAB because they do not only require carbon as a source of energy for growth but also nitrogen source and some specific amino acids (Abbasiliasi *et al*., 2017). Crude fibre are mostly plant polysaccharides that cannot be digested by human dietary enzymes such as cellulose, non-cellulosic polysaccharides like hemicellulose, pectic substances, gums, mucilages and a non-carbohydrate component lignin. It is important because it provides the bulk necessary for proper peristaltic action in the intestinal tract (Ibrahim *et al*., 2016) and the dietary fibre act as prebiotics for growth of probiotics. The crude fibre content (10.31±0.03%) is higher than 5.91±1.42 - 9.65±0.36% determined by Aluko *et al.* (2016) and 5.4 ± 0.3% (Osman, 2004). This shows that the baobab has prebiotics for probiotic growth. Its total sugar content in glucose equivalent (41.52±0.21%) is higher than the range 16.9 - 25.30% presented by Leatherhead (2009). The reducing sugar in glucose equivalent (16.46±0.20) was higher than the range of reducing sugars (2.9 to 8.9) of different species of baobab reported by Ibrahima *et al.* (2013). Nour *et al*. (1980) reported a 23.2% total sugars and 19.9% reducing sugars of baobab pulp and with respect to Murray *et al.* (2001), simple sugars represent about 35.6% of total carbohydrates. The sugar content explains the remarkably sweet taste of the pulp though the sweetness can vary depending on the species, maturity of the fruits, and environmental soil and

climate (Ibrahima *et al*., 2013). The reducing sugar will be used up as a source of energy for the growth of LAB during fermentation. Its pectin content 28.34±1.07% and Vit C (137.44±1.46 mg/100g) falls within the range 23.4 - 33.80% and 74.00 - 163.00 mg/100g respectively, presented by Leatherhead (2009). High pectin content renders juice cloudy with a high viscosity that could pose mixing and pumping problems during processing (fermentation) (Abbasiliasi et al., 2017). LAB also obtain their energy from the metabolism of simple sugars, and glucose is the main carbon source. There is therefore the need for hydrolysis of pectin for the release of more simple sugars for fermentation by LAB.

Some minerals were determined in baobab and amongst potassium (1181.81±0.54 mg/ 100g) was the highest followed by, calcium (180.67±1.89 mg/ 100g), magnesium (85.88±1.08 mg/ 100g), iron (22.96±0.81 mg/ 100g), and sodium (18.85±0.59 mg/ 100g) in descending order. The mineral content of baobab pulp reported by Leatherhead (2009) in descending order was potassium (2010.00 - 2390.00), calcium (257.00 - 370.00), magnesium (126.00 - 179.00), iron (3.95 - 9.13), sodium (7.00 - 31.10) mg/100g. This order is same order realized in the baobab sample analysed but for the fact that the analysed sample, potassium, calcium, magnesium content was lower while the iron content was higher and the sodium content was within the range of that of literature. Also, Ibrahima *et al.,* (2013) realized for different species of baobab pulp, the mineral composition: K (1528 to 3054); Ca (313 – 658); Mg (176 – 255); Fe (4.9 - 16.6); Na (2.3 - 43.4) mg/100g. Potassium is therefore the main mineral present in baobab fruit pulp. Potassium is crucial to heart function and plays a key role in skeletal and smooth muscle contraction, making it important for normal digestive and muscular function. Along with sodium, potassium regulates the water balance and the acid-base balance in the blood and tissues, and plays a critical role in the transmission of electrical impulses in the heart (Pohl *et al.,* 2013). Ca is generally present in small

amounts in fruits and vegetables (Paul and Shaha, 2004; Leatherhead, 2009) but that of baobab is quite high when compared to other dehydrated fruits like apples (19), apricots (61), bananas (22) and peaches (38) mg/100g (Leatherhead, 2009). Baobab can therefore be considered as a natural source of calcium supplementation for pregnant and lactating women, as well as for children and the elderly (Osman, 2004) especially with those with osteoporosis problems. Magnesium is an essential cofactor in many enzymatic reactions in intermediary metabolism while Iron is highly required physiologically for heme formation and to enhance oxygen carrying capacity of red blood cells. The low Na content is good because Na is linked to hypertension in humans (Okwu and Emenike, 2007). These minerals are also important for the growth of LAB as they act as growth factors. The variation in the proximate composition and mineral content of the baobab pulp of this study with that of literature could be due to the soil type and the origin of sample (Aluko *et al*., 2016; Assogbadjo *et al*., 2012).

Some bioactive components; total polyphenol and antioxidant activity were determined and the results presented in table 7.

Table 7: Bioactive composition of baobab pulp

Bioactive component	**Quantity**
Total polyphenol	2619.05±1.69 mg GAE /100g DW
Antioxidant activity	**Quantity (mg TE/100g DW)**
DPPH	3879.00±1.20
FRAP	4640.98±1.18
ABTS	4931.99±3.12

The TPC of baobab pulp of this study is higher (2619.05±1.69 mg GAE /100g DW) than the TPC of baobab pulp reported by Ismail *et al.* (2019) (543.34 ± 2.05 mg GAE/100 g DW) and Tembo *et al*. (2017) (1870± 1.61 mg/100 g FW) who also used 80% methanol in extraction of the bioactive components of baobab. Baobab is a rich source of polyphenol and vitamin C contributes to its high antioxidant content. The 2, 2–diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was 3879.00±1.20 (mg TE/100g DW) higher than 2216.69 ± 7.15 mg TE/100g DW presented by Ismail *et al.* (2019). Baobab pulp therefore has a high free radical scavenging ability. The Ferric reducing antioxidant power (FRAP) assay assesses the electron transfer potential and it was 4640.98±1.18 mg TE/100 g DW. This value is similar to 4564.81 ± 16.45 mg TE/100 g DW) (Ismail et al., 2019) and 4660 ± 11.8 mg TE/100 g FW (Tembo *et al*., 2017). The high FRAP content indicates baobab has a better antioxidant activity. In addition, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical quenching ability of baobab was 4931.99±3.12 mg TE/100 g DW high when compared to 1680± 17.0 mg TEAC/100 g FW presented by (Tembo *et al*., 2017). The difference in the antioxidant activity of baobab pulp when compared to literature can be due to the incubation period for the different assays (Tembo et al., 2017), soil type, geographical location, age of fruit, processing and storage conditions (Kaboré et al., 2011). The baobab pulp is therefore a functional food due to its antioxidant activity and therefore the baobab juice could serve in the functional food market.

2.3.2 Amino acid profile of baobab pulp

Table 8 shows the amino acid profile of baobab pulp. Out of the 10 known essential amino acids, only 6 were detected in baobab pulp with the composition ranging between 0.140 to 15.515 mg/100 g DW. Arginine (15.515±0.168 mg/100 g DW) was the principal essential amino acid determined followed by histidine (5.475±0.197 mg/100 g DW). Other essential amino acids were

present in trace amounts. For the non-essential amino acids, asparagine (50.742±4.840 mg/100 g DW) was the main amino acid detected followed by proline (4.797±0.465 mg/100 g DW) and then serine (2.229±0.156 mg/100 g DW). The other non-essential amino acids were present in trace amounts and others not detected (table 8). The only sulfur containing amino acid present was cysteine (0.949±0.216 mg/100 g DW). Tryptophan (1.301±0.702 mg/100 g DW) was the only aromatic amino acid detected. The results were different from the amino acid profile of baobab pulp analysed by others (Cisse and Montet, 2012; Makawi *et al*., 2019; Osman, 2004). Osman (2004) found high amounts of glutamic acid (23.7 g/100 g protein), aspartic acid (10.3 g/100 g protein), and arginine (8.0 g/100 g protein). Cisse and Montet (2012) obtained varying concentrations for different baobab species. Generally, aspartic acid, arginine, asparagine and methionine were present in high amounts. Makawi *et al.* (2019) detected aspartic acid (224.4 mg/100 g protein) as the main amino acid present followed by serine (68.3 mg/100 g protein) and alanine (55.6 mg/100 g protein).

Since the baobab will be destined for fermentation, the amino acids will be necessary as carbon source for LAB. In addition to fermentable sugars as energy source for fermentation of LAB, other growth factors like specific amino acids are required for their growth (Nsogning *et al.*, 2018). However, the composition of nitrogenous compound concentrations in fruits and their derivate products are poor (de Nadra, 2007). Therefore, mixtures with lemongrass could ferment better due to the high amino acid composition of lemongrass.

Table 8: Amino acid profile of baobab pulp

Amino acid	Composition (mg/100 g DW)
Essential amino acids	
Arginine	15.515±0.168
Valine	0.446±0.034
Methionine	ND
Leucine	ND
Threonine	0.140±0.122
Phenyl alanine	ND
Histidine	5.475±0.197
Isoleucine	ND
Lysine	1.077±0.352
Tryptophan	1.301±0.702
Non-essential amino acids	
Serine	2.229±0.156
Leucine	ND
Asparagine	50.000±4.000
Cystine	ND
Glutamic acid	1.340±0.033
Glycine	ND
Proline	4.797±0.465
Aspartic acid	1.990±0.326
Glutamine	1.468±0.044
Tyrosine	ND
Isoleucine	ND
Hydroxy proline	ND
Cysteine	0.949±0.216
Alanine	0.632±0.012

ND = Not detected

2.3.3 Extraction and characterization of baobab pectin

The yield of baobab pectin was 39.02± 0.59% which is quite high compared to, kiwifruit pomace pectin (ranged between 3.6% and 4.5% w/w) extracted with different extraction methods (Yuliarti *et al.*, 2015), carrot pomace pectin (ranged 5.0 to 15.2%) extracted at different extraction conditions (Jafari *et al*., 2017), orange peel powder pectin (8.78%) (Kute *et al*., 2020). High pectin content increases turbidity and viscosity of fruit juices (Barros et al., 2004). Viscosity influences

the fluidity of juice and this influences the mass transfer effect between the substrate and the microorganism during fermentation (Shen et al., 2009).

The extraction method has a great effect on the pectin yield and for the conventional methods, the type of acid used and extraction conditions affect the pectin yield. Baobab is therefore a good source of pectin. Pectin are used as thickening agent, a gelling agent and a colloidal stabilizer in industries (Raj et al., 2012).

2.3.3.1 Equivalent weight of baobab pectin

Equivalent weight is one of the physical properties used to determine the gelling ability of pectin. The baobab pectin has an equivalent weight of 404.86±1.64 which is lower compared to apple pomace pectin (833.33 to 1666.30) (Kumar and Chauhan, 2010), pomelo peel pectin (540.04 to 711.33) (Roy *et al*., 2017) and within the range of lemon pomace pectin (368±3 to 1632±137) (Azad *et al*., 2014). Baobab pectin therefore has a low equivalent weight and pectin with low equivalent weight are as a result of higher partial degradation of the pectin. Low equivalent weight pectin gel independent on sugar but in the presence of calcium or magnesium ion (Nguyen and Pirak, 2019). The variations in the equivalent weight is due to the amount of free acid present (Devi *et al*., 2014; Yadav *et al*., 2017).

2.3.3.2 Methoxyl content and Anhydrouronic acid content (AUA) of baobab pectin

Methoxyl content describes the ratio of methyl ester to all esterified groups linked to the carboxyl groups of pectin molecules (Yadav *et al*., 2017). Likewise, it is a factor that controls the setting time of pectins and their gel formation ability (Devi *et al*., 2014; Yadav *et al*., 2017). Increase in methoxyl content leads to increase in spreading quality and sugar binding capacity of pectins (Devi *et al.*, 2014). The methoxyl content of baobab pectin was obtained to be 9.15±0.15%.

Pectin with methoxyl contents ≥7 are classified as high methoxyl (Yadav et al., 2017), hence baobab pectin is high methoxyl.

AUA is a parameter that determines the purity of extracted pectin and its value should not be less than 65% (Azad *et al*., 2014; Yadav *et al*., 2017). The baobab pectin has high value of AUA, 95.39±1.06% which implies the pectin was pure and free from protein.

2.3.3.3 Degree of esterification (DE) of baobab pectin

DE is used to classify pectin as low methoxyl pectin (LMP) (DE < 50%) or high methoxyl pectin (HMP) (DE > 50%) as it measures the extent to which carboxyl groups in pectin molecules exist as methyl ester (Yadav *et al*., 2017). The baobab pectin has a DE of 54.42±0.32% and can therefore be classified as high methoxyl pectin. However, DEs values for commercial HM-pectins typically range from 60 to 75% and those for LM-pectins range from 20 to 40%. This implies baobab pectin doesn't fall within the range of commercial pectin. HMP form gels at pH around 3.0 and also requires a minimum amount of soluble solids. The rate of setting of the gels also increases with increase in DE (Raj *et al.*, 2012). Therefore, gels from baobab pectin will set slowly. The DE can also be used as a factor to determine the specific type of pectinase to use for hydrolysis. There are different types of pectinase with specific mechanism of action depending on the structure of the pectin. To further confirm whether the baobab pectin was HMP or LMP, FTIR of the baobab pectin was evaluated.

2.3.3.4 FTIR spectra of baobab pectin

FTIR was used to determine the functional groups present in baobab pectin compared to that of commercial pectin (SIGMA) (figure 13) to confirm if the extracted baobab pectin is indeed a methylated pectin. The peaks at 3794 cm^{-1} and 3333 cm^{-1} for extracted pectin and 3308 for

commercial pectin are due to O-H stetching as a result of inter and intramolecular hydrogen bonds of the galacturonic acid backbone of the pectin molecule (Chaliha *et al.*, 2017). The next band observed at 2936 cm^{-1} for extracted pectin and 2933 cm^{-1} for commercial pectin corresponds to C-H stretching (include CH, CH_2, and CH_3). The bands at 1730 cm^{-1} and 1601 cm^{-1} refer to stretching vibrations of ester carbonyl groups (C=O) and carboxyl groups (COO-), respectively. A stronger absorption at 1730 cm^{-1} and a weaker absorption at 1601 cm^{-1} (Khamsucharit *et al.*, 2018) indicates that the baobab pectin is a high methoxyl pectin (DE > 50) and this same trend was also observed with the commercial pectin, stronger absorbance at 1728 cm^{-1} and weaker at 1604 cm^{-1}. The presence of methyl groups confirms the results obtained in section 2.3.3.3. The band region observed between 1413 cm^{-1} to 410 cm^{-1} is the fingerprint region specific for each polysaccharide and is difficult to assign a band of this region to a specific atom group vibration because they correspond to complex interacting vibrating systems (Manrique and Lajolo, 2002). The spectra at that region were also similar to that of commercial pectin. The results therefore suggest that the FTIR spectrum of commercial pectin is similar to that of the baobab pectin and the baobab pectin is HMP.

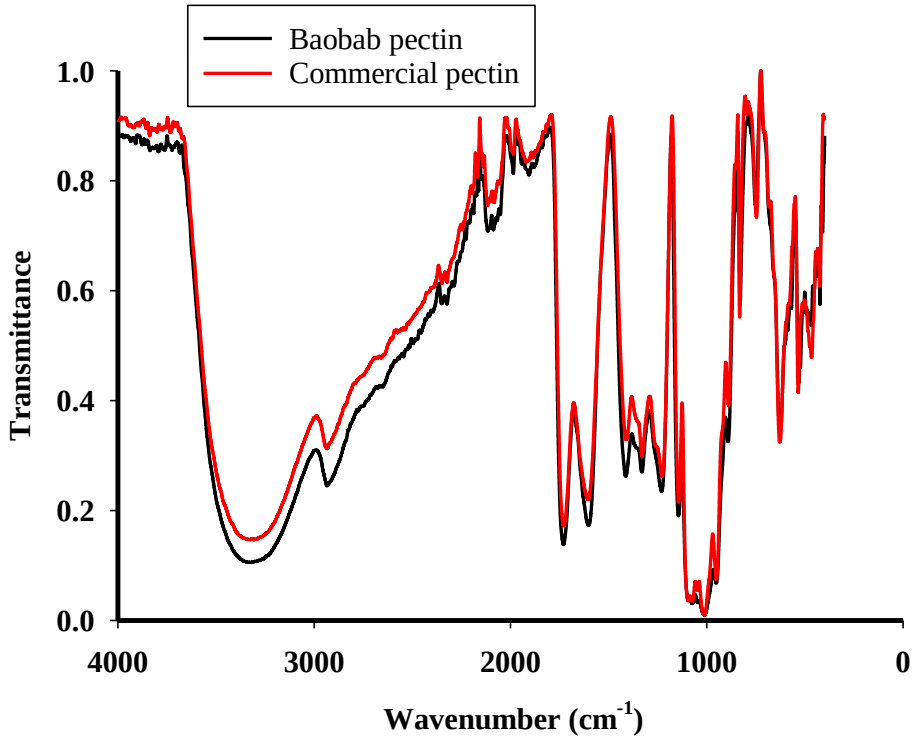


Figure 13: FTIR spectra of extracted pectin and standard pectin

Table 9: Frequencies and intensities of functional groups present on pectin

Wavelength (cm-1)	Functional group
3894-3333	O–H stretching
2936	C–H stretching
1730	C=O, esterified
1601	COO- asymmetric stretching
1413	COO- symmetric stretching
1330	C–H bending
1230-1012	C=O Stretching

2.3.4 Pectin hydrolysis of baobab pectin

Baobab is a fibrous fruit with a high pectin content of 28.34±1.07 g/100g DW. Pectin in its native form is complex for microbial utilisation during fermentation and in order to avoid the use of additives, there was therefore a need to hydrolyse the pectin to release more fermentable sugars and oligosaccharides that will be used as carbon source during fermentation. Pectin also increases the viscosity of juice which could affect the mass transfer effect between substrate and microorganism during fermentation (Shen et al., 2009). The pectin and reducing sugar content of the juice at the different hydrolysis time was therefore determined in order to evaluate the effect of the process parameters on the juice obtained.

2.3.4.1 Effect of process parameters on the degree of hydrolysis of baobab pectin

The degree of hydrolysis for each set of process parameters (enzyme concentration and time), was determined to measure the extent of hydrolysis of the pectin in the baobab juice and the results presented in Figure 14. The DH increased from 0 – 31.69%, 47.10%, 56.73%, and 74.67% respectively for 0.03, 0.07, 0.1 and 0.2% v/v pectinase concentration. At low enzyme concentrations (0.03 and 0.07% v/v), the straight lines indicate a prolonged first order reaction. This is because depletion of pectin is much less at low enzyme concentration due to diffusional limitation between substrate and enzyme active site. There is the formation of less enzyme-substrate complex and therefore less release of products in the medium. For the enzyme concentrations 0.1 and 0.2% v/v, there was a sharp increase in the DH up to 30 min of hydrolysis, followed by a steady increase. Sengupta *et al*. (2018) also realized a sharp increase in the degree of hydrolysis of bael (*Aegle marmelos*) juice in the first 30 min of reaction and further, there was a slow rise. Similar trend was also noticed by Ninga *et al.* (2018) in hydrolysing pectin in guava.

They realized a sharp increase in the degree of hydrolysis within 20 min and later a steady increase. In the first 30 min, the slope is constant (figure 14) which implies constant depletion of pectin present in the baobab due to the fact that the active sites of the pectinase are free and therefore, there is a high DH at that point. There is a significant decrease in the pectin concentration thereby leading to the accumulation of reaction products in the medium. Beyond 30 min, there is change in the gradient of the curve which could be explained by the effect of saturation of enzyme active sites with substrates and also the accumulation of hydrolysed products in the medium could lower the activity of the enzyme.

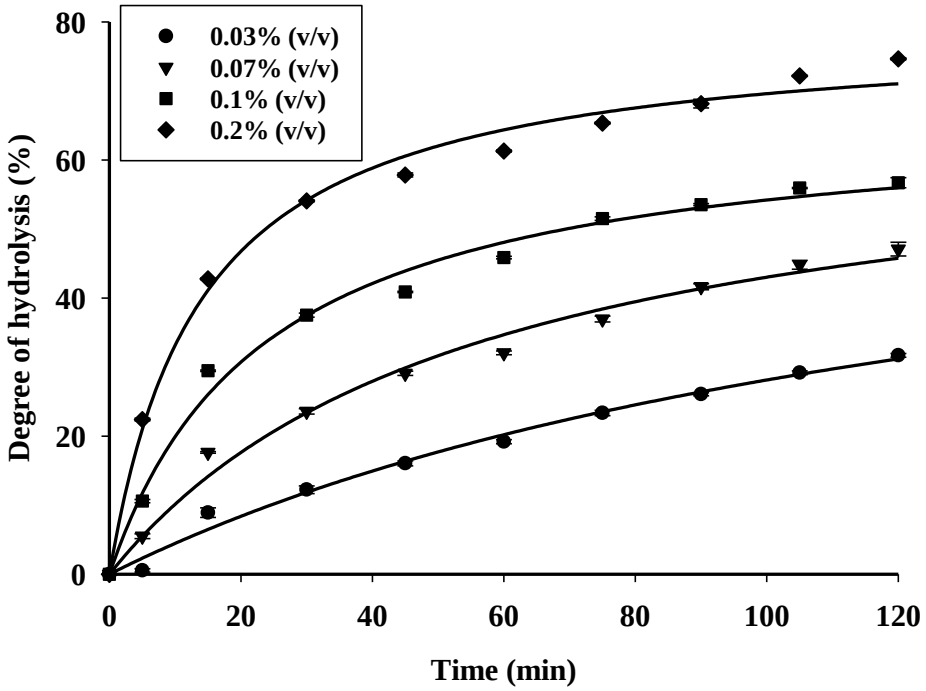


Figure 14: Degree of hydrolysis of baobab juice with respect to different incubation time for different enzyme concentrations

It is as well observed that the DH increased with increasing enzyme concentration from 0.03% to 0.2% regardless of the hydrolysis time. This can be explained by the fact that as the

enzyme concentration increases, there is an increased number of active sites present for the reaction to occur but at low concentrations, there is saturation of the active sites. Enzyme concentration, 0.2% v/v, displayed the highest DH throughout the hydrolysis time. Therefore, 0.2% v/v enzyme concentration and reaction time 120 min, was the optimal pectin hydrolysis conditions. This condition is different from that of Ninga *et al.* (2018) and Sengupta *et al*. (2018) due to the difference in substrates and difference in type of pectinase used. They used polygalacturanase while in this work, a consortium of pectin lyase and polygalacturanase was used. These two enzymes have different mechanisms of action. Pectinase (Pectinex® Ultra AFP, (Novozymes)), isolated from *Aspergillus aculeatus* and *Aspergillus niger*, is a mixture of principally pectin lyase (10%) and polygalacturunase (0.2%). The key enzyme, pectin lyase catalyse the eliminative cleavage of (1,4)-alpha-D-galacturonan methyl ester giving oligosaccharides with 4-deoxy-6-O-methyl-alpha-D-galact-4-enuronosyl groups at their non-reducing ends (Novozyme Product data sheet). The pectin content of the resulting juice thereby decreases and there is the release of galacturonic acid and oligosaccharides in the medium (Combo *et al*., 2012).

Combo *et al.* (2012) established the activity pattern of treatment of polygalacturonic acid with different enzymes (including Pectinex Ultra SP-L from Novozymes), for different hydrolysis time (5 min to 2h). They reported the accumulation of mono, di and trimers of galacturonic acid after 2 h of hydrolysis but there was a high yield of di and trimers of galacturonic acid in the first 5 min. This could explain the reduction of pectin content in the first 5 min in the hydrolysis of baobab pectin. The accumulation of small hydrolytic units at the beginning is due to cleavage of galacturonic acid monomers or digalacturonic acid from the non-reducing end by exo-PG (Nikolic´ and Mojovic, 2007). Pectin lyase (PL) cleaves glycosidic bonds by ß-elimination to yield

oligomers without absolute requirement of Ca^{2+} (Oumer, 2017). They have preference for high degree of methylated pectins and baobab pectin is partially methylated pectin with a degree of methylation of 54.42%. The activity of PL increases with increase in degree of esterification (>50% DE) (Yadav *et al*., 2009) and the activity decreases with partly methyl-esterified oligoGalpA due to the presence of unesterified GalpA residues (van Alebeek *et al*., 2002). Benen, Voragen and Visser (2003) studied the mode of action of pectin lyase A on $(6\text{-}0\text{-}CH_3GalpA)_6$ and they realized the degradation of GalpA6-6CH3 yielded saturated and unsaturated $(GalpA)_2$, $(GalpA)_3$ and $(GalpA)_4$. PLA has at least 8 subsites. On the other hand, polygalacturonases (PGases), hydrolyse α-1,4 glycosidic bonds between two galacturonic acid residues. They can either be endo-PG, catalysing the random hydrolysis of α-1,4- glycosidic bonds of pectic acid or exo-PG catalysing the sequential hydrolysis of α-1,4-glycosidic linkages in pectic acids (Naidu and Panda, 1998). *A. niger* has at least 7 endoPGs (PG I, PG II, PG A, PG B, PG C, PG D, and PG E). They can be grouped in to PG I, PG A, PG C, and PG D which have almost same properties and then PG II, PG B, and PG E. PG D is the only isomer that hydrolyses dimers while the other isomers hydrolyse trimers as their smallest hydrolysable substrates. Hydrolysis of trimers take place slowly from the reducing end at the first glycosidic linkage. However, hydrolysis of tetramer is of greater interest. Hydrolysis can either be by secondary attack or multiple attack/processivity. For secondary attack, both products are released from the enzyme and the larger product serves again as a substrate. For processivity, only one product (the smaller product) is first released while the other is retained at the enzyme and subsequently shifts over the active site for another catalytic event (Benen *et al*., 2003). Endo-PGs act by random depolymerisation of pectic acids thereby, resulting in a rapid decrease in the viscosity while the sequential action of exo-PG cause a large increase in the formation of reducing groups and a slow decrease in viscosity (Wong, 1995).

2.3.4.2 Depectinisation kinetics of baobab pulp pectin

Figure 15 presents three phases and the different phases represent different reaction orders. The first phase represents the zero order in which the rate does not depend on the pectin concentration and in this region, the active sites of the enzymes are getting saturated. It is therefore considered region of low activity (Robinson, 2015). The other phase represents the first order where the rate depends on the pectin concentration. The active sites of the enzymes are free and therefore hydrolysis is highest at that section and is considered as area of full activity (Robinson, 2015) and the last phase is the zero-order phase.

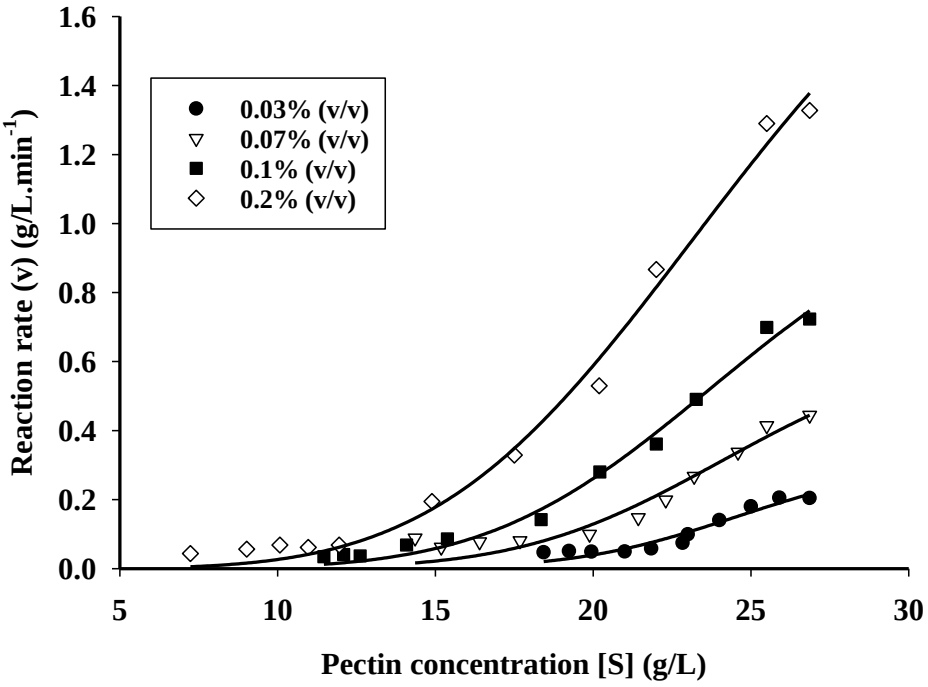


Figure 15: Rate of reaction of depectinisation of baobab pulp pectin at different enzyme concentrations

The curve is semi-sigmoidal which deviates from the hyperbola shape of the Michaelis – Menten kinetics. Hill *et al*. (1977) stated deviations from Michaelis-Menten Kinetics could be as a result of substrate inhibition, substrate activation, random pathways or allosteric effects (Seibert

and Tracy, 2014). The formation of products (galacturonic acid) in the medium can in turn inhibit the activity of the enzymes. Enzymes are made up of active sites with specific ionic groups and these ionic groups must be in a suitable form (acid or base) to function. However, in the course of hydrolysis, there is the variation in the pH of the medium due to release of galacturonic acid which can result in changes in the ionic form of the active site. This thereby leads to changes in the activity of the enzyme and hence the reaction rate. (Doran, 2013). Phenolic acids have been shown to inhibit enzyme activity studied in PG enzymes and the order of inhibition is as follows: cinnamic acid > ferulic acid > *p*-coumaric acid > salicylic acid > chlorogenic acid (Kant *et al.*, 2013). Phenolic compounds like ferulic acid, *p*-coumaric acid, and chlorogenic acid (section 2.3.10) were identified to be present in baobab pulp. This could be one of the factors that led to the sigmoidicity of the curve. Cooperative substrate binding also leads to deviation from the Michaelis-Menten Kinetics (Marangoni, 2003). Cooperative interaction is the binding of one substrate molecule inducing conformational changes in the enzyme that result in altered affinities at other vacant sites (Marangoni, 2003; Seibert and Tracy, 2014). Cooperativity can either be homotropic or heterotropic. In homotropic cooperativity, same substrate molecules can simultaneously bind to the enzyme active site and is referred to as autoactivation. The Hill equation has been used to describe the kinetic behaviour of cooperative enzymes.

2.3.4.3 Effect of enzyme concentration on Hill parameters

The Hill equation (equation 30), a three-parameter function (k', n, Vmax) was fitted to the experimental v versus [S] data, and the estimates of k', n, and Vmax were obtained for the different enzyme concentrations (table 10). The R^2 of the different enzyme concentrations were above 0.9 which shows that the data properly fits to the Hill model.

The value of n which stands for the degree of cooperativity were 7, 6, 5, and 4 respectively for the enzyme concentrations; 0.03, 0.07, 0.1 and 0.2 % v/v. Ninga *et al.* (2018) also realised decrease in the value of n from 7 to 4, with increase in enzyme concentration from 0.033 to 0.1% w/w for pectin hydrolysis of guava pulp. The decrease in the value of n with respect to the enzyme concentration implies there is decrease in the cooperativity and for the case where n=1, there is no cooperativity and the Hill equation reduces to the Michaelis-Menten model (Marangoni, 2003). Values of n greater than 1, depict positive cooperativity which means the binding of the first molecule enhances the ease in the binding of the second molecule to occur (Seibert and Tracy, 2014).

Table 10: Hill model parameters for baobab pectin hydrolysis at different enzyme concentrations

	Enzyme concentration			
	0.03 % v/v	**0.07 % v/v**	**0.1 % v/v**	**0.2 % v/v**
Vmax (g/L.min^{-1})	0.336±0.016	0.729±0.035	1.255±0.030	2.378±0.0678
n	8.801 ≈ 9	6.721 ≈ 7	5.850 ≈ 6	4.861 ≈ 5
Adj R^2	0.921	0.932	0.989	0.989
[S50] (g/L)		25.14		

Active sites which are saccharide-binding-units are generally referred to as subsites. Depolymerizing enzymes like pectinase have multiple subsites which varies from 2 to 14 and are generally labelled from -n to +n (the annotation ‘n’ is different from the Hill coefficient). The non-reducing end are denoted as -n and the reducing end, +n of the sugar moieties (Massa et al., 2007). The subsites are linearly aligned with the active site found somewhere in the array (figure 16). The nature of the active site determines the specificity and mechanism of action of pectinases (Wong, 1995). The active site is in the region of -1 and +1 and therefore catalysis occurs when the substrate

covers that subsite. Binding of substrate to subsite which does not cover the active site leads to unproductive product which is an inhibitory (competitive) complex (Benen *et al*., 2002).

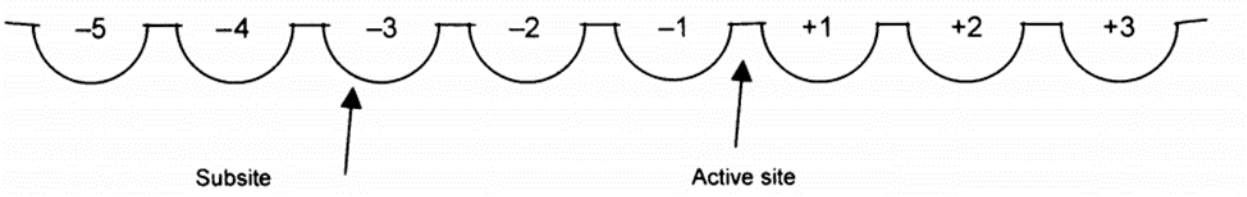


Figure 16: Subsite array of pectinase enzyme showing the subsite and the active site

PLA is made up of eight subsites (-5 to +3) in the substrate-binding cleft. The highest affinity is realised at subsite +3, followed by subsites -2 and -4, while subsites -5 and -3 present the lowest affinities. In order to form a productive product, the substrate at +1 and +3 has to be methyl-esterified because they have hydrophobic pocket for proper fitting of the methyl-esters. Subsite +2 readily tolerates carboxyl groups because it lacks hydrophobic pocket, and same for all negative subsites. This implies for formation of productive enzyme-substrate complex, binding has to be more specific at the positive subsites than at the negative subsite (van Alebeek *et al*., 2002). On the other hand, endo-PGs are processive enzymes and the rate of processivity differ with DP. PG1 is processive at DP ≥ 6 and has a high affinity for subsite -5 which explains its processive nature. PGA and PGC are processive at DP ≥ 7 while PGD is processive at DP ≥ 4. Binding of substrates to the different subsites depends on the affinity of the substrate to the subsite. For hydrolysis of hexamers, the pentamer product binds to subsite -5 to -1, therefore the pentamer has a high affinity for that subsite. Furthermore, when a pentamer is hydrolysed, a productive complex binds subsite -4 to +1 and -3 to +2 and an unproductive complex can also be formed in subsite -5 to -1. Since PGD is processive at DP 4, it has four functional subsites, -3 to +1 and can also hydrolyse dimers (Benen *et al*., 2003).

2.3.4.4 Effect of enzyme concentration on maximum rate of reaction (Vmax)

Figure 17 represents effect of enzyme concentration on maximum rate of reaction (Vmax). There was a monotonic rise of the maximum rate of reaction with increase in enzyme concentration. The Vmax increases from 0.336±0.016 to 2.378±0.0678 g/L.min^{-1} with increase in enzyme concentration from 0.03 to 0.2% v/v. At higher enzyme concentrations, there is the formation of more ES complexes and therefore the release of more products in the medium. This therefore leads to an increase in the maximum velocity.

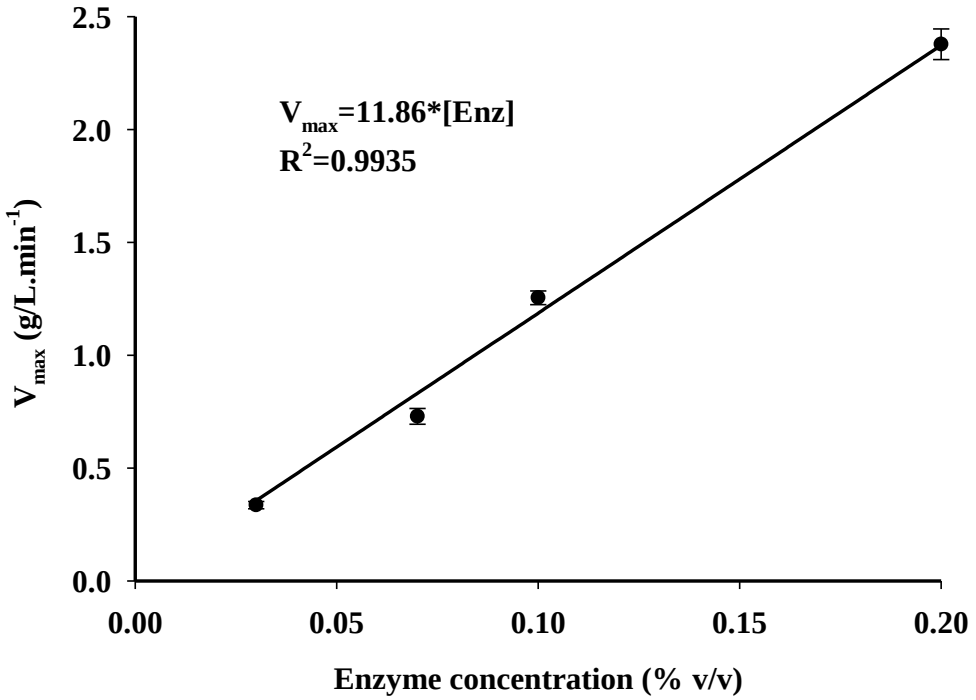


Figure 17: Effect of enzyme concentration on maximum reaction rate (Vmax)

During enzyme hydrolysis, the substrate binds to the active site of the enzyme thereby forming enzyme-substrate (ES) complex. As hydrolysis time increases, there is the formation of more ES complexes thereby leading to saturation of the enzyme active sites. The maximum

velocity (Vmax) of the enzyme, is therefore the point at which the enzyme active sites are bound to substrate, and the amount of ES complex equates the total amount of enzyme (van Oort, 2010).

2.3.5 Effect of enzyme concentration on reducing sugar content of baobab juice

The reducing sugar yield at different incubation times for the different enzyme concentrations were determined and the results expressed in glucose equivalent is represented in figure 18. The reducing sugar content increased gradually with increase in incubation time and also with increase in enzyme concentration from 0.03% to 0.2%. This same trend was realized by Belafi-Bako´ *et al.* (2007) who hydrolysed pure pectin using polygalacturonase from *Aspergillus niger*. Pectinase act by either multiple attack or processivity thereby releasing monomers. PL catalyse random cleavage of methylated pectins by ß-elimination, simultaneously releasing saturated monomers and dimers and 4,5 unsaturated trimer and tetramer. For partly methylated substrates, unproductive enzyme-substrate complexes can be formed, which greatly reduce the overall PLA activity (van Alebeek *et al*., 2002). Conversely, endoPGs release monomers from the reducing end while exoPGs release monomers from the non-reducing end (Benen et al., 2002).

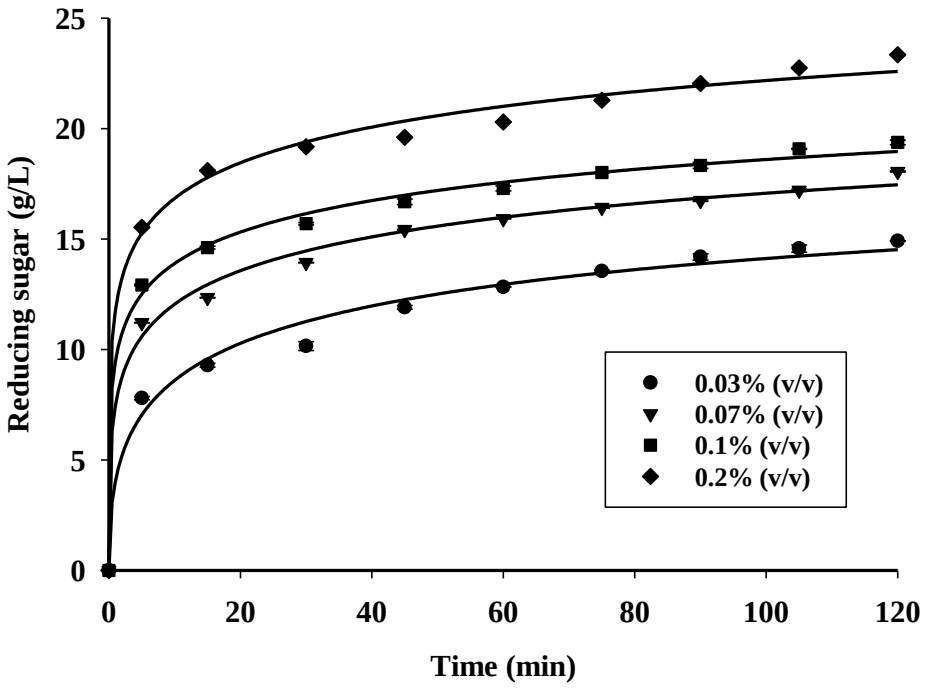


Figure 18: Reducing sugar content of different enzyme concentrations at with respect to different incubation times

The product progression profiles (PPP) for PG1, PGA, PGC and PGD are similar in that at the beginning of the hydrolysis, there is a rapid increase of the monomers. This can explain the increase in reducing sugar content in the first 15 min (figure 18). But as time increases, large products formed at the beginning are being hydrolysed in to smaller products until the smallest substrate that can efficiently be hydrolysed is depleted. This is evident in the product progression profiles of PG II, PGB and PGE (Benen *et al.*, 2002). As the enzyme concentration increases, the number of active sites present in same amount of substrate concentration increases and the quantity of product release is greater. There is also the release of galacturonic acid in to the reaction medium of which increase concentration in the medium leads to product inhibition that reduces the reaction rate (Belafi-Bako´ *et al*., 2007).

2.3.6 Scanning Electron Microscope (SEM) analysis of baobab pulp

The microscopic surface of the baobab pulp before and after treatment with the different concentrations of pectinase are presented in figure 19. The untreated baobab pulp shows smooth, compact surface of the fibres with intact folds. The surface component was greatly destroyed by pectinase which the destruction increased with increase in the enzyme concentrations (figures b, c, d, and e). Alba *et al.* (2020) identified uronic acid as the main monomer of polysaccharide present in baobab pulp. Uronic acid is a component of pectin and pectin are complex polysaccharides present in the primary cell wall and middle lamella of plant cells (Naidu and Panda, 1998). The pectinase hydrolyses these pectin present in the cell wall of the baobab thereby exposing the internal structure. Exposure of the internal structure makes it more accessible for the pectinase and therefore the release of monosaccharides and/or short chain polysaccharides into the medium.

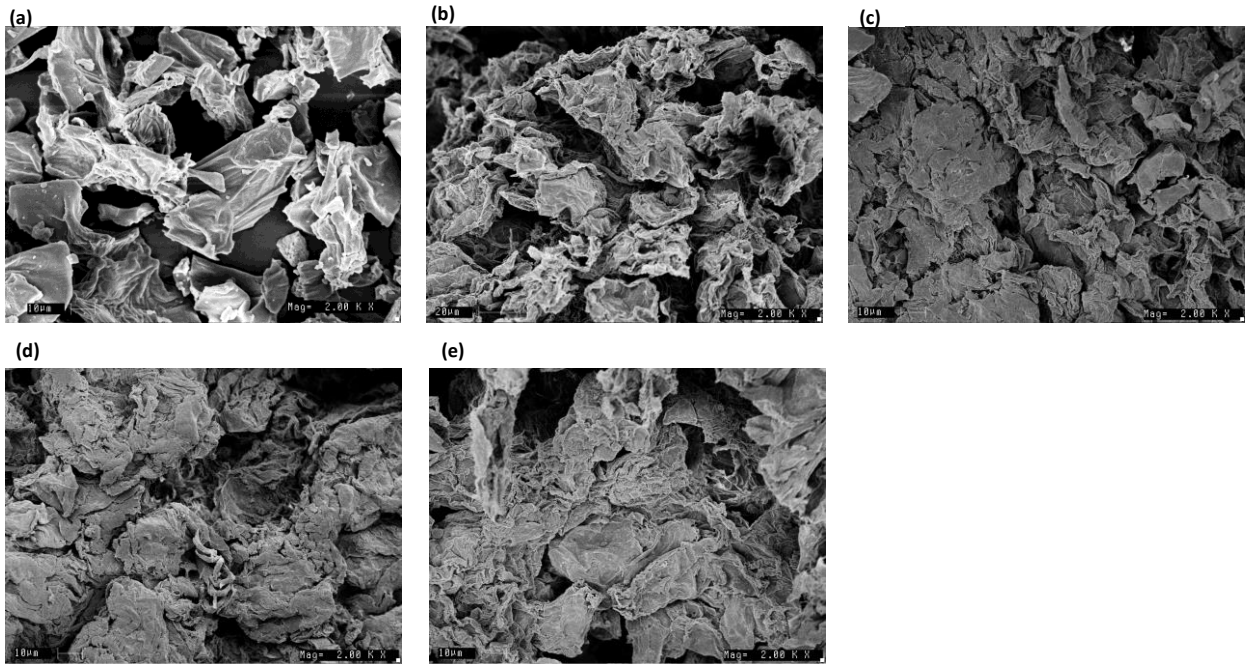


Figure 19: SEM images for baobab samples (a) sample without enzymatic treatment (b) sample treated with 0.03% pectinase for 120 min (c) sample treated with 0.07% (d) sample treated with 0.1% and (e) sample treated with 0.2%

2.3.7 Effect of hydrolysis on rheology of baobab juice

The rheograms of the baobab juice before and after hydrolysis analysed at 25 ^{o}C is illustrated in figures 20. The data was fitted to the Herschel-Bulkley and power law models and the model parameters determined (table 11). The Herschel-Bulkley model reduces to the power law model when the yield stress, $\tau_0 = 0$ and to the Bingham model when n=1 (Keshani *et al.*, 2012). The data best fitted to the Herschel-Bulkley model with $R^2 > 0.98$ and lower values of RSME compared to the Power law model.

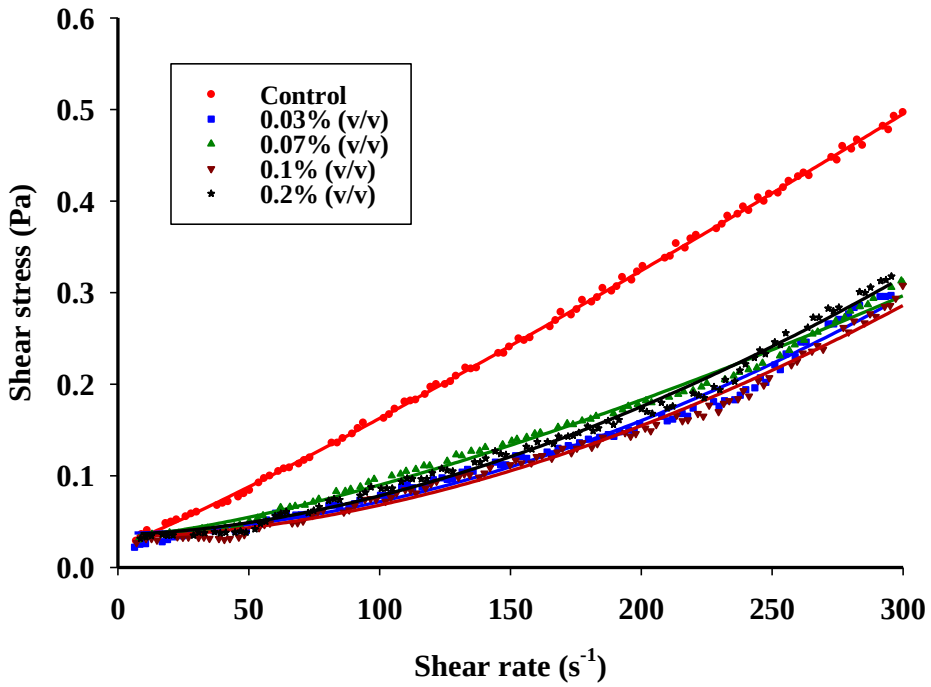


Figure 20: Rheogram of baobab juice before and after hydrolysis with different enzyme concentrations at 25 ^{o}C

The consistency index, K, decreased with hydrolysis. The consistency index of the non-hydrolysed sample was high due to the presence of pectin molecules. Upon hydrolysis of the baobab, the apparent viscosity decreased because of partial degradation of complex pectin molecules present thereby leading to a decrease in the consistency index. The consistency index also decreased with increase in pectinase concentration and this result corroborates that of Sharoba and Ramadan (2009).

The juice presents a non-Newtonian flow behaviour and the values of n are greater than one which implies it is a shear-thickening fluid. This could be as a result of the presence of pectin in the juice due to the fact that a partial hydrolysis (74.67%) was done not a complete hydrolysis. The shear stress for all cases is low, therefore, less stress will be needed to cause the fluid to flow. On the

other hand, the n values for the power law model were lower than that of the Herschel-Bulkley model.

Table 11: Rheological parameters of Herschel-Bulkley and Power law models of hydrolysed baobab juice compared to the non-hydrolysed sample evaluated at 25 ^{o}C

	K	***n***	**τ_0**	**R^2**	**RSME**
			Herschel-Bulkley model		
Control	0.0008	1.1106	0.0236	0.9994	0.0033
0.03%	7.3532E-6	1.8352	0.0373	0.9858	0.0092
0.07%	9.1484E-5	1.3966	0.0332	0.9930	0.0067
0.10%	8.1483e-6	1.8132	0.0335	0.9897	0.0080
0.20%	1.4414e-5	1.7318	0.0363	0.9914	0.0078
			Power law model		
Control	0.0017	0.9918		0.9978	0.0064
0.03%	0.0003	1.2080		0.9615	0.0150
0.07%	0.0008	1.0294		0.9814	0.0110
0.10%	0.0002	1.2664		0.9701	0.0135
0.20%	0.0003	1.2138		0.9728	0.0138

- **Apparent viscosity**

The apparent viscosity of the baobab juice before and after pectinase hydrolysis decreased with increasing shear rate (figure 21) up to a critical shear rate of about 55 s-1. After the critical shear rate, the apparent viscosity remains constant with increase in shear rate. This section is typical for Newtonian fluids where the viscosity of the fluid is independent of shear rate. The

apparent viscosity again increases after application of shear rate above 225 s-1. This is because Newtonian behaviours are kept constant at low temperatures than at high temperatures (Vidal *et al*., 2018). However, from the flow behaviour index, n values (n > 1) (table 11), the fluid generally exhibits a non-Newtonian behaviour. These results are similar with that of Vidal, Carvalho and Pereira (2018) on cashew apple by-products.

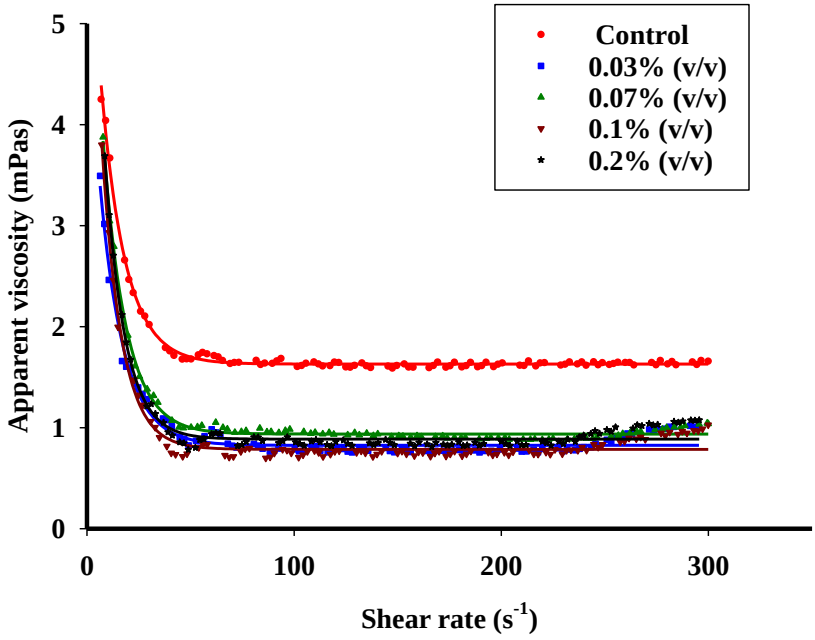


Figure 21: Apparent viscosity curves of non-hydrolysed and hydrolysed baobab juice at different pectinex concentrations measured at 25 ^{o}C

Generally, the apparent viscosity decreased greatly after enzyme treatment. Baobab is rich in pectin which has a cohesive network as a result of its high-water holding capacity (Chauhan *et al*., 2017). The pectin content therefore causes an increase in the apparent viscosity of the non-hydrolysed juice. Upon hydrolysis, there is the degradation of the pectinaceous matter and the release of free water in to the system which leads to a great reduction in the viscosity (Abbes *et al*., 2014). This result corroborates the results of literature (Nso et al., 1998; Abbes et al., 2014;

Borchani et al., 2019; Ninga et al., 2021). Depectinisation therefore has an effect on viscosity of juice, which has a great role to play during mixing in the course of processing. For any agitation system with a shear rate below 55 s^{-1}, the energy required for the hydrolysed samples is lower but at a critical shear rate from 55 s^{-1}, there will be a constant amount of energy required for agitation for both the non-hydrolysed and hydrolysed. It is therefore necessary to hydrolyse the baobab juice before processing in order to decrease the cost of mixing and pumping of the juice.

2.3.8 Microbial analysis of baobab juice before and after hydrolysis

The microbial content of the baobab juice before and after pectinase hydrolysis was determined and the results presented in table 12. After enzymatic treatment, the reaction was stopped by heating at 95 ^{o}C for 5 min which also served as a pasteurization step for the juice. After pasteurization, aseptic techniques were followed during centrifugation to obtain a clear juice. The results show total destruction of the microbial flora present in the baobab juice after pasteurization. This shows that the thermal treatment method and aseptic techniques followed after pasteurization were effective and therefore the juice was ready as substrate for LAB fermentation.

Table 12: Microbial content of baobab juice before and after enzymatic hydrolysis

Microorganism	Before	After
Aerobic mesophile	795±95	0
Coliforms	399±21	0
Yeast and mold	295±55	0
Staphylococcus	0	0
Lactobacillus	435±115	0

2.3.9 Characterization of baobab juice

Some parameters such as pH, TSS, reducing sugar, TPC, antioxidant (DPPH, FRAP and ABTS) and colour were determined for the juice before and after enzymatic treatment to evaluate the effect of enzymatic treatment on the composition of the juice. The results are presented in table 13. The pH of the baobab juice after enzymatic treatment decreased with increase in the enzyme concentration. Pectinase hydrolyse pectin leading to accumulation of galacturonic acid in the medium (Combo *et al*., 2012) which cause a decrease in the pH of the juice. As the enzyme concentration increases, the amount of galacturonic acid released in the medium is higher and so the decrease in pH with increase in enzyme concentration. This can also be confirmed with the results of the organic acids analysed in the baobab juice after hydrolysis which galacturonic acid was the principal acid present in the baobab juice (section 2.3.11). Ninga et al. (2021) also realised a decrease in pH upon pectin hydrolysis of guava.

The TSS of the baobab juice increased from 4% (non-hydrolysed sample) to 5.2 after pectinase treatment and this is a measure of the percentage of soluble solids present in a liquid with respect to its refractive index (Bunphan *et al*., 2015). The reducing sugar measured in glucose equivalent also showed a great increase and the mechanism is as explained in section 2.3.5. The hydrolysis process release monosaccharides and short chain oligosaccharides which will be readily available as carbon source for LAB during fermentation. LAB obtain their energy from the metabolism of simple sugars and amongst the carbon sources required by the LAB, glucose is the main source (Lubeck and Lubeck, 2019). These results are in accord with the results of Ninga *et al*. (2021) The enzymatic hydrolysis had a positive impact on the polyphenol and antioxidant content of the juice. Other authors also noticed an increase in the polyphenol and antioxidant content of fruit juice after enzymatic treatment. The total phenolic content and antioxidant activity

of Black carrot (*Daucus carota* ssp. sativus) juice (Khandare *et al*., 2011); grape juice (Lima *et al*., 2015); pomegranate juice (Rinaldi *et al*., 2013) increased after enzymatic treatment. Some polyphenols are linked to cell wall polysaccharides and these cell wall polysaccharides contain hydrogen groups and glycosidic oxygen atoms that creates hydrogen bonds and hydrophobic interactions with polyphenols (Chamorro *et al*., 2012). In addition, baobab is made up of bound polyphenols as identified in section 2.3.10. Degradation of the cell walls by enzymatic treatment cause a solubility of the entrapped polyphenols and therefore a rise in the polyphenol and antioxidant content of the juice after treatment. That notwithstanding, Khandare *et al.* (2011) realized that higher dose of pectinase concentration (above 0.2%) led to a low yield of important phytochemicals such as anthocyanins and flavonoids and this is due to degradation of the anthocyanin glucosides caused by either polyphenol oxidase activity or ß-glucosidase side activities in the enzyme. Ninga *et al*. (2021) realised a decrease in the polyphenol content and no significant difference with the antioxidant content of hydrolysed guava juice. The difference could be as a result of the difference in substrate and enzyme used.

The colour of the juice before and after pectinase treatment was evaluated by the CIELAB parameter which L* represents lightness from black (0) to white (100), a* from green (-) to red (+) and b* from blue (-) to yellow (+). The raw baobab juice was cloudy and its L* value was 95.92±0.02%. After pectinase treatment, the juice obtained was clear and the lightness increased with increase in enzyme concentration. Pectinase breaks down pectin molecules and as a result, pectin–protein flocs are formed which are removed by centrifugation, thereby removing the colloidal aspect of the juices and at the end, a clear juice is obtained. The increase in the lightness could also be due to absence of enzymatic browning (Arsad *et al.*, 2015). The yellow colour (b*) of the juice decreased with increased in the enzyme concentration and same for the total colour

difference measured with respect to L*, a* and b*. The decrease could be as a result of degradation of pigments present in the baobab.

Generally, the juice obtained using pectinex concentration 0.2% (v/v) present best results when compared to the others and will be advantageous to use it as substrate for fermentation. This therefore, confirms the results of optimum hydrolysis conditions obtained.

Table 13: Composition of baobab juice treated with different concentrations of pectinex

Sample	pH	TSS (ºBrix)	Reducing sugar (g Gluc/ L)	Total polyphenol (mg GAE/100 mL)	DPPH (mg TE/ 100 mL)	FRAP (mg TE/ 100 mL)	ABTS (mg TE/100 mL)
Raw sample	4.2±0.00^{a}	4.0±0.00^{a}	11.84±0.69^{a}	167.70±0.36^{a}	143.62±3.00^{a}	205.67±0.41^{a}	185.86±2.40^{a}
0.03%	3.90±0.02^{b}	5.00±0.00^{b}	16.65±0.15^{b}	171.81±0.54^{b}	173.24±1.52^{b}	225.00±0.93^{b}	213.03±4.17^{b}
0.07%	3.76±0.01^{c}	5.00±0.00^{b}	19.71±0.15^{c}	172.75±0.10^{c}	177.02±6.72^{cd}	217.68±0.38^{c}	217.72±0.97^{b}
0.1	3.72±0.02^{d}	5.10±0.00^{c}	20.88±0.52^{d}	177.40±0.73^{d}	185.01±5.36^{de}	221.11±0.31^{c}	225.96±0.28^{c}
0.2%	3.62±0.01^{e}	5.20±0.00^{d}	25.23±0.27^{e}	184.20±0.20^{e}	164.49±3.80^{f}	219.95±0.56^{d}	235.44±0.67^{d}

Results are expressed as the mean ± standard deviation (n= 3). Values followed by different superscript lowercase letters of same column mean statistically significant differences ($p \leq 0.05$).

Sample	L*	a*	b*	dE*ab
Raw sample	95.92±0.02	-0.23±0.01	10.38±0.01	11.15±0.00
0.03%	96.10±0.06	-0.22±0.01	11.36±0.04	12.01±0.06
0.07%	97.09±0.01	-0.35±0.00	10.28±0.03	10.68±0.02
0.1%	97.20±0.04	-0.37±0.01	10.71±0.01	11.07±0.00
0.2%	97.58±0.09	-0.47±0.00	9.72±0.02	10.02±0.01

2.3.10 Polyphenol profile of baobab pulp

The polyphenol profile of baobab pulp was determined before fermentation to evaluate the effect of fermentation on the polyphenol profile of the fermented beverage. Polyphenols are important because they possess antioxidant properties. They act as hydrogen donors, chelate metals, acceptors of free radicals and chain breakers (Sinha, 2019). A total of 16 phenolic compounds were identified, 8 hydroxycinnamic acid (caffeic, chlorogenic, *p*-coumaric, ferulic, sinapic, trans-cinnamic, trans-ferulic and syringic acid); 3 hydroxybenzoic acids (gallic, vanillic and dihydroxybenzoic acid); 2 flavanols (catechin and epicatechin); 2 flavonols (rutin and quercitin); and 1 flavanone (naringin) (table 14). In the free polyphenol extract, epicatechin (34.76±4.91), vanillic acid (24.48±0.08), gallic acid (20.81±0.11), quercitin (16.86±0.38), syringic acid (13.93±1.58), trans ferulic acid (9.43±0.79), caffeic acid (7.14±0.52), naringin (6.26±0.78), catechin (4.83±0.70), *p*-coumaric acid (0.83±0.05) were quantified and rutin, ferulic and sinapic acid were also identified to be present. The bound polyphenol extract contained caffeic acid (27.53±0.45), gallic acid (14.17±0.44), Dihydroxybenzoic acid (9.66±0.40), catechin (1.49±0.19), syringic acid (1.47±0.37), and p-coumaric acid (0.36±0.03) and chlorogenic acid was also identified to be present. In the bound extract, in addition to some of the polyphenols present in the free extract, Dihydroxybenzoic acid and chlorogenic acid was present. Pectinase hydrolysis had a positive impact on caffeic acid (86.56±6.65), gallic acid (49.33±1.66), and *p*-coumaric acid (6.23±1.55). While negative impact on epicatechin (17.01±1.83), dihydroxybenzoic acid (3.93±0.57), catechin (3.83±0.06), quercitin (3.06±0.13), syringic (2.64±0.33), and vanillic acid (2.64±1.22). Chlorogenic, ferulic and rutin were also identified to be present in the juice. Ismail *et al.* (2019) identified protocatechuic acid, catechin, epicatechin, chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, rutin, ellagic acid, quercetin-3-rhamnoside, and quercetin in

baobab pulp. While Sokeng *et al.* (2019) identified kaempferol, catechin, rutin and procyanidin in baobab pulp. Tembo (2016) on the other hand, identified epicatechin, Procyanidin B2, Epigallocatechin-3-O-gallate, gallic acid, caffeine, and 5-Hydroxymethylfurfural as the predominant polyphenol compounds of baobab fruit pulp.

Table 14: Phenolic compound profile of baobab pulp and baobab juice

Polyphenol compound	**Free polyphenol (mg/ 100 mL)**	**Bound polyphenol (mg/ 100 mL)**	**Baobab juice (mg/ 100 mL)**
Gallic acid	20.81±0.11	14.17±0.44	49.33±1.66
Dihydroxybenzoic acid	ND	9.66±0.40	3.93±0.57
Chlorogenic acid	ND	P	P
Catechin	4.83±0.70	1.49±0.19	3.83±0.06
Vanillic acid	24.48±0.08	ND	2.64±1.22
Caffeic acid	7.14±0.52	27.53±0.45	86.56±6.65
Syringic acid	13.93±1.58	1.47±0.37	2.64±0.33
Epicatechin	34.76±4.91	ND	17.01±1.83
p-coumaric acid	0.83±0.05	0.36±0.03	6.23±1.55
Ferulic acid	P	ND	P
Sinapic acid	P	ND	ND
Rutin	P	ND	P
Trans ferulic acid	9.43±0.79	0.41±0.05	ND
Quercitin	16.86±0.38	ND	3.06±0.13
Trans cinnamic acid	ND	ND	ND
Naringin	6.26±0.78	ND	ND

P= present, ND= not detected

2.3.11 Ascorbic and Organic acid content of baobab

The organic acid content of the baobab pulp compared to that of the juice obtained after pectinase hydrolysis (0.2% v/v) are shown in table 15. In the baobab pulp, D-galacturonic acid

(260.36±8.00) was present in high amount followed by citric (131.20±7.60), Iso-citric acid (60.20±19.48), tartaric (45.12±20.20), malic (44.03±9.40), propionic (30.24±9.98), succinic (8.13±0.75), glutaric (7.07±0.06), and oxalic (3.81±1.38). While in the baobab juice obtained after pectinase hydrolysis, D-galacturonic acid (2276.00±40.26) was present in high amount followed by citric (189.10±14.61), tartaric (162.77±14.81), iso-citric acid (160.76±1.37), malic (125.17±5.57), propionic (15.92±11.74), succinic (11.18±0.19), and glutaric (9.72±1.19). The organic acid content of the juice obtained after pectinase hydrolysis (0.2% v/v) was higher than that of the baobab pulp. This implies hydrolysis released acids in to the medium and therefore the decrease of the pH of the juice after hydrolysis. D-galacturonic acid was the main organic acid identified in baobab pulp and in the juice. The degradation of pectin by pectinase released galacturonic acid in to the medium which could explain the reason of the high quantity of D-galacturonic acid identified in the juice. After hydrolysis, oxalic acid was not identified in the juice and the propionic acid content decreased. Sokeng *et al.* (2019) detected the presence of malic, citric, succinic, lactic, fumaric and formic acids in baobab pulp and malic acid (45.3%) was the most abundant acid, followed by quinic acid (37.9%), citric acid (11.6%), and succinic acid (3.8%). Tembo (2016) identified citric acid (3300.84 ± 0.90 mg/100 g FW pulp) in high amounts in baobab pulp followed by malic acid (2364.98 ± 28.8), ascorbic acid (351.92 ± 7.41), tartaric (173.93 ± 5.50) and dehydroascobic acid (114.65 ± 1.10). The presence of these organic acids influences the acidity and flavour of the baobab pulp and juice and they aid in extending the shelf life of the juice.

The ascorbic acid of the baobab pulp was quite high (137.44±1.46 mg/100 mL) compared to that of the baobab juice (2.49±0.08 mg/100 mL). The great decrease in the ascorbic acid content of the baobab juice could be due to the degradation of the ascorbic acid during pectinase deactivation process. Vitamin C is a water-soluble vitamin generally present in fruits and

vegetables (Igwemmar *et al*., 2013). Vitamin C is heat labile and processing of foods rich in Vit C at temperatures above 60 ^{o}C lead to a drastic loss of the vitamin (Njoku *et al*., 2011; Roig *et al*., 1995).

Table 15: Organic acid content of baobab pulp and baobab juice

Organic acid	**Baobab pulp (mg/100 mL)**	**Baobab juice (mg/ 100 mL)**
D-galacturonic acid	260.36±8.00	2276.00±40.26
Oxalic	3.81±1.38	0.00
DL-Tartaric	45.12±20.20	162.77±14.81
L-malic	44.03±9.40	125.17±5.57
Isocitric acid	60.20±19.48	160.76±1.37
Citric	131.20±7.60	189.10±14.61
Succinic	8.13±0.75	11.18±0.19
propionic	30.24±9.98	15.92±11.74
Glutaric	7.07±0.06	9.72±1.19
L-Ascorbic acid	137.44±1.46	2.49±0.08

Partial conclusion

Baobab pectin was successfully hydrolysed and the depectinisation kinetics of the pectin in the actual juice studied. The optimum hydrolysis conditions were enzyme concentration, 0.2% v/v and time, 120 min at 50 ^{o}C. The experimental data was fitted to the Hill model because of the sigmoidicity of the curve. The Hill parameters, n value which is the degree of cooperativity decreased from 9 to 5 and the extrapolated Vmax increased from 0.336 - 2.378 g/L.min^{-1} with increase in enzyme concentration (0.03 – 0.2% v/v). There was also an increase in the reducing sugar concentration and at the optimum condition, the reducing sugar content was 25.23 g Gluc/L. This would probably favour the growth of LAB during fermentation since pectin is a complex medium to be metabolised by the bacteria. The antioxidant activity of the juice obtained at the optimum condition was higher than in the other cases. The viscosity of the hydrolysed juice decreased which implies during processing of the hydrolysed juice compared to the non-hydrolysed juice, less energy will be used for pumping and mixing of the juice.

Though the reducing sugar content of the baobab juice increased after hydrolysis, its amino acid content is low and therefore would not completely favour the growth of LAB during fermentation. LAB need adequate amounts of carbon, nitrogen and trace elements in the medium for growth and the carbon: nitrogen ratio needs to be controlled (Erkman and Bozoglu, 2016). This implies, in addition to baobab, a different nitrogen source has to be added. For this reason, lemongrass which has a high nitrogen content with a lemon scent had to be mixed with baobab juice before fermentation to enrich the baobab juice with amino acids and improve the aroma. In order to mix the lemongrass, an extract had to be prepared.

CHAPTER 3: OPTIMISATION OF EXTRACTION CONDITIONS OF BIOACTIVE COMPONENTS FROM LEMONGRASS (*Cymbopogon citratus* Stapf.)

Abstract

The aim of this section was to determine the optimal decoction conditions to recover phenolic compounds from *C. citratus* leaves. The extraction variables, lemongrass powder concentration (2-5 g /100 mL), temperature (85 -95 °C), and time (5-10 min) were assessed by central composite design for process optimization. Antioxidant activity (DPPH) and total polyphenol content (TPC) were monitored as responses. At the optimal conditions, the phenolic and aroma profile of the extract was evaluated. The TPC and DPPH were 71.98±0.33 mg GAE/100 mL extract and 80.63±0.49 mg TE/100mL extract respectively under optimal conditions (lemongrass powder concentration 5 g/100 mL, temperature 93.8 °C and time 11.3 min). The phenolic, organic and aroma profile of the lemongrass extract, extracted at optimum condition was analysed. The results revealed the main phenolic compounds of *C. citratus* extract to be caffeic (20.81±0.003 mg/100mL) and syringic acids (18.63±7.390 mg/100mL) while citral and geraniol were the primary volatile compounds. Propionic acid (20.137±0.163 g/L) was identified as the principal organic acid in the extract. The lemongrass had a protein content of (8.40±0.05%) and a high amino acid content which can serve as nitrogen source during fermentation. The results achieved herein suits the potential use of lemongrass extract as natural source of antioxidant, aroma compounds and nitrogen source that can be used in different industrial sectors.

Keywords: *Cymbopogon citratus*; decoction; optimization; polyphenols; natural antioxidants.

3.1 Introduction

Cymbopogon citratus (lemongrass) is a tall perennial grass of the family Poaceae commonly cultivated in humid tropical and subtropical regions of the world (Mabai *et al*., 2018). Lemongrass is rich in minerals, vitamins, and macronutrients (carbohydrate, protein, and small amounts of fat). Its high nitrogen content could serve as a nitrogen source for LAB during fermentation. These leaves also are good sources of various bioactive compounds including alkaloids, terpenoids, flavanoids, phenols, saponins and tannins that confer lemongrass leaves pharmacological properties. The use of natural plant extracts in the food industry is gaining interest because they help in improving the antioxidant properties, sensory and extend the shelf life of products they are incorporated in. The properties are gained due to the bioactive components present in the plant extracts. Moreover consumers are now health conscious and therefore go in for products with natural preservatives (Caleja *et al*., 2016). To that effect, lemongrass could possibly be used as a nitrogen source and also to extend the shelf life of probiotic baobab juice.

Lemongrass is used either fresh leaves, dried powdered, concentrated extract, or essential oil depending on the application. Up to date, the extract from infusion method remains the widely employed methods owing to its lower cost and simplicity. Some process parameters, like, process time, water/substrate ratio, and temperature has been declared as the principal factors for efficient extraction of bioactive components from plants (Roseiro *et al*., 2013; Thangam *et al*., 2014). The application of unsuitable conditions during extraction procedures could lead to the degradation of target compounds and reduce extraction efficiency. Therefore, optimizing the extraction process can aid in choosing suitable process conditions for improving the extraction yield of bioactive compounds. Hence, the objective of this section was to optimize the polyphenols and antioxidants extraction from lemongrass by decoction method using central composite design.

.2 Materials and methods

3.2.1 Materials

C. citratus (photo 2) leaves were harvested from a farm in Bini-dang, Ngaoundere (7°19'0" N and 13°34'60" E), Adamawa region, Cameroon in June, 2018.

Photo 2: Lemongrass (*Cymbopogon citratus*) plant

Fresh lemongrass harvested in the morning to avoid loss of essential oils, was washed and withered by exposing at 25 ^{o}C for 24 h. This was to reduce the moisture content of the leaves in order to condition the leaves for the next step of the process, drying as described in figure 22. After withering, the leaves were cut into sizes of about 2cm because essential oil content and active principal concentration are high at this length (Rocha et al., 2012). The cut leaves were then dried at 60 ^{o}C for 3 h (Wiyeh, 2016). Furthermore, the dried leaves were ground and sieved using a 1mm sieve to obtain the granulated lemongrass. They were then packaged and stored at -40 ^{o}C until analyses. The lemongrass was in granules not fine powder to avoid the formation of clump/mass that could affect the extraction process by impeding the free flow of solvent. The granules create accessibility of the solvent to the solute during decoction (Chanioti *et al*., 2015).

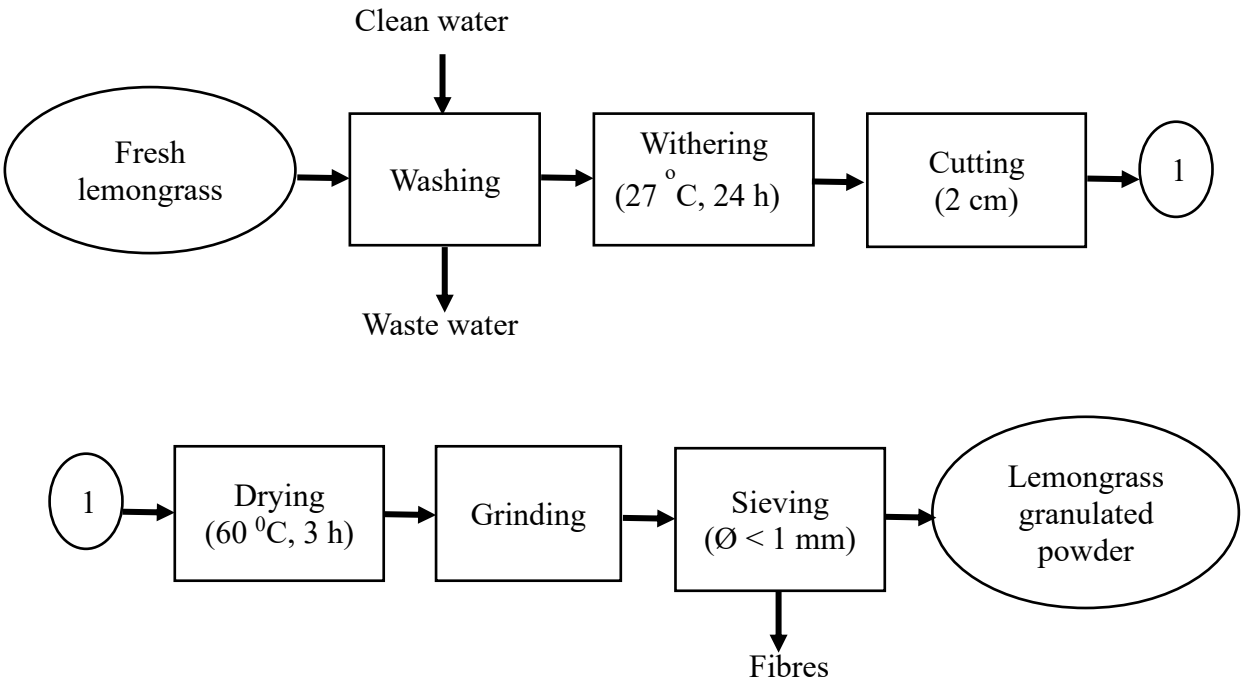


Figure 22: Block diagram for production of granulated lemongrass powder

3.2.2 Methods

3.2.2.1 Determination of proximate composition of lemongrass leaves

3.2.2.1.1 Determination of moisture content of lemongrass leaves

The moisture content of lemongrass leaves was analysed according to the method described in section 2.2.3.1.1.

3.2.2.1.2 Determination of total ash content of lemongrass leaves

The total ash content of lemongrass leaves was determined with respect to the method in section 2.2.3.1.2.

3.2.2.1.3 Determination of crude protein of lemongrass leaves

The crude protein of lemongrass leaves was determined according to the assay in section 2.2.3.1.3.

3.2.2.1.3 Determination of fat content of lemongrass leaves

The fat content of lemongrass leaves was determined according to the method in section 2.2.3.1.4.

3.2.2.1.4 Determination of crude fibre of lemongrass leaves

The crude fibre content of lemongrass leaves was done with respect to the method in section 2.2.3.1.5.

3.2.2.1.5 Determination of carbohydrate content of lemongrass leaves

The content of total carbohydrates was calculated by subtracting the sum of moisture, protein, fat, ash and crude fibre from 100 (AOAC, 2000).

3.2.2.2 Determination of reducing and total sugars of lemongrass leaves

The reducing and total sugar content of lemongrass leaves was extracted and determined as in section 2.2.3.3.

3.2.2.3 Determination of mineral content of lemongrass leaves

The minerals present in lemongrass leaves were digested and analysed according to the method in section 2.2.3.4.

3.2.2.4 Extraction of bioactive components of lemongrass leaves

The bioactive components of lemongrass leaves were extracted in 80% methanol as described in section 2.2.3.5.

3.2.2.4.1 Determination of total polyphenol content (TPC)

The TPC of the lemongrass extract was then determined by the method in section 2.2.3.5.1.

3.2.2.4.2 Determination of antioxidant activity

The antioxidant activity (DPPH, FRAP, ABTS) of the lemongrass extract was analysed by the methods described in section 2.2.3.5.2.

3.2.2.5 Decoction of lemongrass powder

Decoction, a process of boiling herb in a specified volume of water at a particular time (Olorunnisola *et al*., 2014) was used because it can be easily controlled than infusion. During decoction process, plant capillaries remain open for the set time for proper extraction of solutes in to the solvent.

The factors; lemongrass powder/water ratio, temperature and time (table 16) were chosen with respect to literature (Juntachote *et al*., 2006; Ramos *et al*., 2017; Sah *et al*., 2012; Sherwani *et al*., 2013) and preliminary works to ensure maximum extraction of bioactive components and total reduction of microbial load. The lemongrass powder was extracted with distilled water following the central composite design (CCD) matrix (table 17). In brief, the water bath (Julabo TW8, Germany) was set at the temperature, as imposed by the CCD matrix. The conical flask containing distilled water was deposited in the water bath until reaching the set temperature. The lemongrass powder was then introduced in the conical flask and agitated at 150 rpm in order to facilitate the process of mass transfer during decoction. Agitation increases turbulent diffusion and the transfer rates of material from the surface of the particles to the bulk of the solution (Chanioti et al., 2015). After extraction, samples were cooled immediately in ice water bath to reach room temperature (28-30 ^{o}C). The slurry was further filtered using Whatman paper N° 4 paper. The filtrate was rinsed three times with distilled water, and all three extracts were mixed. The volume of the total extract was adjusted to 100 mL with distilled water and referred to as lemongrass extract.

Table 16: Domain of study of decoction of dried lemongrass powder

Factors	**Range**
Lemongrass powder concentration	2-5 g/100 mL
Temperature	85-95 °C
Time	5-10 min

3.2.2.5.1 Experimental design, modelling, validation of model, and optimization

The orthogonal quadratic central composite design (CCD) was utilized to scrutinize the decoction process. Table 17 presents the factors and their coded levels utilized for the CCD. The independent factors studied were lemongrass powder concentration (X_1), decoction temperature (X_2), and extraction time (X_3). The CCD consisted of 20 trials, and each trial was done in triplicate, and the average responses (TPC and DPPH) were reported.

Table 17: Matrix of coded and real values for the study of decoction of dried lemongrass

Exp. N°	Coded variables			Real variables		
	Lemongrass powder concentration (w/v)	Temperature (°C)	Time (min)	Lemongrass powder concentration (w/v)	Temperature (°C)	Time (min)
1	0	0	0	3.5	90	7.5
2	1.52	0	0	5.78	90	7.5
3	1	-1	-1	5	85	5
4	1	1	1	5	95	10
5	-1	-1	-1	2	85	5
6	-1.52	0	0	1.22	90	7.5
7	0	0	-1.52	3.5	90	3.7
8	0	-1.52	0	3.5	82.4	7.5
9	0	0	1.52	3.5	90	11.3
10	0	0	0	3.5	90	7.5
11	-1	1	-1	2	95	5
12	0	0	0	3.5	90	7.5
13	0	1.52	0	3.5	97.6	7.5
14	1	1	-1	5	95	5
15	1	-1	1	5	85	10
16	-1	-1	1	2	85	10
17	-1	1	1	2	95	10
18	0	0	0	3.5	90	7.5
19	0	0	0	3.5	90	7.5
20	0	0	0	3.5	90	7.5

3.2.2.5.2 Mathematical model

The mathematical model employed was a second-degree polynomial model with linear, quadratic, and interaction terms (equation 34).

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (34)$$

Where Y is the response, β_0 is the constant term, β_i are the linear coefficient terms, β_{ii} are the quadratic coefficient terms, β_{ij} are the interaction coefficient terms and x_i and x_j, the factors.

From the coded variables, equation 35 was used to transform them into real values to realize experiments in the laboratory.

$$X_i = X_{0i} + x_i \Delta X_i \quad (35)$$

The value of α and the number of experiment N were calculated in order to respect the orthogonality criterion and using the equation 36 and 37:

$$\alpha = \left(\frac{2^k \left(\sqrt{2^k + 2k + n_0} + \sqrt{2^k} \right)^2}{4} \right)^{\frac{1}{4}} \quad (36)$$

$$N = k^2 + 2k + n_0 \quad (37)$$

Where: k is the number of variables, n_0 is the number of trials in the centre.

The CCD matrix was obtained by Minitab 19.2 (2019 Minitab, LLC All rights reserved). R-square (R^2), R^2-square adjusted (adj-R^2), absolute average deviation (AAD) (equation 38), the bias factor (B_f) (equation 39), and the accuracy factor (A_f) (equation 40) were utilised to validate the models.

$$AAD = \frac{\left[\sum_{i=1}^{N}\left(\frac{\left|Y_{i,\exp} - Y_{i,cal}\right|}{Y_{i,\exp}}\right)\right]}{N} \quad (38)$$

$$B_f = 10^{\frac{1}{N}\sum_{i=1}^{N}\log\left(\frac{Y_{i,cal}}{Y_{i,\exp}}\right)} \quad (39)$$

$$A_f = 10^{\frac{1}{N}\sum_{i=1}^{N}\left|\log\left(\frac{Y_{i,cal}}{Y_{i,\exp}}\right)\right|} \quad (40)$$

Where $Y_{i,exp}$ is the responses, $Y_{i,cal}$ the calculated responses, and N is the number of experiments used in the calculation.

Lastly, optimization was executed in one hand using Minitab 19.2 (2019 Minitab, LLC All rights reserved). The conditions fixed were to maximize both total polyphenol content and DPPH Radical Scavenging Activity. A composite optimal was considered for the two responses and the responses at the composite optimal verified.

Analyses

The TPC and antioxidant activity (DPPH, FRAP, AND ABTS) of the different lemongrass extracts were determined with respect to the methods described in sections 2.2.3.5.1 and 2.2.3.5.2 respectively.

3.2.2.6 Microbial analysis of lemongrass extract

The microbial load of the lemongrass extract before and after decoction was analysed with respect to the method in section 2.2.3.10.

3.2.2.7 Colour analyses of lemongrass

The colour of the lemongrass extract and the lemongrass leaves was measured in Minolta colorimeter (CM-5, Konica Minolta, Tokyo, Japan).

3.2.2.8 Determination of phenolic acid compounds of lemongrass extract

The free and bound phenolic compounds were extracted with respect to the method of section 2.2.3.11 and the phenolic compounds of the different extracts were then determined by HPLC.

3.2.2.9 Determination of organic acid profile of lemongrass extract

The organic acid content of the lemongrass extract was determined according to the method of section 2.2.3.12.

3.2.2.10 Determination of Vitamin C content of lemongrass extract

The vitamin C present in the lemongrass leaves were extracted and analysed by the method of section 2.2.3.13.

3.2.2.11 Evaluation of lemongrass extract aroma compounds

Extraction and analysis of volatile compounds in lemongrass extract was done according to the method described by Siewe *et al.* (2020) with some modifications. The solid-phase microextraction (SPME) method was used in extraction of volatile compounds from lemongrass extract. 5 mL of lemongrass extract in well-sealed glass vials (20 mL) were heated at 50 ^{o}C for 20 min in a water bath. The aroma compounds trapped in the headspace of the vials were adsorbed for 30 min with the 2 cm, 50/35 µm Carboxen / polydimethylsiloxane / divinylbenzene (CAR/PDMS/DVB) fibre (Supelco Inc., Bellefonte, USA). The adsorbed fibre was directly

introduced into a 7890B Agilent GC injector port (Agilent Technologies, Santa Clara, California, USA) at 250 ^{o}C for 3 min to desorb the volatile compounds. The RT-WAX capillary column (60×0.25 mm, 0.25 µm; J & W Scientific, Folsom, CA) assisted in separating the volatile compounds. The program of the GC column was set at 40 ^{o}C for 3 min, then the temperature was increased with an increment of 5 ^{o}C /min to 235 ^{o}C, and maintained for 10 min. The carrier gas, helium was used at a flow rate of 1.8 mL/min. The temperature and electron voltage of the mass spectrometric detector was operated at 230 ^{o}C and 70 eV respectively with the transfer line temperature of 250 ^{o}C. The chromatogram was recorded in the range of 40–450 amu of the total ion current.

The mass spectra of the volatile compounds obtained were identified by comparing with the mass spectra database of the US National Institute of Standards and Technology (NIST). Calculations of the relative percentage (% area) were based on the ratio between the peak area of each compound and the sum of areas of all compounds (Pino and Barzola-Miranda, 2020).

3.2.3 Statistical analysis

The statistical analyses of the experimental design data and plotting of surface plot were realised using the softwares Minitab 19.2 and OriginPro 2019b (9.6.5.169, OriginLab Corporation). ANOVA test was utilised to obtain the statistical significance of the regression coefficient on the level of significance declared at $p \leq 0.05$. Differences between mean were analysed by Duncan test at the significance level of $p \leq 0.05$. The results were expressed as a mean ± standard deviation.

3.3 Results and discussion

3.3.1 Proximate composition and bioactive composition of lemongrass

The proximate composition of lemongrass leaves is presented in Table 18. Lemongrass leaves contain nutrients (proteins, carbohydrate and fibres) which explains its use. The moisture content of dried lemongrass, 10.09±0.06%, lower than 11.35% determined by Uraku *et al*., (2016) shows it is desirable for longer periods of storage and less attack of microorganisms.

Table 18: Proximate composition of *C. citratus* leaves

Macronutrients (g/100g DW)	
Moisture content	10.09±0.06
Ash	8.06±0.05
Fat	4.45±0.10
Protein	8.39±0.05
Crude fibre	30.32±0.65
Reducing sugar	1.02±0.00
Total sugar (as glucose)	30.15±0.42
Micronutrients (mg/100g DW)	
Calcium	283.57±0.54
Iron	58.06±0.27
Potassium	1175.87±1.08
Magnesium	93.44±1.08
Sodium	50.50±1.89
Vitamin C (mg/100 mL)	
Vit C	15.60±3.37

The value of ash content 8.06±0.05% indicates the presence of minerals in lemongrass leaves (Asaolu *et al*., 2009). The protein content (8.40±0.05%) was higher compared to 4.56% reported by Asaolu *et al*. (2009) and in agreement with 8.51% reported by Ojo (2017). The protein content of lemongrass (8.40±0.05%) is higher than that of baobab (2.89±0.08%) which will supplement the baobab as a source of Nitrogen for the growth of LAB.

The crude fibre content (30.32±0.65%) indicates lemongrass leaves are an adequate source of crude fibre compared to other conventional plant leaves (Asaolu *et al*., 2009; Nambiar and Matela, 2012). The carbohydrate content of 44.16 g/100 g indicates it is a good energy source. The reducing sugar and total sugar contents of 1.02±0.00 and 30.15±0.42% is different from that obtained by Assous *et al*. (2012) whom realized a reducing sugar content of 6.91% and a total sugar content of 9.01% for fresh lemongrass leaves. The total sugar content is quite low compared to that of baobab (41.52±0.21%), which implies the main source of carbon for fermentation of the juice will be supplied by baobab. The vitamin C content of the lemongrass (15.60±3.37 mg/100g) was higher than 2.43±0.06 mg/100 g (Uraku *et al*., 2016) and 2 mg/100g (Radali and Alka, 2018). The variations in the composition of lemongrass leaves with literature could be due to differences in geographical location and maturity stage of the plant (Ranjah *et al*., 2018).

The lemongrass leaves are also made up of minerals such as potassium (1175.87±1.08), calcium (283.57±0.54), magnesium (93.44±1.08), iron (58.06±0.27) and sodium (50.50±1.89) mg/100g DW in descending order. This implies potassium was the main mineral present in the lemongrass leaves. Geetha and Geetha (2015) also realized potassium (60.2 mg/100 g) as the main mineral in lemongrass leaves, then sodium (56.3 mg/100 g), calcium (39.4 mg/100 g), and iron (0.034 mg/100 g). The high mineral content of the lemongrass leaves compared to literature could be as a result of the wet digestion method used for analysis, geographical location and maturity stage of the

plant. Minerals play an essential role in microbial growth in general and have a special effect on bacterial enzymatic activity. Essential metal ions serve bacteria in a number of functions: 1) as activators or cofactors of a variety of enzymes, 2) in membrane transport, and 3) as components of molecules or structural complexes (Hayek and Ibrahim, 2013). Mg^{2+} is an essential element for the growth and metabolic activities of LAB. Mg^{2+} stimulates the growth of LAB and improve LAB survival complexes (Hayek and Ibrahim, 2013). A deficiency in this ion can lead to delayed cell division and growth (Boyaval, 1989). The main function of iron in aerobic metabolism is in the reduction of oxygen by means of the cytochrome chain and concomitant generation of chemical energy but anaerobic species may also contain high quantities of iron. Despite that, all lactic acid bacteria lack cytochromes and derive their energy from fermentation (Boyaval, 1989). However, MacLeod and Snell (1947) found no enhanced growth of several lactobacilli on supplementation with iron in an iron deficient medium. Potassium is the main mineral required for microbial growth. In the works of MacLeod and Snell (1947) all the tested LAB (*Lb. arabinosus, Lb. delbrueckii, Lb. casei, Lb. pentosus, Lb. fermenti, Streptococcus faecalis*, and *Leuconostoc mesenteroides* required potassium for their growth. In addition to potassium, they also require manganese and phosphate. MacLeod and Snell (1947) also realized Ca^{2+} ions were not essential for growth of several lactic acid bacteria analysed. Nonetheless, they stimulate the growth of some lactic acid bacteria. The growth of LAB in the presence of Na depends on the concentration of the Na^{+} present (MacLeod and Snell, 1947). High concentrations cause inhibition. Thus, LAB in general requires Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, K^{+}, and Na^{+} as essential or stimulatory factors for nutrient transportation and enzymatic activity (Hayek and Ibrahim, 2013). All these minerals are present both in lemongrass and baobab but higher in lemongrass than in baobab (except potassium which

is higher in baobab). Thus, the minerals can thereby sustain the fermentation process of the baobab-lemongrass probiotic beverage.

Phenolic compounds are one of the vital group of compounds acting as main antioxidants which contributes to the medicinal value of various plants (Kouassi *et al*., 2017). The total polyphenol content (table 19) of the methanol extract of lemongrass was found to be 1656.30 ± 4.08 mg GAE/100 g DW. The antioxidant activities, DPPH and FRAP activities were found to be 1261.30 ± 3.28 and 1920.92 ± 2.59 mg TE/100 g DW, respectively. The total polyphenol content and antioxidant activities of lemongrass leaves were different from those stated in literature (Kouassi *et al*., 2017; Nambiar and Matela, 2012; Sah *et al*., 2012). The values of 118.14 ± 1.05 mg GAE/g and 178.069 ± 1.57mM TE/mL for TPC and antioxidant was observed by Kouassi *et al.* (2017). 1324.9 ± 31.06 mg GAE/100 g, 15.96 ± 0.53 and 23.40 ± 1.19 µmol TE/g DW for TPC, DPPH and FRAP respectively was realized by Nambiar and Matela (2012). The variations in the polyphenol content and antioxidant activities of lemongrass leaves compared to that of literature could be as a result of the geographical location, maturity stage and extraction method (Adeyemo *et al*., 2018; Ranjah *et al*., 2018).

Table 19: Bioactive composition of *C. citratus* leaves

Bioactive component	**Quantity**
Total polyphenol	1656.30 ± 4.08 mg GAE /100 g DW
Antioxidant activity	**Quantity (mg TE/100 g DW)**
DPPH	1261.30 ± 3.28
FRAP	1920.92 ± 2.59
ABTS	1792.81 ± 3.85

3.3.2 Amino acid profile of lemongrass

The amino acid profile of lemongrass is as shown in table 20. Lemongrass is a plant rich in protein and also has a rich amino acid profile. It contains 9 essential amino acids except for methionine which was not detected. The essential amino acid composition ranges from 2.423 to 31.728 mg/100 g DW. The most abundant being threonine, followed by valine, tryptophan, phenylalanine. On the other hand, the composition of the non-essential amino acid ranges from 1.01 to 1517.666 mg/100 g DW, and cystine was the only non-essential amino acid not detected. Asparagine, proline, aspartic acid, tyrosine, threonine, serine and glutamic acid were the most abundant in descending order. They have as values: 1517.666±11.134; 97.171±0.332; 35.384±0.323; 33.809±0.046; 31.728±0.045; 24.283±0.141; 22.437±0.104 mg/100 g DW respectively.

Amino acid is essential for the growth of lactic acid bacteria in addition to other growth factors such as fermentable sugars, fatty acids, vitamins and purines. Amino acid catabolism plays an important role in the growth of LAB. This is because carbohydrate metabolism by LAB leads to acidification and therefore, low pH of the medium which is detrimental to the growth of the LAB. The low pH shifts lactic metabolism from hexose fermentation to utilization of amino acids. Glutamine, glutamate, and arginine play a major role in pH homeostasis and stationary phase survival of LAB (Ganzle, 2015). The amino acid composition of lemongrass is high when compared with that of baobab (section 2.3.2). Therefore, the mixture of the baobab juice with lemongrass extract could favour the growth of LAB during fermentation.

Table 20: Amino acid profile of lemongrass leaves

Amino acid	Composition (mg/100 g DW)
Essential amino acids	
Arginine	2.423±0.003
Valine	23.395±0.393
Methionine	ND
Leucine	6.713±0.032
Threonine	31.728±0.045
Phenyl alanine	10.618±0.046
Histidine	2.437±0.054
Isoleucine	6.596±0.013
Leucine	6.713±0.032
Tryptophan	11.199±0.056
Non-essential amino acids	
Serine	24.283±0.141
Asparagine	1517.000±11.000
Cystine	ND
Threonine	31.728±0.045
Glutamic acid	22.437±0.104
Glycine	1.547±0.060
Proline	97.171±0.332
Aspartic acid	35.384±0.323
Glutamine	8.159±0.002
Lysine	7.331±0.023
Tyrosine	33.809±0.046
Hydroxy proline	5.777±0.014
Cysteine	1.017±0.058
Alanine	5.873±0.007

3.3.3 Mathematical modelling of decoction process of lemongrass leaves

The results of the 20 experimental runs with their responses are presented in appendix 3, while the ANOVA results are put forward in Table 21.

Table 21: Analysis of variance for the regression models of TPC and DPPH

	TPC (mg GAE/100 mL extract)			DPPH (mg TE/100 mL extract)		
Source	Sum of squares	F-value	P-value	Sum of squares	F-value	P-value
Model	3239.34	62.97	< 0.0001	6169.45	213.63	< 0.0001
X_1	2804.15	1210.94	< 0.0001	5909.96	2203.64	< 0.0001
X_2	54.35	23.47	0.0047	27.65	10.31	0.0237
X_3	103.35	44.63	0.0011	89.13	33.24	0.0022
$X_1 X_2$	1.25	0.54	0.4960	0.22	0.081	0.7878
$X_1 X_3$	8.44	3.65	0.1145	8.85	3.298	0.1290
$X_2 X_3$	27.57	11.91	0.0182	14.77	5.507	0.0658
X_1^2	163.71	70.698	0.0004	58.40	21.774	0.0055
X_2^2	39.07	16.87	0.0093	50.38	18.786	0.0075
X_3^2	36.14	15.61	0.0108	10.04	3.74	0.1107
Residual	57.16			32.09		
Lack of fit	45.58	3.94	0.0794	18.68	1.39	0.3625
Pure error	11.58			13.41		
Cor total	3296.50			6201.54		

X_1, X_2, and X_3 represents the linear effects (lemongrass powder concentation, temperature and time respectively); X_{12}, X_{13} and X_{23} are the different interactions and $X_{1,}^2$ $X_{2,}^2$ and X_3^2 the quadratic effects.

The significance of the model terms and model equations were validated with respect to the *p*-value ($p \leq 0.05$). The models of both responses were highly significant ($p < 0.0001$). The linear coefficients (X_1, X_2, X_3), the interaction (X_2X_3) and the quadratic terms (X_1^2, X_2^2, X_3^2) were the significant model terms ($p \leq 0.05$) for TPC while for DPPH scavenging activity, the linear terms (X_1, X_2, X_3), and the quadratic terms (X_1^2, X_2^2) were significant ($p \leq 0.05$).

The model validation terms, Lack of fit, coefficient of determination (R^2), adjusted R^2 (adj-R^2), AAD, B_f and A_f are presented in Table 22. The lack of fit for both models were not significant ($p > 0.05$), which implies that no considerable improvement was achieved by the inclusion of the statistically parametric values. The coefficient of determination R^2 were 0.9829 and 0.9948 for TPC and DPPH, respectively; indicating that both mathematical models can explain 98.29% and 99.48% (respectively for TPC and DPPH) experimental observations as a function of independent variables.

Table 22: Model validation parameters

Model	**R^2**	**R_{adj}**	**AAD**	**B_f**	**A_f**
TPC	0.9827	0.9671	0.0011	1.0004	1.0034
DPPH	0.9948	0.9902	0.0016	1.0012	1.0218

Adj-R^2 of both models (0.9674 and 0.9900 for TPC and DPPH, respectively) were within close range to their respective coefficient of determination indicating that the variability of each response can be explained by the independent variables involved in the process. Joglekar and May (1987) suggested that R^2 should be at least 80% for model fit; therefore, the empirical models of TPC and DPPH fits the actual data models. Baranyia *et al*. (1999) and Ross (1996) stated, in

addition to R^2, other validation model terms, AAD, bias and accuracy factors are of great interest to be considered. They measure the relative average deviation of predicted and observed responses. An AAD of 0 and a bias factor and accuracy factor of 1 indicate model adequacy. In this study, all the validation terms fell within the accepted range of model validity which affirms the validity of the models (Table 23). The empirical equations developed for TPC (equation 41) and DPPH (equation 42) activity are as follows:

$$TPC = 54.735 + 9.806x_1 + 1.365x_2 + 1.883x_3 - 1.692x_1^2 - 0.827x_2^2 - 0.795x_3^2 - 0.171x_1x_2 + 0.445x_1x_3 + 0.803x_2x_3 \quad (41)$$

$$DPPH = 58.377 + 14.237x_1 + 0.974x_2 - 1.748x_3 - 1.011x_1^2 - 0.939x_2^2 + 0.419x_3^2 - 0.071x_1x_2 + 0.455x_1x_3 + 0.588x_2x_3 \quad (42)$$

3.3.3.1 Singular and quadratic effects on TPC and DPPH

In this case, to view the effect of a singular factor, the other factors were fixed at their minimal value to lessen their contribution. That means for lemongrass powder concentration (X_1), for decoction temperature (X_2) and extraction time (X_3), the respective fixed values were: 1.22; 82.4 ^oC and 3.7 min.

- **Effect of lemongrass powder concentration (X_1)**

The lemongrass powder concentration (X_1) had a significant impact on TPC and DPPH (Table 21). It is observed from Figure 23 that, at an initial ratio value of 1.22 g/100 mL, the values of 29.72 mg GAE/100 mL and 36.62 mg TE/100 mL were obtained for TPC and DPPH respectively. An increase of that ratio until 5.78 g/100 mL, generated a significant increase of TPC and DPPH respectively to 58.27 mg GAE/100 mL and 78.13 mg TE/100 mL. At a fixed decoction temperature of 82.4 ^oC and a fixed extraction time of 3.7 min, an increase in ratio led to increase in the amounts

of TPC and DPPH activity in the extract. The lemongrass powder concentration was found to be the essential factor ($p \leq 0.0001$) that affected the yield of TPC and DPPH activity of the lemongrass leaves extract. As the lemongrass powder/ water ratio increased, the TPC and DPPH activity increased significantly.

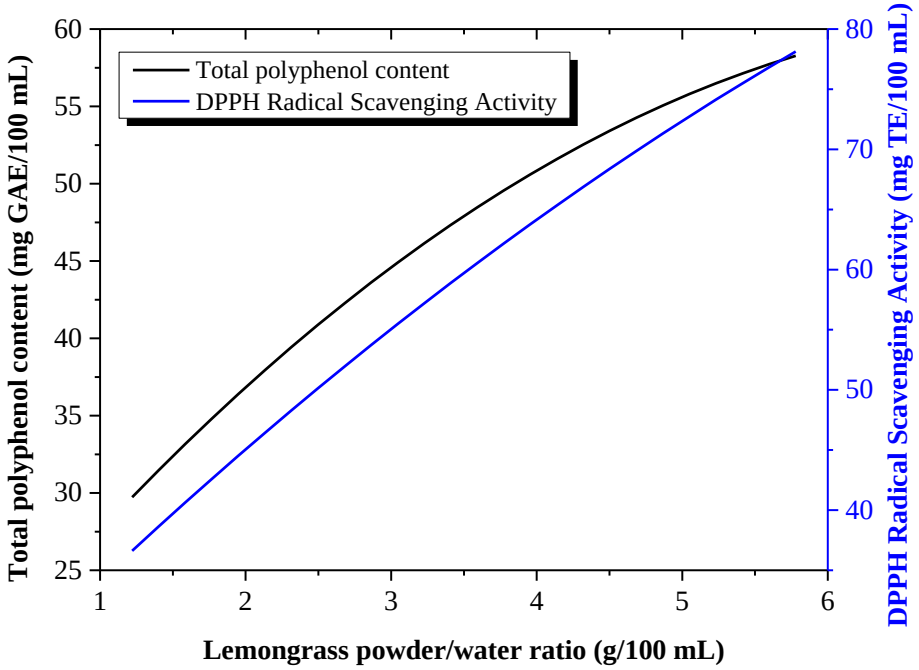


Figure 23: Evolution of Total polyphenol content and DPPH radical scavenging activity as a function of lemongrass powder/water ratio. Decoction temperature and extraction time respectively fixed at 82.4 ^{o}C and 3.7 min.

The gradient of the curve which is a representation of mass transfer is constant but, changes at the level the lemongrass powder concentration was extended beyond the critical limit (quadratic effect). This implies at that level; mass transfer is diminishing and therefore there is a decrease in concentration of bioactive components. The rate of diffusion increases with concentration gradient between solid and liquid (Gertenbach, 2001; Norshazila *et al.*, 2017) because it is a driving force

of mass transfer (Norshazila *et al*., 2017). This implies more polyphenols permeates out of the matrix in to the solvent but at very high concentrations, diffusion decreases as a result of steric hindrance in the medium.

- **Effect of decoction temperature (X_2)**

The decoction temperature has a significant positive and negative impact on TPC and DPPH (Table 21). Firstly, it is observed (Figure 24) that, at an initial decoction temperature of 82.4 °C, the values of 29.72 mg GAE/100 mL and 36.62 mg TE/100 mL were obtained for TPC and DPPH respectively. These values increased to reach a max of 32.29 mg GAE/100 mL for TPC at 91.2 °C and, 39.08 mg TE/100 mL for DPPH at 90.5 °C. After that, a significant decrease was obtained up to 30.95 mg GAE/100 mL for TPC and 37.19mg TE/100 mL for DPPH, when increasing the decoction temperature at 97.6 °C.

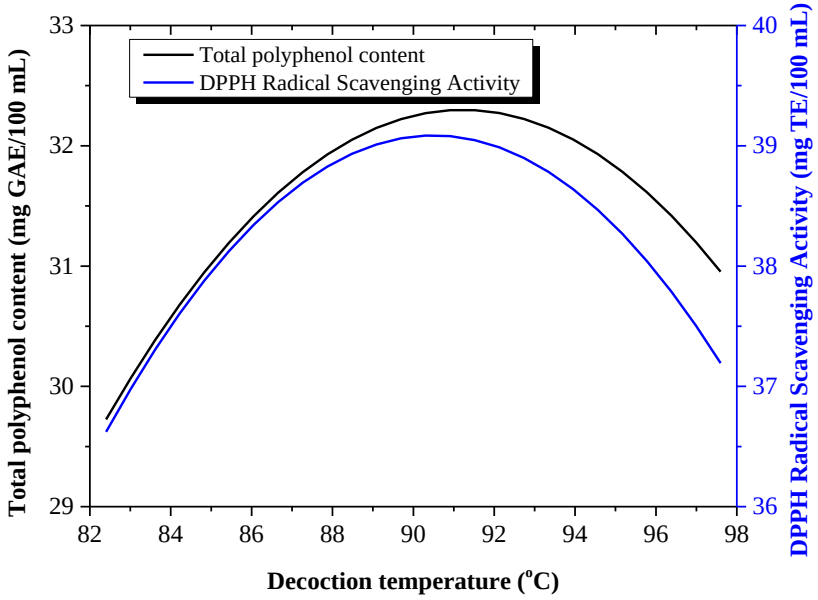


Figure 24: Evolution of Total polyphenol content and DPPH radical scavenging activity as a function of decoction temperature. Lemongrass powder/water ratio and extraction time respectively fixed at 1.22 and 3.7 min.

The TPC and DPPH activity increased with the rise of extraction temperature and then level off at high temperature. The increment in TPC and DPPH activity is due to the fact that high temperatures soften cell wall tissue thereby enhancing the solubility of polyphenols into the solvent (Cacace and Mazza, 2003; Irakli *et al*., 2017). The solvent thereby penetrates the plant matrix and results in the mass transfer of compounds from the matrix into the solvent (Jovanovic´ *et al*., 2017). Temperature has been shown in studies to be a dependent factor in the extraction of polyphenols from different sources (Vuong *et al*., 2013). However, a further increase of the temperature led to the decrease of both TPC and DPPH activity suggesting that high temperature may have caused the degradation of phenolic compounds resulting in a decrease in DPPH activity. The decrease could also be due to the binding of polyphenols to proteins thereby leading to a decrease in both total polyphenol content and antioxidant activity. Polyphenols bind to proteins either by covalent or non-covalent bonds. The main type of interaction, non-covalent interactions can either be by hydrogen, hydrophobic and ionic bonding (Brudzynski and Maldonado-Alvarez, 2015; Zhang *et al*., 2014). Binding engages the use of functional groups involved in redox cycling, metal chelation, or electron donor which thereby affects the antioxidant property of polyphenols (Brudzynski and Maldonado-Alvarez, 2015). Many works have been done on the binding of polyphenols to proteins which affect their biological activity (Bandyopadhyay *et al*., 2012; Gallo *et al*., 2013; Jakobek, 2015).

- **Effect of extraction time (X_3)**

The extraction time has a negative significant impact on DPPH while, it is noted in the first hand a positive significant and after a significant negative impact on TPC (Figure 25). For TPC, the value of 29.72 mg GAE/100 mL was obtained at 3.7 min extraction time and increased with a

rise in extraction time to reach a max of 31.54 mg GAE/100 mL at 7.47 min extraction time. After that, TPC decreased to 29.68 mg GAE/100 mL at 11.3 min extraction time.

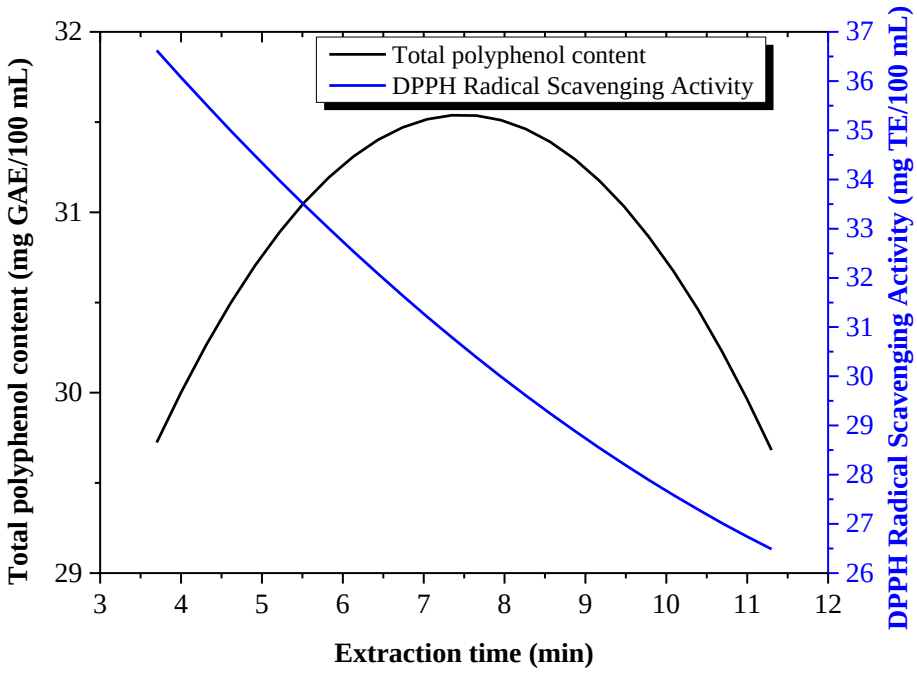


Figure 25: Evolution of Total polyphenol content and DPPH radical scavenging activity as a function of extraction time. Lemongrass powder/water ratio and decoction temperature respectively fixed at 1.22 and 82.4 °C

This could be explained by that, as the lemongrass get in to contact with the hot liquid, there is immediate dissolution of the phenolic compounds, and diffusion of target analytes from materials to outside solvent. A relatively lengthy extraction time contributed to a positive influence on the TPC. However, extended extraction time led to degradation of polyphenols and lowering of DPPH activity due to the thermo labile feature of phenolic compounds. This finding is in accord with that of Jeszka-Skowron and Zgoła-Grześkowiak (2014) who realized that extended extraction time of polyphenols from *Camellia sinensis* caused a decrease in rutin and chlorogenic acid

content. Vuong *et al.* (2013) also reported that extended extraction time of polyphenols and antioxidants from *Carica papaya* leaf led to a decrease in the values.

- **Effect of interaction decoction temperature/extraction time (X_2X_3) on TPC**

The synergistic effect of temperature and time generated a positive impact (the increase) on the TPC. With increase in temperature at a short time (Figures 26), the plant matrix is fragilized and, the solvent enters the cell leading to a mass transfer of soluble compounds from the matrix into the solvent (Jovanovic´ *et al*., 2017).

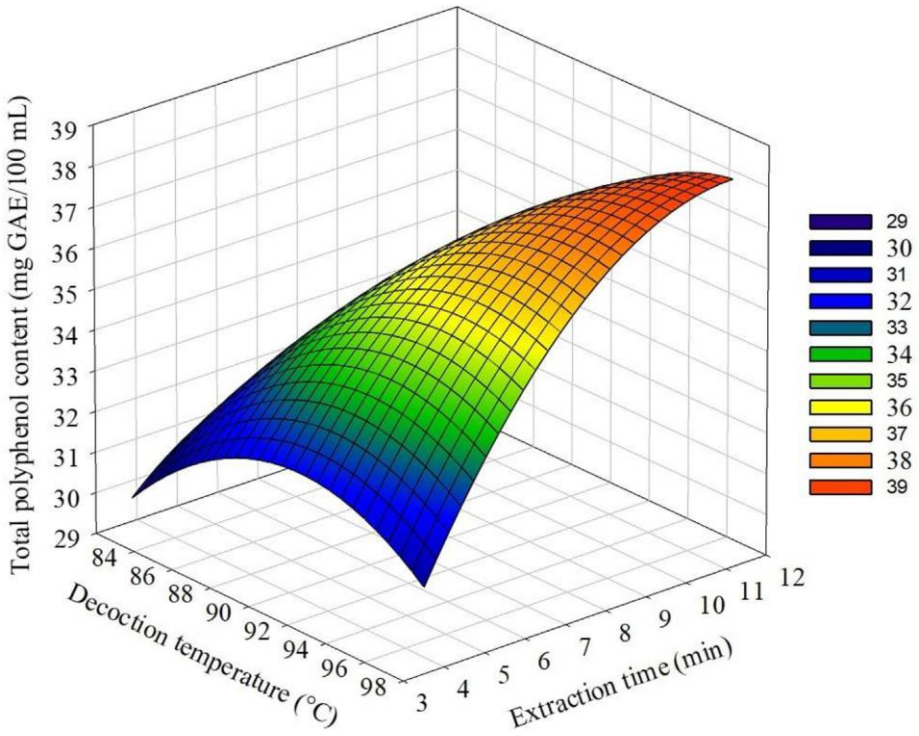


Figure 26: Mesh plot of the evolution of Total polyphenol content as a function of decoction temperature and extraction time. Lemongrass powder/water ratio fixed at 1.22

3.3.3.2 Determination of optimal conditions

Composite desirability was effectuated to find the composite optimum by maximizing the TPC and DPPH (table 23). Lemongrass was therefore extracted at the composite optimum

(lemongrass powder concentration; 5 g/100 mL, temperature: 93.8 ^{o}C and time: 11.3 min) and analysed. The experimental values of TPC and DPPH obtained at the optimal conditions were 71.98±0.33 mg GAE/100 mL of extract and 80.63±0.49 mg TE/100 mL of extract, respectively. Compared to the predicted response of 73.00 mg GAE/100 mL extract for polyphenol and 87.75 mg TE/100mL extract for DPPH, the experimental data was in conformity.

Table 23: Composite desirability of total polyphenol and DPPH activity of *Cymbopogon citratus* extract

Lemongrass powder concentration	Temperature (^{o}C)	Time (min)	Predicted TPC (mg GAE/100 mL)	Predicted DPPH (mg TE/100 mL)	Desirability value
5	93.8	11.3	73.00	87.75	0.92

Comparing to the results of Oboh *et al*. (2010), they obtained a total polyphenol content of 0.5 mg GAE/g and DPPH radical scavenging activity of 70% for hot water extracts of lemongrass at conditions (lemongrass concentration 10 g/100 mL, temperature 100 ^{o}C and time 10 min). The differences could be as a result of the different extraction conditions used and the extraction temperature has a major effect on the polyphenol content as described above.

3.3.4 Effect of independent variables on FRAP, reducing and total sugar of lemongrass extract

Apart from TPC and DPPH studied in RSM, FRAP, reducing sugar and total sugar were analysed for the 20 experience. FRAP ranged from 42.27±0.70 to 142.44±1.09 mg TE/100 mL extract, the reducing sugar from 25.25±0.53 to 132.79±0.16 mg Gluc/100 mL, and total sugar from 51.49±0.33 to 163.11±0.91 mg Gluc/100 mL of extract (table 24). The sugar content of lemongrass

extract is quite low and will therefore supply a limited carbon source for the fermentation of the baobab-lemongrass beverage.

Table 24: FRAP, reducing sugar and total sugar of the 20 runs

Exp. N°.	FRAP (mg TE/100mL extract)	Reducing sugar (mg Gluc/100mL extract)	Total sugar (mg Gluc/100mL extract)
19	95.34±0.63	86.23±0.77	112.05±1.03
10	142.44±1.09	132.79±0.16	163.11±0.91
2	125.59±0.50	118.25±0.59	157.75±0.25
8	130.35±0.17	116.75±0.74	154.57±0.83
1	59.17±0.80	45.03±0.93	71.67±0.80
9	42.27±0.70	25.25±0.53	51.49±0.33
13	97.60±0.64	86.48±1.67	112.31±0.90
11	97.37±0.70	79.16±0.20	117.55±0.47
14	81.11±0.66	91.37±0.44	122.18±0.81
18	98.94±1.08	89.20±0.77	116.65±0.59
3	66.77±1.47	50.59±0.86	88.62±1.18
17	84.79±0.13	88.18±0.65	113.86±1.06
12	101.57±0.84	87.44±1.82	122.75±0.66
4	137.23±0.24	125.31±0.44	154.63±0.35
6	129.49±0.87	122.34±1.31	154.39±0.50
5	62.39±1.00	44.61±0.27	68.11±0.10
7	53.23±1.13	57.65±1.38	78.25±0.51
15	95.50±1.41	84.37±1.23	113.16±1.75
16	97.14±1.12	89.33±1.10	113.56±0.78
20	96.38±0.98	89.65±0.28	114.26±0.97

3.3.5 Microbial profile of lemongrass extract before and after extraction

The microbial quality of the lemongrass extract was determined by analysing the aerobic mesophile, coliforms, yeast and fungi, staphylococcus, and Lactobacillus, before and after extraction. The results (table 25) show that the lemongrass powder initially was made up of high amounts of aerobic mesophile and in addition to that, they had coliforms and staphylococcus. There was no Lactobacillus and yeast and fungi identified in the lemongrass leaves. After extraction at the optimal conditions of (lemongrass powder concentration; 5 g/100 mL, temperature: 93.8 ^{o}C and time: 11.3 min), there was a complete destruction of the microbial flora thereby making the extract safe from microbes. The lemongrass extract is therefore good for the fermentation process which only the LAB introduced will have total action in the medium.

Table 25: Microbial quality of the lemongrass extract

Microorganism	Before (cfu/mL)	After (cfu/mL)
Aerobic mesophile	26850±11150	0
Coliforms	20±6	0
Yeast and fungi	0	0
Staphylococcus	4	0
Lactobacillus	0	0

3.3.6 Colour of lemongrass powder and extract at optimal condition

The colour of lemongrass powder and that of extract was analysed and results presented in table 26. ‘L’ ranges from lightness to darkness and the higher it is, the lighter the sample. ‘a’, is an indicator of greenness for minus value and redness for plus value, while ‘b’, is for blueness (minus value) and yellowness (plus value). The lemongrass powder was darker (45.88±0.16), with a tint

red (1.69±0.10) and a yellow colour (22.99±0.30) when compared to the target. The lemongrass extract was little lighter (85.18±0.11), red (3.91±0.09) and highest yellow (45.95±0.20) when compared to the target.

Table 26: Colour difference of lemongrass powder and extract

	Lemongrass powder	**Target**	**Lemongrass extract**	**Target**
L	45.88±0.16	97.29	85.18±0.11	100
a	1.69±0.10	-0.21	3.91±0.09	0
b	22.99±0.30	-0.13	45.95±0.20	-0.02
dE*ab	56.40±0.23	**/**	48.46±0.23	/

3.3.7 Polyphenols of lemongrass extract

` Polyphenols of free and bound portions of lemongrass and lemongrass leaf extract are presented in Table 27. The free extract of the lemongrass leaves was made up principally of catechin (278.68±9.48 mg/100 mL), followed by caffeic acid (79.19±31.43 mg/100mL), vanillic acid (54.37±19.84 mg/100 mL), naringin (20.45±0.17), epicatechin (18.98±1.95), quercitin (11.82±1.41), and in small amounts *p*-coumaric acid (3.90±0.32), trans ferulic acid (1.11±0.12), trans cinnamic acid, and syringic acid (0.21±0.02). On the contrary, the bound portion was made up mainly of, epicatechin (338.22±31.64), *p*-coumaric acid (281.14±0.79), catechin (21.98±16.43), quercitin (19.44±1.07) and trans ferulic acid (13.07±0.48).

Table 27: Phenolic acid compounds of free, bound and lemongrass extract

Phenolic compound	Free polyphenol (mg/100 mL)	Bound polyphenol (mg/100 mL)	Lemongrass extract (mg/100 mL)
Gallic acid	ND	ND	3.932±0.515
Dihydroxybenzoic acid	ND	ND	3.411±0.121
Chlorogenic acid	ND	ND	ND
Catechin	278.68±9.48	21.98±16.43	9.43 ± 5.49
Vanillic acid	54.37±19.84	ND	5.98±2.17
Caffeic acid	79.19±31.43	ND	20.82±0.00
Syringic acid	0.21±0.02	ND	18.63±7.39
Epicatechin	18.98±1.95	338.22±31.64	3.76±1.24
p-coumaric acid	3.90±0.32	281.14±0.79	0.88±0.39
Sinapic acid	P	ND	ND
Rutin	P	P	P
Trans ferulic acid	1.11±0.12	13.07±0.48	0.58±0.17
Quercitin	11.82±1.41	19.44±1.07	6.07±0.33
Trans cinnamic acid	1.04±0.18	ND	0.25±0.01
Naringin	20.45±0.17	ND	ND

ND = Not detected; P = Present

The polyphenols present in the lemongrass leaf extract have a lower content than that of the free and bound portions. The difference could be as a result of the difference in extraction solvents and methods. The extraction temperature (93.8 ^{o}C) and time (11.3 min) might have led to the degradation of some phenolic compounds. Caffeic (20.816±0.003 mg/100 mL) and syringic (18.635±7.390 mg/100 mL) acids were the prominent phenolic compounds in the lemongrass extract. Caffeic acid has been shown to have a potent antioxidant activity due to the

electrochemical behaviour of the two hydroxyl groups that are in the ortho position on the ring (Gulcin, 2006; Mercado-mercado *et al*., 2020). Other phenolic compounds (Gallic acid, dihydroxybenzoic acid, catechin, vanillic acid, epicatechin, *p*-coumaric acid, trans-ferulic acid, quercitin, and trans-cinnamic acid) were present in small amounts. Caffeic acid was also reported the main phenolic acid compound present in lemongrass infusion (Coelho *et al*., 2016). Otherwise, Rodrigues *et al.* (2015) instead detected a high quantity of chlorogenic acid in hot and cold extracts of lemongrass. Kouassi *et al.* (2017) also detected the presence of protocatechuic acid, caffeic acid, rutin, *p*-coumaric acid, ferulic acid, quercetin, kaempherol in ethanol and methanol extracts of lemongrass. Harvest region and seasons might account for the differences in profile and content in phenolic compounds (Costa *et al*., 2016). Likewise, the difference in profile and content might be the main factors that drive the difference in antioxidant activities of lemongrass extract. When compared with the phenolic acid profile of baobab juice (section 2.3.10), the lemongrass extract was made up of higher contents of catechin, vanillic acid, syringic acid and quercitin. While baobab was made up of higher content of gallic acid, caffeic acid, epicatechin and *p*-coumaric acid. A mixture of baobab and lemongrass will thereby give a wide range of phenolic compounds. Polyphenols are strong antioxidants due to their low redox potential and capacity to donate electrons or hydrogen atoms (Zhang *et al*., 2014). Polyphenols have several mechanisms of action, they inhibit the formation of lipid radicals, disrupt the propagation of chain auto-oxidation reactions, suppress singlet oxygen, reduce hydrogen peroxides to stable compounds, chelate transition metal ions, inhibit endogenous prooxidative enzymes, and activate endogenous antioxidant enzymes (Seczyk *et al*., 2019).

3.3.8 Organic acid profile of lemongrass extract

An organic acid is an organic compound with acidic properties containing carbon, and known to affect particularly taste formation and many physiological processes (Theron and Lues, 2011). Organic acids (oxalic, tartaric, malic, iso-citric, ascorbic, citric, succinic, propionic and glutaric acids) were determined and the concentration of each organic acid of the lemongrass leaves and lemongrass leaf extract are as shown in table 28. The main acid in the lemongrass leaves is glutaric acid (12.906±0.072) and the other acids are present in small amounts. While, propionic acid was the dominant organic acid in lemongrass extract with a concentration of 20.137±0.163 mg/mL, followed by glutaric acid, succinic acid, iso-citric acid, tartaric acid, citric acid, malic acid, oxalic acid and ascorbic acid in descending order.

Generally, the organic acid content in the lemongrass leaf extract was higher than in the lemongrass leaves. Water has a high dielectric constant and therefore the easy dissociation of acids in to water (Ardestani *et al*., 2015) which can be increased at higher temperatures. However, Aktas and Yildiz (2011) reported the decomposition of organic acids by heat which explains the decrease in some of the organic acids. The difference in the organic acid content could also be as a result of the difference in the extraction process for the determination of the organic acid content. The extraction condition therefore has a great effect on the extraction of organic acids.

Organic acids are naturally used as food preservatives because they exhibit antimicrobial inhibitory activities and also act as acidulants. Propionic acid, the main acid found in lemongrass is known to have antimicrobial activity primarily against moulds and bacteria (Theron and Lues, 2011), this contributes to the antibacterial effect of lemongrass. Succinic acid plays a significant role in the Krebs cycle and tartaric acid is generally used as acidulant. Citric acid is the main acid found in fruits and in lemongrass, it is found in small quantity. It has a fresh acidic flavour and a

pleasant taste. Malic acid has a smooth lingering taste, tart taste but not sharp as that of citric acid (Theron and Lues, 2011). Ascorbic acid is considered an antioxidant because it acts as an oxygen scavenger by removing molecular oxygen in polyphenol oxidase reactions.

Table 28: Organic acid compounds of lemongrass leaves and lemongrass extract

Organic acid compound	Lemongrass leaves (mg/mL)	Lemongrass extract (mg/mL)
Oxalic	0.003±0.000	0.009±0.002
DL-Tartaric	0.108±0.008	0.131±0.022
L-malic	0.053±0.003	0.038±0.009
Isocitric acid	0.241±0.003	0.217±0.025
Citric	0.015±0.000	0.059±0.008
Succinic	0.060±0.002	0.259±0.007
propionic	0.253±0.002	20.137±0.163
Glutaric	12.906±0.072	0.459±0.106
Ascorbic acid	0.156±0.034	0.030±0.001

3.3.9 Aroma profile of lemongrass extract

Lemongrass is widely used in perfumery and in beverages and taken as tea due to its lemon flavour (Haque *et al*., 2018). The volatile profile of lemongrass extract was therefore analysed to identify the volatile compounds present. A total of 37 aroma compounds were identified in the extract and arranged in different chemical groups (table 29). Amongst the group, aldehydes were the most prominent that made up to 37.52±13.96%. The principal aldehyde identified was citral, 35.02±3.98%, the main compound in lemongrass that gives its lemon scent and has antimicrobial properties (Fattah *et al*., 2010; Li *et al*., 2018).

Table 29: Volatile compound content of lemongrass leaf extract

Composition	RT	% composition
Hydrocarbons		**4.04±0.31**
Undecane	5.431	2.32±0.71
Naphthalene, decahydro-1,6-dimethyl-	25.664	1.71±0.35
Esters		**11.12±3.38**
Oxalic acid, allyl ethyl ester	3.64	0.90±0.20
Acetic acid, butyl ester	5.14	1.51±0.42
Linalyl acetate	24.058	8.57±2.20
Methyl 2-undecynoate	39.794	0.15±0.00
Alcohols		**10.48±0.70**
2-Nonanol	5.641	1.43±0.49
6-methyl-5-Hepten-2-ol	20.367	1.02±0.07
Verbenol	24.493	0.86±0.04
cis-Verbenol	24.678	3.05±0.04
5,8,10-Undecatrien-3-ol	26.944	0.71±0.12
cis-p-mentha-1(7),8-dien-2-ol	31.091	1.00±0.49
3,7-dimethyl-6-Octen-1-ol	32.116	1.42±0.36
Selina-6-en-4-ol	37.924	0.99±0.16
Aldehydes		**37.52±13.96**
3,7-dimethyl-2,6-Octadienal	22.502	0.60±0.06
2-Decenal, (E)	27.119	0.37±0.20
Citral	30.306	35.02±3.98
2-Undecenal	30.986	0.30±0.12
2,4-Decadienal	32.496	1.23±1.09
Acids		**5.36±0.33**
Acetic acid	19.481	0.48±0.14
Hexanoic acid	33.391	1.08±0.20
Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl,	34.162	1.12±0.89
Octanoic acid	36.698	0.97±0.34
Nonanoic acid	37.928	0.64±0.16

n-Decanoic acid	39.044	0.19±0.04
Benzoic acid	40.439	0.28±0.10
Dodecanoic acid	41.025	0.59±0.17
Terpenes and terpenoids		**19.34±7.03**
Citronellol	31.861	2.33±0.43
2,6,10-trimethyl-Dodecane	32.852	0.67±0.09
Geraniol	33.632	16.34±2.84
Ketone		**7.37±3.13**
6-methyl-5-Hepten-2-one	13.974	6.82±3.65
6-methyl-3,5-Heptadien-2-one	25.133	0.56±0.25
Others		**4.76±0.63**
Tetrahydro-3-Furanol	4.815	0.32±0.05
Methylene chloride	2.674	1.75±0.33
1,1'-oxybis- Octane	31.491	1.65±0.28
4-Acetonylcycloheptanone	39.399	0.73±0.18
Ethylene oxide	2.439	0.31±0.07

Terpenes and terpenoids were also present in appreciable amounts (19.34±7.03%). In this group, geraniol was the main component, followed by citronellol, and then 2,6,10-trimethyl-Dodecane. Coelho *et al.* (2016) also analysed the volatile components of lemongrass extract and citral was the major aroma compound identified, followed by geraniol. Other groups of compounds were present in small amounts such as esters, aromatic alcohols, ketones, and aromatic acids.

Partial conclusion

The RSM was successfully used to optimize the decoction conditions of lemongrass leaves. The aim was to maximize the extraction of total polyphenols and antioxidants, and the optimal conditions generated were: lemongrass powder/water ratio (5 g/100 mL), temperature (93.8 ^{o}C) and time (11.3 min). This yielded a TPC of 71.98±0.33 mg GAE/100 mL extract and 80.63±0.49 mg TE/100 mL respectively for TPC and DPPH. When compared to the predicted values, it was in agreement, therefore suitability of the models. The extract was rich in a range of phenolic compounds and the main aroma compound citral and geraniol was not loss during extraction. The extract was made up of principally propionic acid. Propionic acid and citral are known to have antimicrobial properties. The extract was also free of microbes and therefore ready to be supplemented in baobab juice for fermentation. In addition, the lemongrass powder presented a high amino acid content which implies the lemongrass extract could be used as nitrogen source during fermentation, since baobab has a low amino acid content.

To that note, baobab was therefore mixed with lemongrass to undergo fermentation which gives the objective of the next chapter.

CHAPTER 4: MODELLING FERMENTATION KINETICS OF BAOBAB-LEMONGRASS PROBIOTIC BEVERAGE

Abstract

The aim of this section was to determine the kinetic parameters of growth and substrate utilisation models of lactic acid fermentation of baobab-lemongrass beverage. A kinetic study for the production of baobab-lemongrass probiotic beverage was carried out using autochthonous *Lb. fermentum* from baobab pulp. Baobab juice obtained from pectin hydrolysis and lemongrass extract were mixed in different proportions, 50/50, 75/25 and 100/0 baobab and lemongrass. Furthermore, a batch fermentation was done at controlled pH and at the end, the biomass, substrate utilised and product formed were evaluated. The experimental data were fitted to the Monod, Logistic, and Leudeking-Piret equations to determine the fermentation kinetic parameters. The parameters were determined by the non-linear regression method using the Levenberg-Marquardt algorithm in MATLAB. Correspondingly, the R^2 and RSME were used to choose the best model and compare the results. In addition, the effect of fermentation on the rheology of the probiotic juice, antioxidant activity, organic acid profile, polyphenol profile and aroma were also evaluated. Moreover, the viability of the *Lb fermentum* in the probiotic juice was determined for 28 days during storage at 4 and 28 ^{o}C. The results showed that the substrate composition had a great effect on the growth of the LAB. Growth was best in the 50/50 baobab-lemongrass probiotic beverage followed by the 75/25 and then the 100% baobab. The Logistic model fitted best than the Monod model. The 50/50 baobab-lemongrass probiotic beverage fitted best the logistic model with a μ_{max} and X_m of 0.0731 h^{-1} and 13.9100 cfu/mL respectively. While for the substrate utilisation and product formation models, the 75/25 baobab-lemongrass probiotic beverage showed a better fitting than the other cases. The parameters $Y_{x/s}$, m_s, α and β were respectively 2.9675 g/g, 0.0003 g/L. g^{-1}, 0.0118 and 0.0038. Furthermore, the Herschel-Bulkley model fitted best the rheological data and the apparent viscosity increased after fermentation. The flow behaviour of the baobab-lemongrass probiotic beverage presented a shear thickening behaviour at 25. The consistency index and yield stress increased after fermentation due to the presence of the bacteria cells in the juice. The fermentation had a positive impact on the antioxidant activity of the probiotic juice. The organic acid profile showed the presence of lactic and acetic acids amongst other acids after fermentation. The aroma of the juice was contributed by acids, aldehydes, terpenes, ketones and alcohol. At the end of storage for 28 days, the viability of the *Lb. fermentum* in the 50/50, 75/25 and 100/0 baobab-lemongrass probiotic beverage were 8.31, 7.65 and 4.79 respectively at 4 °C and 8.56, 8.56 and 4.67 respectively at 25 °C.

Keywords: LAB fermentation, probiotic beverage, kinetic parameters, baobab juice and lemongrass extract

4.1 Introduction

Baobab pulp is mostly used in the production of baobab nectar (Cisse and Montet, 2012; Cisse *et al*., 2009) and baobab juice (Tembo, 2016). In addition to the transformation of baobab pulp to nectar and juice, fermentation by lactic acid bacteria can be regarded as a simple and profitable biotechnology for maintaining and/or improve the nutritional, sensory and shelf-life properties of the fruit. In order for the lactic acid bacteria to realize fermentation, they require both carbon and nitrogen source (Abbasiliasi *et al*., 2017). Though baobab is a rich carbohydrate source, it has a low protein content which makes it a poor nitrogen source. However, lemongrass has a higher protein content compared to baobab and can eventually supplement the nitrogen source for the fermentation process. In addition, it can contribute in imparting the aroma of the juice. The intrinsic characteristics of fruit matrix play an important role in the lactic acid fermentation and in order to obtain desirable properties, the selection of lactic acid bacteria is an important step to be considered (Di Cagno *et al*., 2009). Compared to allochthonous strains, autochthonous cultures may ensure better performance as they may prolong shelf life and contribute in the increase of targeted nutritional, rheological and sensorial properties (Di Cagno *et al.*, 2009, 2011, 2013)

Fermentation process can be better understood by modelling the kinetics. Mathematical models predict the influence of fermentation operating parameters on the rate of cell growth, substrate utilization and lactic acid (LA) production (Jaiswal and Abu-ghannam, 2013). Probiotic activity is measured by strain viability and its ability to attain high cell number. It is therefore important to determine the optimal conditions for the growth of the microbial cells that would ensure the accumulation of a high concentration of viable cells. This section therefore has as objective to study of the batch fermentation kinetics of baobab and lemongrass juice using autochthonous LA bacteria.

4.2 Materials and methods

4.2.1 Materials

Baobab juice obtained from pectinase hydrolysis (0.2% v/v) and lemongrass extract obtained at optimal conditions was used for the fermentation process.

4.2.2 Methods

4.2.2.1 Isolation and identification of LAB from baobab pulp

LAB was isolated from baobab pulp using a selective media, MRS (Man Rogosa and Sharpe) for LAB. Baobab powder (0.5 g) was dissolved in 9 mL of MRS (Man Rogosa and Sharpe) broth, incubated at 37 ^{o}C for 24 h followed by streaking on MRS agar. Single isolated colonies were successively streaked three times on MRS agar to obtain pure colonies. Biochemical assay was done on the isolate to confirm if it was LAB. DNA was further isolated and the PCR product sequenced. The PCR product was sequenced at Sakhala Enterprises (Bangalore, India) and the obtained sequences were merged with BioEdit (version 7.0.5.3) and BLAST searched by comparison to known sequences in the National Center for Biotechnology Information (NCBI) GenBank database (http://blast.ncbi.nlm.nih.gov).

4.2.2.2 Fermentation of baobab-lemongrass juice

Different mixtures of the baobab juice and lemongrass extract were prepared. In a conical flask and following aseptic techniques, 150 mL of different mixtures of the juice were prepared: 50/50 baobab-lemongrass, 75/25 baobab-lemongrass and 100% baobab juice. The pH of the mixture was adjusted to pH 5±0.6 in order to favour the growth of the *Lb. fermentum*.

Strains of *Lb. fermentum* isolated from baobab was propagated in MRS broth at 37 ^{o}C for 12 h. The 12 h cells were harvested by centrifugation (8000 *g*, 10 min at 4 ^{o}C), washed twice with saline

and then Milli-Q water and re-suspended in Milli-Q water. To the different mixtures 5% of the *Lb. fermentum* culture suspended in Milli-Q water was immediately inoculated into the different baobab-lemongrass mixtures. Fermentation was carried out in a batch mode in which the vessel was prepared with an initial charge of inoculum and medium, the process left to run and was not altered by further nutrient addition or removal. This mode is simple and widely used in the laboratory to determine cell growth and product formation because it can be easily operated and contamination is limited. Contamination can have a great effect on the fermentation process if not controlled (Macauley-Patrick and Finn, 2008). Fermentation was carried out in 25 mL test tubes with a volume of 10 mL each of the fermentation broth. It was incubated in an incubator shaker (Excella E24, New Brunswick Scientific, US) under agitation (150 rpm) at 37 ^{o}C. Gentle agitation during fermentation is important because it suspends cells in the medium for proper growth, enhance oxygenation, proper heat transfer and also aids the mass transfer of nutrients without damaging the structure of cells (Dutta, 2008; Uzir and Don, 2007). Improper mixing can cause substrate concentrations drop to zero especially in areas where cells are out of suspension (Uzir and Don, 2007). The agitation created a turbulence in the medium which thereby annuls the effect of external mass transfer. In this light, external mass transfer has no effects on the growth curves. A fermentation kinetics was followed for 72 h where samples were taken at 4 h time interval, 0, 4, 8, 12, 16, 20, 24, 28, 36, 48th h and then 72nd h. The viable cell count, pH and reducing sugar were determined.

4.2.2.2.1 Viable cell count determination in baobab-lemongrass probiotic beverage

Viable cell counts were determined at the different time intervals by serial dilution of the fermented beverage with sterile saline water until 10^{-10} dilution. Aliquots of 0.1 mL of 3 different

dilutions were plated on MRS agar by pour plate method. The plates were incubated for 48 h at 37 ^{o}C and plates containing 20–350 colonies were counted and recorded as colony forming units (cfu) per mL of solution.

4.2.2.2.2 pH analysis of the baobab-lemongrass probiotic beverage

The pH of the fermented juice was directly measured on the pH meter and recorded.

4.2.2.2.3 Analysis of reducing sugar in baobab-lemongrass probiotic beverage

The reducing sugar content of the fermented juice was determined as described in section 2.2.3.3.

4.2.2.3 Growth kinetics of fermentation of baobab-lemongrass probiotic beverage

Kinetics of growth, substrate utilization and product formation were determined and modelled according to the Monod (Altıok *et al*., 2006), logistic, substrate utilization and Leudeking-Piret models (Liu *et al*., 2003). The models are described as follows:

- **Growth models**

The simplest growth model is the Malthus model (Ghosh *et al*., 2012) given as:

$$\frac{dX}{dt} = \mu X \qquad (43)$$

Where X is the concentration of biomass, μ the specific growth rate and t is time.

The specific growth rate, μ, can be expressed as a function of the limiting substrate, S, by the Monod equation which is the most frequent used model to describe microbial growth. The equation is given as:

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (45)$$

Where μ_{max} is the maximum specific growth rate when S >> K_S and the concentrations of all other essential nutrients are not limiting; K_S is the substrate utilisation concentration when $\mu=\mu_{max}/2$. To describe growth kinetics at both the exponential and stationary phases, the logistic equation is used (equation 46).

$$\frac{dX}{dt} = \mu X \left(1 - \frac{X}{X_m}\right) \quad (46)$$

Where X_m is the maximum biomass concentration. The integrated form of the equation (equation 47) using $X = X_0$ (t = 0) gives a sigmoidal variation of X as a function of t which may represent both an exponential and a stationary phase:

$$X = \frac{X_0 X_m e^{\mu_m t}}{X_m - X_0 + X_0 e^{\mu_m t}} \quad (47)$$

- **Product formation model**

The Leudeking-Piret equation (equation 48) describes the product formation model originally developed for the formation of lactic acid by *Lb. delbrueckii*. The rate of the product formation depends on both the instantaneous biomass concentration, X, and the growth rate, dX/dt, in a linear manner:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (48)$$

Where α and β are the growth and non-growth associated constants respectively.

- **Substrate utilisation model**

The substrate utilisation model is the Leudeking-Piret like equation (equation 49) where the amount of carbon substrate used for product formation is assumed to be negligible:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - m_s X \qquad (49)$$

Where $Y_{X/S}$ is the biomass yield based on substrate utilisation and m_s, the maintenance coefficient. The kinetic model parameters were determined by curve fitting method using the nonlinear regression analysis based on the Levenberg-Marquardt algorithm with the MATLAB (MATLAB, R2015a) program. The accuracy of the models was assessed with respect to the coefficient of determination R^2 and RSME.

The probiotic baobab juice before and after fermentation (for 36 h) was further analysed to determine effect of fermentation on the juice.

4.2.2.4 Determination of rheological parameters of baobab-lemongrass probiotic beverage

The rheology of the baobab-lemongrass beverage before and after fermentation was determined according to the method of section 2.2.3.9.

4.2.2.5 Determination of TPC of baobab-lemongrass probiotic beverage

The TPC of the baobab-lemongrass probiotic beverage before and after fermentation was determined according to the method described in section 2.2.3.5.1.

4.2.2.6. Determination of antioxidant activity of baobab-lemongrass probiotic beverage

The DPPH, FRAP and ABTS assay of the baobab-lemongrass beverage before and after fermentation were determined with respect to the method described in section 2.2.3.5.2.

4.2.2.7 Analysis of colour of baobab-lemongrass probiotic beverage

The colour of the baobab-lemongrass probiotic beverage before and after fermentation was measured with a Minolta colorimeter (CM-5, Konica Minolta, Tokyo, Japan) as described in section 2.2.3.10.

4.2.2.8 Determination of phenolic acid profile of baobab-lemongrass probiotic beverage

The phenolic acid profile of the baobab-lemongrass probiotic beverage was determined by HPLC according to the method of section 2.2.3.11.

4.2.2.9 Determination of organic acid and vitamin C content of baobab-lemongrass probiotic beverage

The organic acid and vitamin C content of the baobab-lemongrass probiotic beverage was done with respect to the procedure in section 2.2.3.12 and 2.2.3.13 respectively.

4.2.2.10 Analysis of aroma profile of baobab-lemongrass probiotic beverage

The aroma profile of the baobab-lemongrass probiotic beverage before and after fermentation was analysed following the method described in section 3.2.2.11.

4.2.2.11 Effect of storage on viability of *Lb. fermentum* in baobab-lemongrass probiotic beverage

The fermented juice samples (fermented at 37 ^{o}C for 36 h) were stored at 4 ^{o}C and at room temperature, 28 ^{o}C, for four weeks. At weekly interval, viabilities of probiotic culture in fermented baobab-lemongrass juice were investigated and expressed as colony forming units (cfu/ mL).

4.3 Results and discussion

4.3.1 Isolation and identification of LAB from baobab pulp

Lactobacillus fermentum (appendix 5) was isolated from baobab fruit pulp and the probiotic attributes evaluated showed it had some probiotic properties. The isolate was acid tolerant (appendix 6) and bile tolerant (appendix 7) up to 1% oxgall concentration. The isolate showed good resistance to gastrointestinal transit (appendix 8, 9, and 10) and possessed some antibacterial properties (appendix 11). It further showed aggregation properties to different pathogens and was susceptible to a good number of antibiotics (appendix 12). Thereupon, baobab can serve as a potential source of isolation of LAB and the isolate could be eventually used as a starter for the fermentation of the baobab-lemongrass beverage.

4.3.2 Fermentation of baobab-lemongrass juice

Fermentation was carried out in a batch mode under agitation and the biomass, reducing sugar concentration, lactic acid concentration and pH for each process parameter was measured and represented in figure 25, 26 and 27 respectively.

4.3.2.1 Effect of fermentation on growth rate

The growth curve for the fermentation of the baobab lemongrass juice with autochthonous *Lb. fermentum*, yields a typical microbial growth curve (figure 27). The fermentation characteristics of the different mixtures are similar but for the 100% baobab which was slightly different. There are three phases, the acceleration, exponential and stationary phase. The first four hours of incubation represent the acceleration phase where *Lb. fermentum* adapts to the complex medium of the beverage and therefore a slight growth is observed and there is no significant difference in growth with respect to the different beverage mixtures.

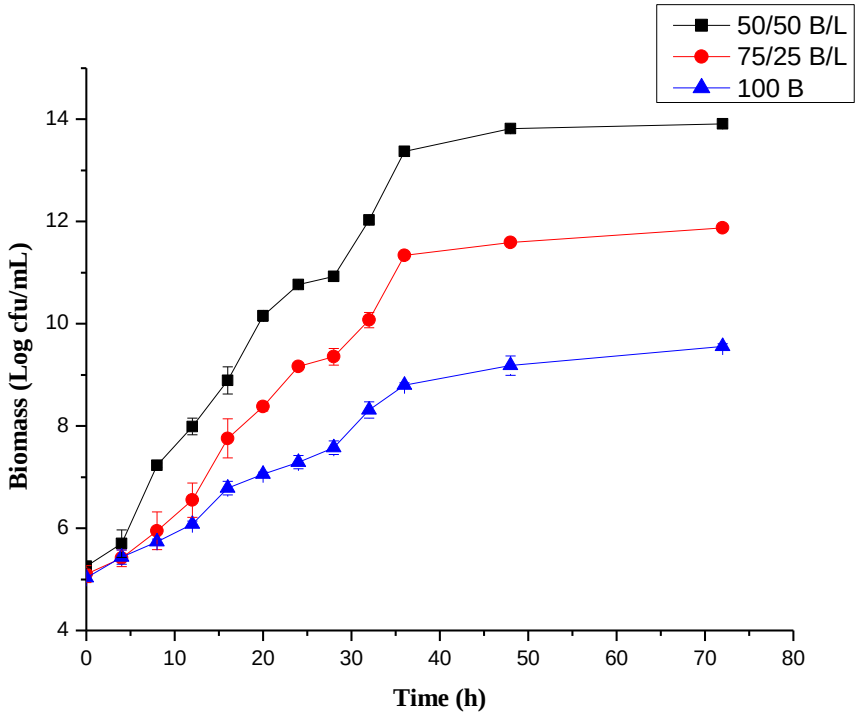


Figure 27: Effect of fermentation on biomass of the fermented probiotic baobab-lemongrass beverages

The initial probiotic concentration ranged from 5.029 to 5.256 Log cfu/mL, and after 4 h of fermentation, it increased to 5.701±0.270, 5.415±0.163 and 5.436±0.138 Log cfu/mL respectively for 50/50 mixture, 75/25 mixture and 100% baobab. For process engineering, the length of lag phase is important and the duration has to be minimized in order to obtain high productivity within a short duration. The length of this phase depends on nutrient composition of medium, the size, age and type of inoculum, and process conditions (Bailey and Ollis, 1986; Dutta, 2008; Shuler and Kargi, 2002). In order to minimize the lag phase, the *Lb. fermentum* culture was grown for about 12 h (within the exponential phase) before inoculating and an inoculum concentration of 5% was used.

There was a subsequent exponential growth phase realized from the 4th hour of incubation to the 36th hour for the 50/50 and 75/25 beverage mixtures, but for the 100% baobab, the growth increased to the 72 h. During this phase, the microorganism uptake nutrients (carbohydrates, nitrogen, vitamins and minerals) from the medium thereby excreting metabolic products (Ray, 2003). The growth rate is constant and is independent of substrate concentration (El-Mansi *et al*., 2012). The number of cells increased to 13.372±0.058, 11.335±0.062, and 9.559±0.051 Log cfu/mL for 50/50 mixture, 75/25 mixture and 100% baobab respectively. A diauxic growth was observed between the 24th and 28th h of fermentation due to a mixture of substrates. Diauxic growth is a biphasic exponential growth with intermittent lag phase (Chu and Barnes, 2016). This is as a result of generally two different types of sugars present in the medium. The microbe first metabolises the most efficient substrate and after its exhaustion, a lag phase is observed before the metabolism of the next substrate. The short lag phase is the time taken to produce enzymes for the metabolism of the second substrate (Aziza and Amrane, 2012).

The growth was highest with the 50/50 mixture, followed by 75/25 and then 100% baobab. This could be due to the presence of growth factors in lemongrass which favours the growth of the *Lb. fermentum*. *Lactobacillus fermentum* carries out fermentation through the heterofermentative metabolism, that converts sugar through the phosphoketolase pathway and gives rise to equimolar mixtures of lactic acid and ethanol or acetic acid and one molecule of ATP (Hayek and Ibrahim, 2013; Lubeck and Lubeck, 2019). Although carbohydrates are the most preferred source of energy, nitrogen, vitamins and minerals are required for both growth and product formation (Ray, 2003; Sauer *et al*., 2017). In most cases the presence of a nitrogen source is crucial for bacteriocin production (Abbasiliasi *et al*., 2017). Even though the reducing sugar content is higher in baobab juice than in lemongrass extract, the amino acid composition of lemongrass is higher than that of

baobab. Amino acids are nitrogenous source essential for the growth of LAB. The different amino acids are being catabolised through different pathways during fermentation (Fernandez and Zuniga, 2006). Hebert *et al*. (2000) evaluated the growth of *Lb. helveticus* CRL 1062 in specific growth mediums and they realized the amino acids; arginine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, or valine, lysine and serine were essential for growth because the microorganism was unable to grow in the absence of these amino acids. Nsogning *et al*. (2018) also noticed the depletion of amino acids, arginine, lysine and glutamic acid during malt wort fermentation with different strains of *Lb. plantarum*, *Lb. brevis* and *Lb. amylolyticus* caused cell death. The high amino acid composition of lemongrass therefore explains the higher rate of growth in the 50/50 mixture followed by 75/25 mixture and then 100% baobab juice. In addition, minerals and vitamins have been proven to be growth factors for LAB. Snell (1945) showed that the addition of vitamins and nitrogen source to growth medium boosted the growth of LAB and specifically *Lb. fermentum* grew best when the medium was supplemented with pantothenic acid, nicotinic acid, biotin and thiamine. LAB in general requires Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, K^{+}, and Na^{+} as essential or stimulatory factors for nutrient transportation and enzymatic activity (Hayek and Ibrahim, 2013). But iron is required in limited amount because lactic acid bacteria grow anaerobically, do not contain cytochrome, and are catalase-negative. All these minerals are present both in lemongrass and baobab but higher in lemongrass than in baobab (except potassium which is higher in baobab). Thus, the higher growth with mixtures containing higher concentration of lemongrass.

After 36 h, growth slows down to 72 h which can be due to nutrient depletion, production of toxic substances and presence of stress factors such as pH. However, the stationary phase had not yet been attained for the 100% baobab probiotic beverage. The biomass increases only gradually or

remains constant during this stationary phase, although the composition of the cells may change. Growth increased from 13.372±0.058 to 13.910±0.071 Log cfu/mL for 50/50 mixture and 11.335±0.062 to 11.878±0.049 Log cfu/mL for 75/25 baobab-lemongrass mixture. At the stationary phase, there is slow growth of survivors that get energy from new substrates and metabolites are formed, often of great biotechnological interest (Pumphrey and Julien, 1996). In this phase, microorganisms activate the stringent response mechanism for survival (Jaishankar and Srivastava, 2017) and thereby divert their resources away from growth toward synthesizing proteins to promote survival (Champomier-Verges *et al*., 2002; Jaishankar and Srivastava, 2017). Under stress conditions, LAB adapt metabolically by selection of alternative fates of pyruvate, the utilization of other carbon sources, the activation of the proteolytic system, and/or the catabolism of free amino acids (FAA) by deamination and decarboxylation (Papadimitriou *et al*., 2016).

4.3.2.2 Effect of fermentation on the reducing sugar content

Substrate utilization during the fermentation of baobab-lemongrass beverage is presented in figure 28. The initial sugar concentration varied for the three different beverage mixtures and the medium was not supplemented with sugar before fermentation. The reducing sugar content was highest in the 100% baobab than the other mixtures. Lemongrass extract is a poor source of reducing sugar and therefore a decrease in the sugar content when the baobab juice was mixed with lemongrass extract. The reducing sugar content decreased constantly as the biomass concentration increased during fermentation. This is because *Lb. fermentum* metabolise the sugar for growth. The percentage decrease was highest in the 100% baobab probiotic beverage because it was the principal nutrient source present in baobab while in the other mixtures, lemongrass supplemented with a nitrogen source also necessary for growth of LAB. The growth in the beverage without supplements implies that the beverage has a rich nutrient medium (especially

when mixed with lemongrass) for the growth of *Lb. fermentum* isolated from baobab. Since the *Lb. fermentum* was isolated from baobab, it was already accustomed to the matrix and thereby fermentation made easy.

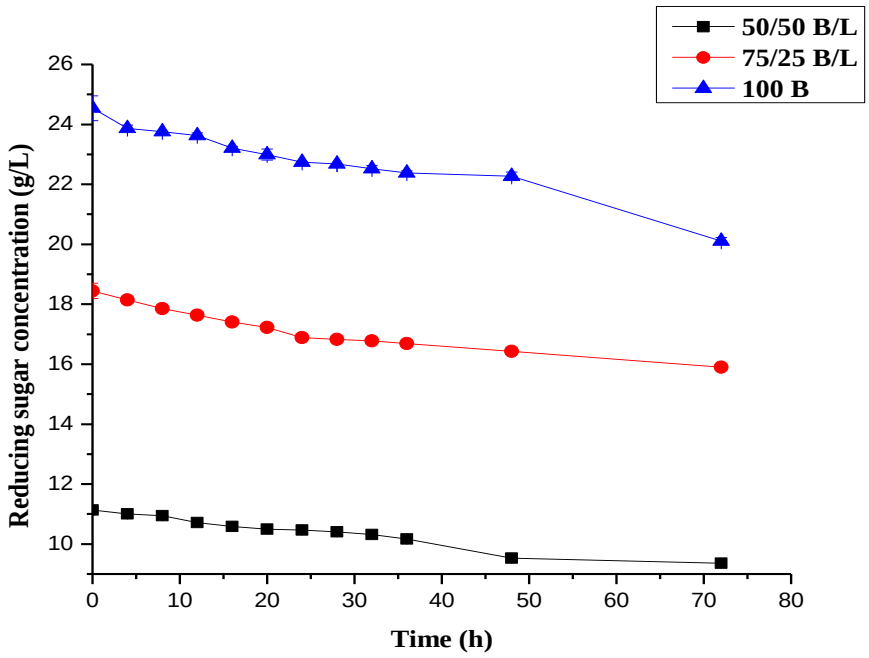


Figure 28: Evolution of reducing sugar content during fermentation of the baobab-lemongrass beverage

4.3.2.3 Effect of fermentation on pH

The initial pH of the different beverage concentrations was low and varied from one mixture to another. Low pH does not support growth of LAB and pH also influence the fermentation characteristics (Peng *et al*., 2021). The pH was therefore adjusted to a pH of about 6.6 to favour the growth of the *Lb. fermentum* in the beverage. For the 50/50 probiotic beverage, there was a sharp decline in the pH from the 4 – 24 h of growth and then slow decline to 72 h (figure 29). The sharp decrease was due to the fact that, at the beginning of fermentation, the medium was rich in nutrients and the metabolism of the nutrients release organic acids. After 24

h, there is nutrient depletion and therefore a decline in pH. While for 75/25 baobab-lemongrass probiotic beverage, there was a slight decrease from 4 – 28 h followed by a sharp decrease to 72 h. The slight decrease of pH could be as a result of the slow metabolism of nutrients in the medium. For the 100% baobab, there was a slow decrease from the 4 – 48 h. The decrease in pH is with respect to the production of organic acids due to growth characteristics of *Lb. fermentum* in the different baobab-lemongrass mixtures as explained above.

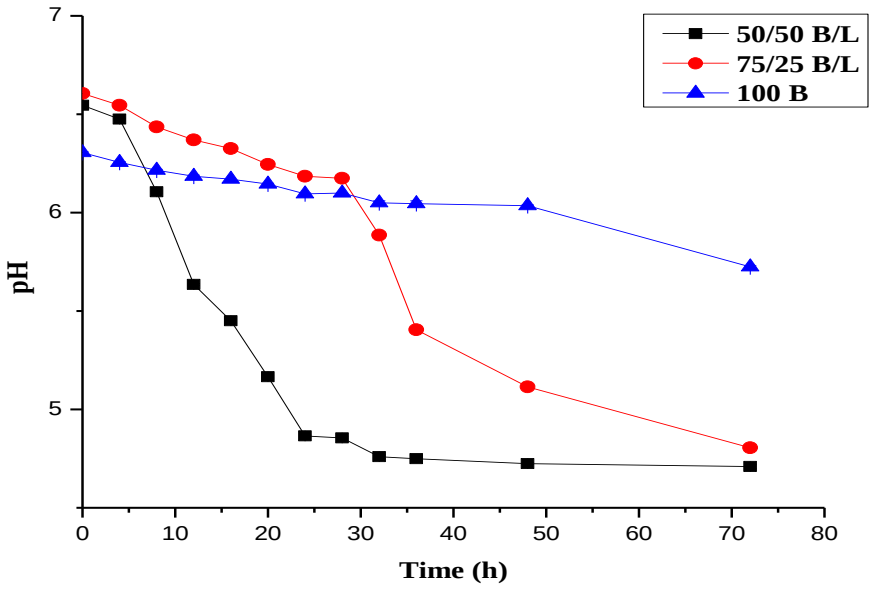
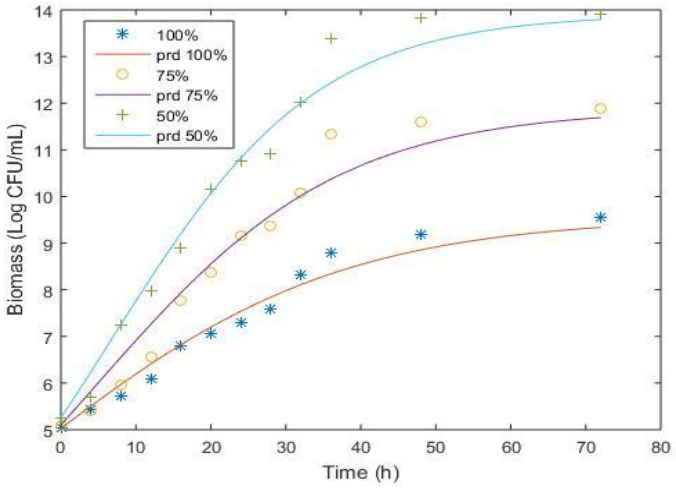


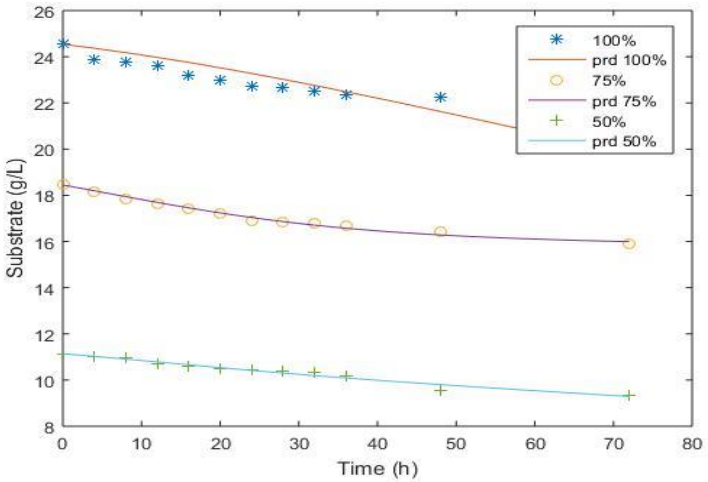
Figure 29: Evolution of pH during fermentation of the baobab-lemongrass

4.3.2.4 Determination of kinetic fermentation parameters of baobab-lemongrass probiotic beverage

The fermentation kinetics of baobab-lemongrass probiotic beverage were fitted to growth, substrate utilisation and product formation models (figure 30). Several growth models have been used to describe the sigmoidal curve of microorganisms among which are the Monod and Logistic models. The experimental data of the fermentation kinetics of biomass was thereby fitted with the Monod and Logistic models (table 30).



(a)



(b)

(c)

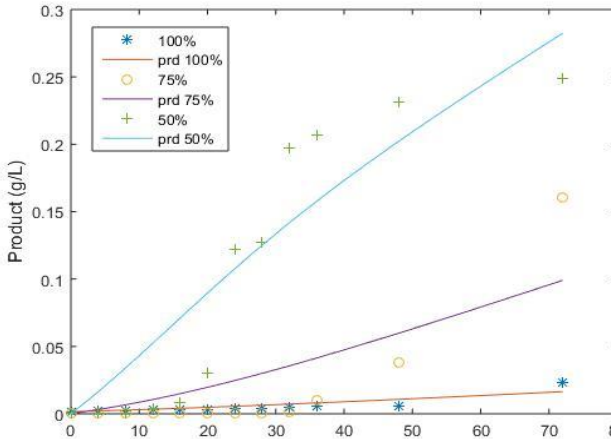


Figure 30: Fitting of fermentation kinetics to (a) Logistic model, (b) substrate utilization model and (c) product formation model

The R^2 of both models were above 0.80 which shows a good fit for both models. However, the Logistic model fitted best than the Monod model, because of its lower RSME values. The values of the maximum specific growth rate, μ_{max}, were generally low for all the cases but were found to be higher with the Logistic model than that of the Monod model. The 50/50 baobab-lemongrass probiotic fermented beverage had the highest μ_{max} (0.0731 h^{-1}) which indicates a suitable cell growth rate and its statistical analysis were best when compared with the other cases. Its R^2 was 0.9800 and RSME, 0.2580. When compared with μ_{max} values of similar or different LAB, it fell within the range of 0.0531 to 0.0769 h^{-1} for growth of *Bifidobacterium lactis* in cabbage juice (Buruleanu et al., 2014). Goranov *et al.* (2015) realised a μ_{max} value of 0,062 h^{-1} for Monod model and 0,0312 h^{-1} for logistic model for the growth of *Lactobacillus delbrueckii* ssp. bulgaricus B1 in a batch fermentation. Specifically, the μ_{max} values for growth of *Lb. fermentum* investigated were lower than those reported in literature. Charalampopoulos *et al*. (2002) obtained a μ_{max} of 0.62 ± 0.04, 0.43 ± 0.05 and 0.53 ± 0.05 in the growth of *Lb. fermentum* in malt, barley and wheat respectively. Ardestani *et al*. (2015) obtained μ_{max} of 0.134 and 0.417 h^{-1} for Monod and logistic models respectively for the growth of *Lb. fermentum* PTCC1744.

The Ks values were high when compared with other reported values (Buruleanu *et al*., 2014; Goranov *et al*., 2015) but lower than the value obtained by Ardestani *et al*. (2015) for *Lb. fermentum* PTCC1744. Ks is the substrate concentration when the specific growth rate is half of its maximum value (Nath *et al*., 2008). The Ks values increased with increase concentration of baobab in the mixture. This could be because of other growth nutrients lemongrass contributes during fermentation. The high Ks values suggest the sugar requirement of the *Lb. fermentum* is high and increases with increase in baobab concentration.

Table 30: Kinetic parameters of growth, substrate utilization and product formation models

Model	sample	μ_m (h^{-1})	X_m (cfu/mL)	R^2	RSME
Modified logistic	50/50	0.0731	13.9100	0.9800	0.2580
	75/25	0.0614	11.8780	0.9630	0.4640
	100%	0.0505	9.5590	0.9700	0.4230
		μ_m (h^{-1})	K_s (g/L)	R^2	RSME
Monod	50/50	0.0367	11.4909	0.9116	4.540
	75/25	0.0264	18.7721	0.8241	6.364
	100%	0.0273	26.0669	0.9431	4.746
		$Y_{X/S}$ (g/g)	m_s	R^2	RSME
Substrate utilisation	50/50	12.8620	0.0014	0.9610	0.1120
	75/25	2.9675	0.0003	0.9850	0.0949
	100%	2.7655e+02	0.0083	0.8470	0.4360
		α (g/g)	β (h^{-1})	R^2	RSME
Product formation	50/50	0.0173	0.0002	0.9340	0.0435
	75/25	0.0118	0.0038	0.9630	0.0093
	100%	0.0028	5.5017e-03	0.9070	0.0019

- **Substrate consumption kinetics**

During fermentation, microbes generally use up the available nutrients present in the medium and at the end multiply and synthesize products. The Leudeking-Piret equation best describes the substrate consumption model and the parameters determined are as shown in table 30. $Y_{X/S}$, the yield constant of microbial growth decreased from 50/50 baobab-lemongrass probiotic beverage to 100% baobab. The decrease could be because, the rate of sugar consumption depends on the growth rate, lactic acid production and the rate of substrate uptake for cell maintenance (Sharma

and Mishra, 2013). The maintenance energy refers to the energy consumed for functions other than synthesis of new cells (Pirt, 1965). The maintenance energy was higher for the 100% fermented baobab juice followed by the 50/50 and then the 75/25 baobab-lemongrass mixture. However, from the R^2 and RSME, the 75/25 mixture best fitted to the Leudeking-Piret model followed by the 50/50 mixture and then the 100% baobab. The low maintenance energy could be due to the stationary phase of the bacteria since it is associated to the growth rate of the bacteria (Pirt, 1965).

- **Kinetics of lactic acid production**

The Leudeking-Piret equation for product synthesis was used to fit the data to get the parameters of lactic acid production. The growth associated term, α, for all the cases was greater than the non-growth associated term, β and it also decreased with increase in concentration of baobab. This implies lactic acid production was growth associated since the β was close to zero. The growth and non-growth-associated constants vary with the substrate, product, temperature, and pH at optimal conditions (Ghimire *et al*., 2020). The R^2 values were greater than 0.90, meaning there is a good fit. In addition, the RSME values were low implying the errors were minimal. From the R^2 and RSME, the 75/25 mixture best fits the model.

These kinetic parameters are of great interest because they describe the fermentation process with respect to the environmental conditions. They are used in understanding and predicting the fermentation process ensuring process conditions are respected and which in turn reduce process cost. Furthermore, they are necessary for scaling up from laboratory scale to industrial scale and for designing bioreactors. The design of bioreactors takes in to account optimal operating conditions gotten from modelling of fermentation process.

4.3.3 Rheology of fermented baobab-lemongrass probiotic juice

Rheological study of food is generally important because rheological parameters help in the prediction of engineering parameters like heat and mass transfer, mixing, aeration of fluids, coefficients for product development, design and evaluation of manufacturing processes, as well as packaging and storage strategies (Diamante and Umemoto, 2015; Doran, 2013). The rheological behaviour of the different mixture concentrations of baobab-lemongrass before and after fermentation were therefore evaluated at 25 ℃ over a shear rate of 0.1-300 s^{-1}. The flow curves are as shown in figures (31 a and b).

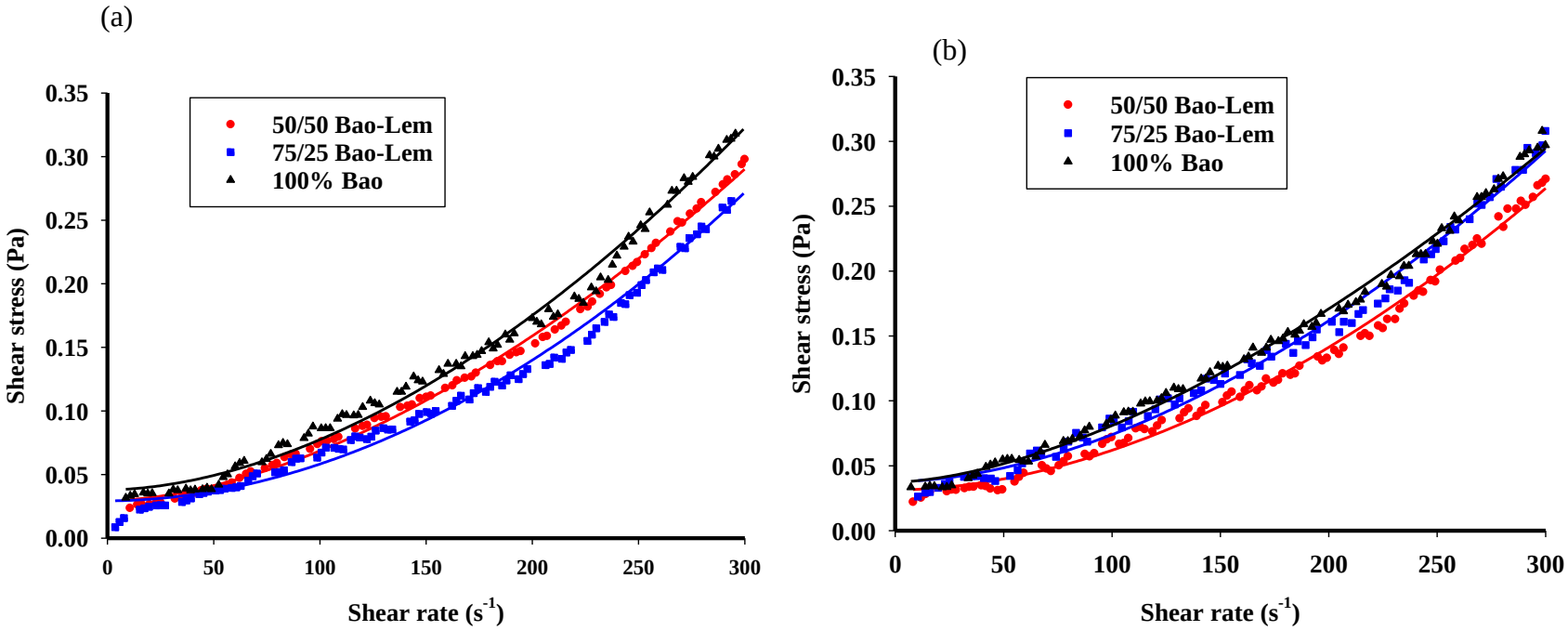


Figure 31: Rheogram of baobab-lemongrass mixture before (a) and after (b) fermentation at 25 °C

For each case, the shear stress increases with increasing shear rate. At high temperatures, there is the decrease in viscosity and is greatly seen in the flow behaviour of the beverage before and after fermentation (figures 31 a and b). The rheological properties of fermented beverages are affected by the presence of cells, substrates, products, and gas (Doran, 2013). Fermentation also modifies the structure of the product (Altay *et al*., 2013). This explains the change of the flow behaviour of the beverage after fermentation. The presence of biomass and exopolysaccharides released during fermentation led to increase in viscosity of the different beverages. The experimental data was fitted to the Herschel-Bulkley and power law models and the results compared with respect to their R^2 and RSME values. The data accurately fitted to the Herschel-Bulkley model for all cases than the power law model. The R^2 ranged from 0.9840 to 0.9971 and RSME, 0.0044 to 0.0092 for Herschel-Bulkley model while for power law model, the R^2 ranged from 0.9693 to 0.9835 and RSME, 0.0104 to 0.0138. The consistency index, K, which represents the level of viscosity, were low (table 31 and 32). This could be as a result of the effect of temperature on the flow behaviour of the fermented beverage. Intermolecular forces of foods greatly affect their viscosity due to the inter-molecular spacing and strength of hydrogen bonds of the foods. These bonds are affected by temperature (Deshmukh *et al*., 2013). A rise in temperature leads to a rise in thermal energy of the molecules which therefore cause a rupture of the bonds present in the juice thereby leading to molecular distancing due to the reduction of the intermolecular forces hence viscosity of fluid decreases (Borchani *et al*., 2019; Deshmukh *et al*., 2013; Santos *et al*., 2016). The K values also increased with increase baobab concentration. This could be as a result of soluble pectin present in the baobab juice due to the fact that a partial hydrolysis was done on the baobab pulp with a degree of hydrolysis of 74.04%. The value increased after fermentation due to increase in consistency of the product. The juice presents a

non-Newtonian flow behaviour and the values of the flow behaviour index, n, are greater than one which implies it is a shear-thickening fluid. Yield stress (τ_0) is defined as the force a fluid must be exposed to in order to start flowing (Björn *et al*., 2012). The values of the yield stress are low which implies the beverage will require a minimum stress to flow. Yield stress is also an important parameter to consider during mixing because it affects the physico-chemical characteristics of the fluid and impede flow even at relative low stresses (Björn *et al*., 2012). Beverages with Pseudoplastic behaviour flow easily than those with dilatant behaviour.

Table 31: Rheological parameters of Herschel-Bulkley model of baobab-lemongrass juice before and after fermentation

	K	*n*	τ_0	R^2	RSME
Baobab-lemongrass beverage before fermentation (25 °C)					
50/50	1.3935E-5	1.7246	0.0294	0.9971	0.0044
75/25	3.8882E-6	1.9357	0.0293	0.9840	0.0092
100%	1.0014E-5	1.7979	0.0379	0.9889	0.0092
Baobab-lemongrass beverage after fermentation (25 °C)					
50/50	6.1724E-6	1.8473	0.0314	0.9940	0.0056
75/25	1.0059e-5	1.7779	0.0378	0.9909	0.0076
100%	2.5439e-5	1.6168	0.0374	0.9950	0.0056

Table 32: Rheological parameters of Power law model of baobab-lemongrass juice before and after fermentation at 25 °C

	K	*n*	R^2	RSME
Baobab-lemongrass before fermentation				
50/50	0.0002	1.2803	0.9835	0.0104
75/25	0.0002	1.2951	0.9734	0.0113
100%	0.0003	1.2138	0.9728	0.0138
Baobab-lemongrass after fermentation				
50/50	0.0002	1.2853	0.9735	0.0117
75/25	0.0003	1.1947	0.9693	0.0138
100%	0.0005	1.1126	0.9764	0.0120

4.3.4 Effect of fermentation on bioactive composition of probiotic beverage

The baobab-lemongrass beverage before and after fermentation with autochthonous *Lb. fermentum* was assayed for TPC, antioxidant (DPPH, FRAP, and ABTS) activity and colour and the results presented in figures 32 and 33. Generally, compared to the un-started juice, fermentation had a significant increase ($p < 0.05$) on the phytochemical component of the juice.

Phenolic compounds have antioxidant properties and are able to prevent many diseases such as cancer, cardiovascular and degenerative diseases when consumed in adequate amounts in food (Rodríguez *et al.*, 2009). They inhibit oxidative processes that promote spoilage and hence extend shelf life of foods (Veron *et al*., 2017). There was an increase in the TPC of the fermented juice when compared to the juice before fermentation. The percentage increase was highest with the 75/25 (33.83%) mixture followed by 50/50 (31.19%) and then 100% (8.12%) baobab probiotic beverage. Wijayanti *et al*. (2017) stated that increase in TPC after fermentation depends on the

type of starter used. The autochthonous *Lb. fermentum*, which is already adapted to the intrinsic characteristics of the baobab, could therefore contribute in increase in the polyphenol content of the juice after fermentation.

The principle of ABTS, DPPH and FRAP assay is based on electron transfer and involves reduction of a coloured oxidant. These assays are limited as they use non-physiological radicals and therefore two or three methods are necessary to evaluate the antioxidant activity of a sample (Floegel *et al*., 2011). The ABTS assay is based on the generation of a blue/green $ABTS^{+}$ that can be reduced by antioxidants (Re *et al*., 1999); whereas the DPPH assay is based on the reduction of the purple DPPH to 1,1-diphenyl-2-picryl hydrazine (Floegel *et al*., 2011). The FRAP assay is based on the reduction of ferric iron ($Fe^{3+)}$ to ferrous iron (Fe^{2+}) resulting in colour development indicating the presence of reductant/antioxidant (Benzie and Strain, 1996).

Generally, the antioxidant activity of the fermented beverage increased significantly when compared to the control. The percentage increase of the antioxidant activity was highest with the 50/50 mixture (41.14, 44.46, 82.38% respectively for DPPH, FRAP and ABTS), followed by 75/25 mixture (25.99, 17.63, 47.76% respectively for DPPH, FRAP and ABTS) and then the 100% baobab (11.40, 4.89, 4.18% respectively for DPPH, FRAP and ABTS) probiotic beverage. The increase in the phytochemical composition can be attributed to the release of bioactive compounds (Hur *et al*., 2014; Septembre-Malaterre *et al*., 2018) such as exopolysaccharides during fermentation that enhance the antioxidant activity of the foods. Fermentation at 50/50 baobab-lemongrass beverage was better than the other substrate composition which could be the reason for the high percentage increase in the antioxidant activity. LAB have antioxidant properties as they display metal chelating ability (Hur *et al*., 2014). The increase could also be because of variations of the phenolic compound profiles (Di Cagno *et al*., 2016) in the baobab and lemongrass.

These results are in agreement with the findings of Veron *et al.* (2017) who also noticed an increase in the antioxidant activity of cactus (*Opuntia ficus-indica*) fruit juice fermented using autochthonous starters but that fermented with allochtonous *Lb. fermentum* ATCC 9338, resulted in a decrease of about 30% in antioxidant activity of juice (Panda et al., 2017). There was an increase in antioxidant property of pineapple (*Ananas comosus* L. Merr.) fermented with autochthonous starters (Di Cagno *et al*., 2010). There was no difference in the TPC of unstarted and started leek (*Allium ampeloprasum* var. porrum) fermented with autochthonous lactic acid bacteria starter but there was an increase in the antioxidant activity (Wouters *et al*., 2013). Fermentation of quinoa flour with selected autochthonous lactic acid bacteria also led to an increase in antioxidant activity (Rizzello *et al*., 2017).

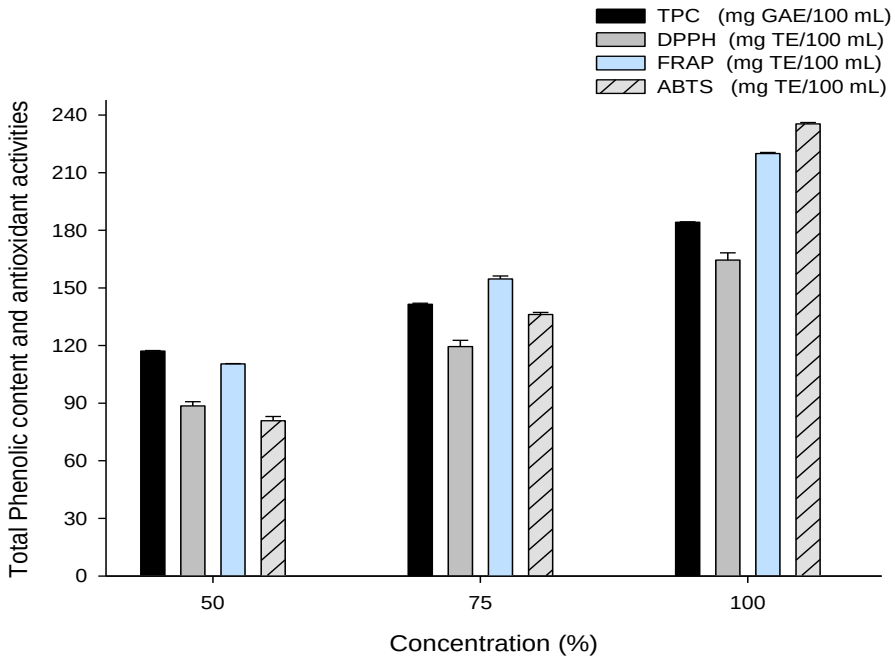


Figure 32: TPC and antioxidant activity of baobab-lemongrass beverage before fermentation

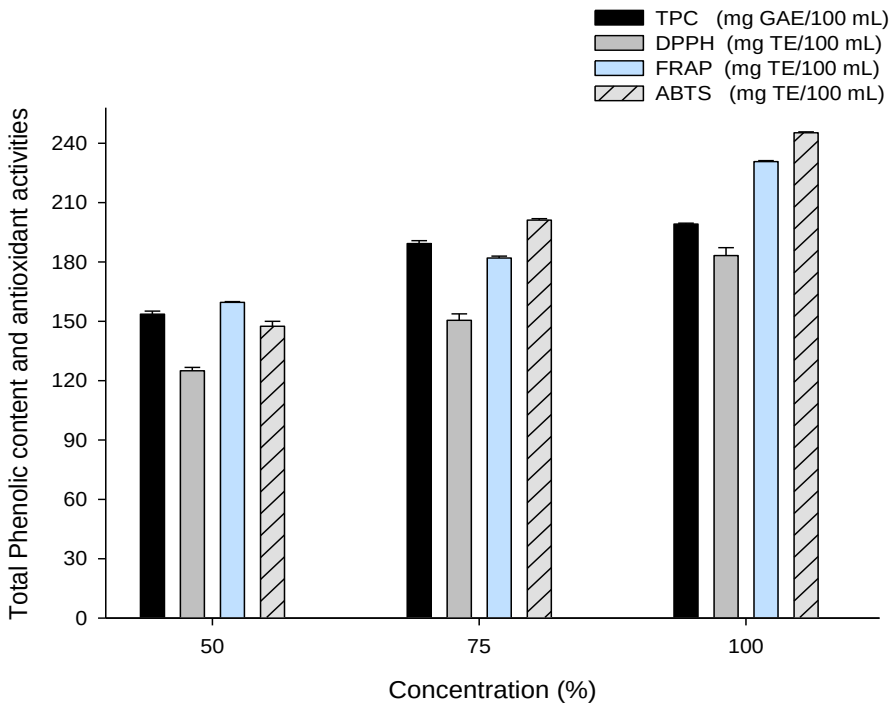


Figure 33: TPC and antioxidant activity of baobab-lemongrass juice after fermentation

4.3.5 Effect of fermentation on colour of probiotic beverage

The colour of the non-fermented and fermented juice analysed with respect to (L*, a* and b*) and the total colour difference (ΔE) results are presented in table 33. There was a decrease in L* and an increase in a*, b* and ΔE for fermented 50/50 baobab-lemongrass beverage and 100% baobab compared to the non-fermented juice. While no difference in L* and a* but decrease in b* and ΔE for 75/25 fermented baobab-lemongrass beverage compared to the non-fermented beverage. The decrease in the brightness (L*) is due to the increase in the biomass *of Lb. fermentum* in the juice thereby resulting in a turbid juice. In the course of the fermentation, the redness and yellowness of the juice increase as the pH decreases. ß-carotene is the main colour pigment present in baobab pulp powder (Aluko et al., 2016) and in lemongrass, there is ß-carotene, chlorophyll a and b (Thorat *et al*., 2017). The increase in the colour ΔE is due to the significant decrease in the lightness and the change in the carotenoid and chlorophyll pigment in the samples.

Table 33: Colour of the different concentrations of baobab-lemongrass probiotic beverage before and after fermentation

	Before fermentation				After fermentation			
Sample	**L***	**a***	**b***	**dE*ab**	**L***	**a***	**b***	**dE*ab**
50%	92.34±0.29	1.63±0.03	27.28±0.07	28.37±0.12	86.60±0.10	4.44±0.02	34.31±0.07	37.11±0.10
75%	90.39±0.16	2.67±0.01	32.25±0.04	33.76±0.03	90.62±0.24	2.63±0.03	28.76±0.07	30.38±0.14
100%	97.58±0.09	-0.47±0.00	9.72±0.02	10.02±0.01	89.64±0.06	3.25±0.02	24.88±0.06	27.14±0.08

4.3.6 Effect of fermentation on organic acid content

During fermentation, organic acids are produced while some are used in the fermentation pathway. The organic acid profile of the juice before and after fermentation is as shown in table 34. For the 50/50 and 75/25 baobab-lemongrass probiotic beverage, the organic content increased significantly ($p < 0.05$) after 36 h of fermentation. This was also confirmed with the high sum of aroma compounds contributed by acids (section 4.3.7). Oxalic acid was not present in the juice which is good because it is considered an antinutrient. It reduces the bioavailability of calcium (Rodrigo *et al*., 2012). There was the production of both lactic and acetic acid attributing to the heterofermentative nature of *Lb. fermentum*. The lactic acid concentration present in the fermented beverages were greater than the concentration of acetic acid and the lactic acid concentration decreased from the 50% to the 100% baobab. The difference could be because of the composition of the medium that affects the production of organic acids (Nguyen *et al*., 2019). Lactic acid improves the savouriness taste and texture of fermented foods whereas acetic acid gives a vinegar-like taste and aroma (Bujna *et al*., 2018). However, Xiong *et al.* (2014) stated that moderate amount of acetic acid improves the stability and organoleptic quality of fermented foods and the concentration of acetic acid present in the baobab-lemongrass fermented juice is not high. Lactic acid bacteria are also known to metabolize citric acid, by the enhancement of ATP production via the acetate kinase pathway producing lactic acid, diacetyl, acetoin and acetic acid (Bujna *et al*., 2018; Xiong *et al*., 2014). Malic acid can also be decreased in the course of fermentation by malolactic enzymes in LAB (Xiong *et al*., 2014). This explains the decrease in the concentration of citric and malic acid after fermentation for 100% baobab juice. The concentration of citric and malic acid increased for 50/50 baobab-lemongrass fermented probiotic juice. While for 75/25 baobab-lemongrass fermented juice, the concentration of malic acid increased and that of citric

acid decreased. This could be attributed to the concentration of lemongrass present, which was greater in the 50/50 than in the 75/25 baobab probiotic fermented beverage. These organic acids are important because of their antimicrobial properties. Acetic acid, lactic acid, and propionic acid exert the most effective antimicrobial properties (Anyasi *et al.*, 2017). Some organic acids are short-chain fatty acids (SCFAs) like acetic and propionic acids (Ricke, 2003). The presence of these acids is essential for human health because they maintain intestinal and immune homeostasis in the human body. SCFAs play a very important role in regulating pH, increasing the absorption of calcium, iron, magnesium, and are beneficial for glucose and protein metabolism in the liver (Markowiak-Kopeć and Śliżewska, 2020). These acids in the juice are therefore important because they act as preservatives thereby limiting the use of synthetic preservatives which are detrimental to humans.

Table 34: Organic acid profile of baobab-lemongrass beverage before and after fermentation

Organic acid	50/50 before (g/L)	50/50 after (g/L)	75/25 before (g/L)	75/25 after (g/L)	100% before (g/L)	100% after (g/L)
D-galacturonic acid	9.622±0.017[a]	11.652±0.236[b]	14.523±0.188[a]	15.641±0.053[b]	22.760±0.403[a]	18.995±0.228[b]
Oxalic	ND	ND	ND	ND	ND	ND
DL-Tartaric	0.731±0.003[a]	0.967±0.020[b]	1.006±0.009[a]	1.409±0.039[b]	1.628±0.148[a]	1.918±0.010[b]
L-malic	0.414±0.004[a]	1.370±0.018[b]	0.628±0.012[a]	0.967±0.053[b]	1.252±0.056[a]	1.002±0.004[b]
Isocitric	0.408±0.017[a]	1.028±0.022[b]	0.498±0.007[a]	1.289±0.047[b]	1.608±0.014[a]	1.406±0.031[b]
L-Ascorbic	0.023±0.002[a]	0.034±0.001[b]	0.031±0.003[a]	0.035±0.001[a]	0.025±0.001[a]	0.021±0.004[a]
Lactic	ND	0.281±0.006	ND	0.170±0.021	ND	0.080±0.001
Acetic	ND	0.096±0.004	ND	0.070±0.001	ND	0.058±0.005
Citric	0.840±0.001[a]	1.336±0.025[b]	1.230±0.224[a]	0.850±0.008[b]	1.891±0.146[a]	1.665±0.002
Succinic	0.191±0.266[a]	11.618±0.456[b]	0.142±0.001[a]	1.258±0.001[b]	0.112±0.002[a]	0.116±0.009[b]
Propionic	10.366±0.025[a]	11.652±0.236 [b]	5.232±0.054[a]	5.465±0.020[a]	0.159±0.117[a]	0.218±0.010[b]
Glutaric	0.111±0.017[a]	0.062±0.006[b]	0.116±0.001[a]	0.141±0.003[b]	0.097±0.012[a]	0.219±0.033[a]

ND-Not detected

Values in the same row with different superscript letters differ significantly ($P < 0.05$)

4.3.7 Effect fermentation on the aroma profile

Volatile compounds were determined both for non-fermented and fermented probiotic beverage and grouped according to the following class: acids, aldehydes, terpenes, ketones, alcohols, and other compounds (table 35). For the 50/50 baobab-lemongrass beverage before fermentation, a total of 25 aroma compounds were detected. Six acid compounds with a sum of 12.42±0.88, 4 aldehydes (21.81±5.88), 5 terpenes (42.75±9.34), 3 ketones (17.26±6.92), alcohols (2.02±0.28), and 3 other compounds (3.74±0.63). While for 50/50 baobab-lemongrass fermented probiotic beverage, a total of 29 compounds were also detected: 10 acids (21.17±0.88), 7 aldehydes (18.79±4.55), 3 terpenes (22.99±6.43), 2 ketones (5.71±0.08), 6 alcohols (28.95±3.52), and one other compounds (2.77±1.81). For the 75/25 baobab-lemongrass beverage before fermentation, a total of 16 aroma compounds were identified. Six acids (17.72±1.36), 4 aldehydes (17.82±3.95), 3 terpenes (33.81±9.30), one ketone (11.10±0.25), one alcohol (16.17±0.91), and one other compound (3.38±1.41). With respect to the fermented beverage, 27 aroma compounds were identified. Ten acids (38.53±3.58), 5 aldehydes (10.60±0.56), 3 terpenes (24.92±8.82), one ketone (1.67±0.03), 6 alcohols (19.78±2.42), and two other compounds (4.49±0.31). For the 100% baobab beverage before fermentation, 17 aroma compounds were identified. Seven acids (62.37±6.81), 5 aldehydes (22.30±4.33). One ketone (1.97±0.11), and 4 other aroma compounds (18.23±1.11). No alcohol and terpenes were detected. While for the fermented beverage, 29 aroma compounds were identified. Ten acids (61.58±6.39), 8 aldehydes (12.75±1.23), 3 ketones (6.16±0.91), 7 alcohols (15.91±1.39) and one other compound (3.60±1.86). No terpene compound was also detected. Generally, the number of aroma compounds increased after fermentation. This is because, during fermentation, lactic acid bacteria synthesize flavours as secondary metabolites by using flavour precursors present in the medium. The type of amino acids and sugars present in the medium

influence the type of flavour compounds formed (Petrovici and Ciolacu, 2018). The increase in the flavour compounds contributed by acids after fermentation could be because *Lb. fermentum* at the end of fermentation, release organic acids into the medium which therefore imparts on the aroma of the juice. However, baobab pulp is naturally acidic and that could be the reason of the high acidic content before fermentation. Some aldehydes were developed after fermentation while others were lost. Terpenes were only identified in the samples containing lemongrass because they are the principal flavour compounds responsible for the aroma of lemongrass. 2.3-Butanedione is a diacetyl with a buttery flavour. It has also been identified in other lactic acid fermented juice like apple juice (Wu *et al*., 2020), and pomegranate juice (Di Cagno *et al.*, 2017). Alcohols have also been identified in other lactic acid fermented juice (Szutowska, 2020).

Table 35: Aroma profile of non-fermented and fermented baobab-lemongrass beverage

Aroma compounds	RT	% Composition 50/50	% Composition F-50/50	% Composition 75/25	% Composition F-75/25	% Composition 100%	% Composition F-100%
Acids		**12.42±0.88**	**21.17±0.88**	**17.72±1.36**	**38.53±3.58**	**62.37±6.81**	**61.58±6.39**
L-Lactic acid	12.04	ND	0.59±0.00	1.56±0.04	ND	ND	ND
Acetic acid	19.47-19.52	ND	2.85±0.07	ND	2.55±0.35	ND	2.75±0.48
Hexanoic acid	33.40-35.24	ND	2.27±1.03	ND	1.04±0.22	3.62±1.19	1.91±0.03
Heptanoic acid	35.23	ND	1.42±0.72	ND	ND	ND	1.41±0.02
Octanoic acid	36.69-36.70	2.70±0.64	3.86±0.64	4.51±1.88	3.64±0.35	12.42±5.44	5.42±0.78
Nonanoic acid	37.93-37.94	ND	2.39±1.23	ND	1.87±0.53	4.79±0.12	2.92±0.98
n-Decanoic acid	39.04-39.05	3.45±0.77	ND	ND	1.06±0.23	ND	1.62±0.82
Neric acid	39.21-39.23	2.18±0.37	1.60±0.13	1.32±0.47	ND	ND	ND
Geranic acid	39.65	ND	1.97±0.34	3.46±1.94	1.46±0.39	ND	ND
Undecanoic acid	40.06-40.07	1.78±0.48	ND	ND	ND	4.41±1.65	ND
2-Hydroxy-1-hexadecanoic acid	40.30	ND	ND	ND	ND	4.87±0.44	ND
Dodecanoic acid	41.01-41.03	1.70±0.51	1.45±0.79	2.15±0.73	2.74±1.16	8.19±2.88	5.12±2.22
Octadecanoic acid	42.40-42.90	0.60±0.05	ND	ND	13.56±3.73	24.07±4.38	23.87±0.13
Tetradecanoic acid	43.09-43.10	ND	2.77±1.49	4.72±1.14	4.40±2.09	ND	8.73±0.25
cis-13-Octadecenoic acid	42.48-43.23	ND	ND	ND	6.22±1.61	ND	7.83±0.50
Aldehyde		**21.81±5.88**	**18.79±4.55**	**17.82±3.95**	**10.60±0.56**	**22.30±4.33**	**12.75±1.23**
3-Methyl-butanal	1.90	ND	1.09±0.14	ND	ND	ND	ND
Acetaldehyde	2.41	ND	0.71±0.00	ND	ND	ND	ND
Hexanal	3.73-6.11	ND	0.62±0.19	2.94±0.02	2.77±0.37	ND	4.52±0.21
Pentanal	1.87-5.85	5.62±1.21	ND	ND	1.47±0.36	13.09±0.00	ND
Heptanal	4.40-8.02	0.50±0.11	0.57±0.01	ND	ND	ND	0.93±0.04
2,2-Dimethylocta-3,4-dienal	21.05	0.70±0.17	ND	ND	ND	ND	ND
2-Decenal, (E)-	27.12-27.14	ND	1.03±0.47	ND	ND	2.00±0.08	1.20±0.02
Citral	29.97-30.17	14.99±2.12	13.82±3.08	11.22±0.01	2.15±0.58	2.75±0.15	ND
2-Undecenal	30.98	ND	0.95±0.13	1.42±0.86	1.39±0.25	ND	1.10±0.01
2,4-Decadienal	32.50-32.51	ND	ND	ND	ND	1.97±0.22	077±0.02
9-Octadecenal	36.99-37.34	ND	ND	2.23±0.81	1.08±0.31	2.48±0.76	
DodecanaL	37.71	ND	ND	ND	ND	ND	0.82±0.11
9,17-Octadecadienal, (Z)-	37.75	ND	ND	ND	ND	ND	2.52±0.01
cis-11-Hexadecenal	39.09	ND	ND	ND	ND	ND	0.89±0.09
Terpenes		**42.75±9.34**	**22.99±6.43**	**33.81±9.30**	**24.92±8.82**	**0**	**0**
D-Limonene	7.82	0.67±0.15	ND	ND	ND	ND	ND

3,7-dimethyl-1,3,7-Octatriene	9.57	0.55±0.15	ND	ND	ND	ND	ND
Linalyl acetate	23.92-24.03	15.82±0.25	4.63±1.54	7.85±0.97	3.12±1.26	ND	ND
Citronellol	31.75-31.81	2.36±1.39	1.76±0.06	1.97±0.34	1.07±0.16	ND	ND
Geraniol	33.55-33.58	23.36±8.38	16.60±3.98	23.98±8.73	20.736±76.35	ND	ND
Ketones		**17.26±6.92**	**5.71±0.08**	**11.10±0.25**	**1.67±0.03**	**1.97±0.11**	**6.16±0.91**
2,3-Butanedione	3.31	ND	2.93±0.78	ND	ND	ND	3.21±1.28
6-Methyl-5-hepten-2-one	13.72-13.97	15.54±1.90	2.77±1.81	11.10±0.25	1.67±0.03	ND	ND
2-Nonanone	15.63	0.56±0.02	ND	ND	ND	ND	ND
2-Undecanone	25.32	1.16±0.34	ND	ND	ND	ND	ND
n-Octyl phenyl ketone	35.90	ND	ND	ND	ND	ND	0.98±0.08
2-Dodecanone	38.77	ND	ND	ND	ND	1.97±0.11	ND
2-Nonadecanone	38.80	ND	ND	ND	ND	ND	1.96±0.47
Alcohol		**2.02±0.28**	**28.95±3.52**	**16.17±0.91**	**19.78±2.42**	**0**	**15.91±1.39**
Ethanol	2.80	ND	2.69±0.13	ND	2.65±0.13	ND	5.26±0.42
2-propyl-1-Heptanol	5.80	ND	ND	ND	2.41±0.33	ND	ND
2-methyl-1-Pentanol	6.60	ND	ND	ND	ND	ND	1.10±0.13
1,3-dichloro-2-Propanol	9.51	ND	ND	ND	ND	ND	2.97±0.42
6-methyl-5-Hepten-2-ol	20.36-20.38	0.73±0.28	2.65±0.15	ND	1.22±0.29	ND	ND
3,7-dimethyl-1,6-Octadien-3-ol	24.01-24.03	ND	ND	ND	3.62±2.23	ND	
Verbenol	24.24	1.30±0.40	ND	ND	ND	ND	ND
2-methyl-6-methylene-, (E)-3,7-Octadien-2-ol	26.934	ND	0.77±0.25	ND	ND	ND	
1-Nonanol	28.14	ND	ND	ND	ND	ND	1.26±0.04
cis-Verbenol	28.32-28.37	ND	6.00±2.54	ND	ND	ND	ND
2,6-dimethyl-1,5,7-Octatrien-3-ol	30.94	ND	0.65±0.08	ND	ND	ND	ND
3,7-dimethyl-2,6-Octadien-1-ol	32.53-32.65	ND	11.57±1.40	16.17±0.91	8.41±1.36	ND	ND
11-Hexadecyn-1-ol	33.862	ND	ND	ND	ND	ND	2.11±0.17
1-Hexadecanol	35.713	ND	ND	ND	ND	ND	0.98±0.03
3-Methyl-1-dodecyn-3-ol	37.353	ND	ND	ND	1.47±0.40	ND	ND
9-Tetradecen-1-ol, acetate, (E)-	37.754	ND	ND	ND	ND		2.22±0.31
Others		**3.74±0.63**	**2.39±0.13**	**3.38±1.41**	**4.49±0.31**	**18.23±1.11**	**3.60±1.86**
Ethyl Acetate	2.319	ND	ND	ND	ND	6.15±1.05	ND
2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	10.40	2.04±0.52	ND	ND	ND	ND	ND
4,5-dimethyl-2,6-Octadiene	11.54	0.50±0.11	ND	ND	ND	ND	ND

1,1'-oxybis-Octane	31.43-31.47	1.19±0.24	2.39±0.13	3.38±1.41	1.93±0.32	4.10±1.20	3.60±1.86
2-heptyl-1,3-Dioxane	41.25	ND	ND	ND	ND	3.11±1.28	ND
Geranyl vinyl ether	42.73	ND	ND	ND	2.56±0.98	62.37±6.81	ND

F = Fermented sample

4.3.8 Effect of storage on fermented probiotic baobab-lemongrass beverage

The probiotic baobab-lemongrass beverage was fermented with the pH adjusted to about 6 for 50/50, 75/25 and 100/0 baobab-lemongrass beverage. After 36 h of fermentation at 37 ^{o}C, the *Lb. fermentum* count in the fermented beverage was 11.09±0.35, 10.34±0.25, and 6.31±0.05 respectively for 50/50, 75/25 and 100/0 baobab-lemongrass probiotic fermented beverage (table 36).

Table 36: *Lb. fermentum* count in fermented baobab-lemongrass beverage stored at 4 ^{o}C and 28±2 ^{o}C

Week	**50/50**	**75/25**	**100%**			
0	11.09±0.35	10.34±0.25	6.96±0.05			
	C-50/50	**R-50/50**	**C-75/25**	**R-75/25**	**C-100%**	**R-100%**
1	10.52±0.71^{a}	9.66±0.92^{a}	9.39±0.09^{b}	8.33±0.27^{b}	6.37±0.10^{c}	6.31±0.51^{c}
2	10.01±0.39^{a}	9.15±0.49^{a}	9.04±0.52^{b}	6.73±0.05^{c}	5.87±0.09^{d}	5.22±0.08^{e}
3	9.19±0.39^{a}	8.61±0.11^{a}	8.35±0.32^{b}	7.24±0.06^{b}	5.98±0.08^{c}	5.29±0.14^{c}
4	8.31±0.29^{a}	8.56±0.11^{a}	7.65±0.28^{b}	6.68±0.58^{b}	4.79±0.38^{c}	4.67±0.33^{c}

Different superscript letters in the same row within same substrate concentration indicate significant difference ($p \leq 0.05$).

The respective beverages were stored at 4 ^{o}C and at room temperature (28±2 ^{o}C) for 4 weeks and the results presented in table 37. Generally, there was decrease in the *Lb. fermentum* count with storage time. However, there was no significant difference ($p < 0.05$) between the different beverage types stored at 4 and 28±2 ^{o}C for the different storage weeks but for week 2 which there was a difference ($p > 0.5$) for 75/25 and 100% baobab fermented beverage. However, the juice stored at room temperature were contaminated by yeast and mould after the second week of storage. At the end of the storage time the viable cell count ranged from 6.68±0.58 to 8.56±0.11

for 50/50 and 75/25 baobab beverage mixture but lower for 100% baobab fermented beverage. The range 6 – 8 Log cfu/mL falls within the acceptable probiotic range for consumption of fermented probiotic products for maximum health benefits (Pereira *et al*., 2011). These results are in accord with that of Gupta and Bajaj (2017) who also realized a decrease in the viability of *Lb. plantarum* in fermented oat flour beverages stored at 4 ^{o}C and room temperature. The viable cell counts of *L. acidophilus, L. plantarum, L. casei*, and *L. delbrueckii* decreased after 4 weeks of cold storage at 4 ^{o}C in fermented mango juice (Reddy *et al*., 2015).

Table 37: pH variation of the different baobab-lemongrass fermented probiotic beverage stored at 4 ^{o}C and 28±2 ^{o}C

Week	**50/50**	**75/25**	**100%**			
0	4.84	4.90	5.69			
	C-50/50	**R-50/50**	**C-75/25**	**R-75/25**	**C-100%**	**R-100%**
1	4.81	4.84	4.90	4.91	5.66	5.64
2	4.84	4.73	4.45	4.85	5.25	5.34
3	4.23	4.45	4.00	4.63	4.99	5.01
4	3.99	4.01	3.80	4.02	4.63	4.89

The decrease in viability could be because of depletion of nutrients and decrease in pH (table 37), accumulation of organic acids, inhibitors produced during fermentation and storage temperature (Moghaddam *et al*., 2018). Optimal temperature for growth of LAB is 37 ^{o}C, when kept at temperature below the growth temperature, the cells die. This is due to physiological changes such as a decrease in membrane fluidity and a stabilization of secondary structures of RNA and DNA resulting in a reduced efficiency of translation, transcription and DNA replication (van de Guchte *et al*., 2002). In order to survive the changes, microorganisms have developed a cold-shock

response. This includes synthesizing cold induced proteins (Papadimitriou *et al*., 2016; van de Guchte *et al*., 2002); to improve membrane fluidity, the proportion of shorter and/or unsaturated fatty acids in the lipids are increased; and for DNA supercoiling, negative supercoiling is reduced and transcription and translation needed for cellular adaptation (van de Guchte *et al.*, 2002).

Partial conclusion

The probiotic fermented baobab-lemongrass beverage was successfully produced, where the biomass concentration at the end of fermentation was more than 10^6 cfu/mL, which is the limit of viability of probiotics. The fermentation data was fitted with growth, substrate utilisation and product formation models and the $R^2 \geq 0.8$ and RSME < 1 (except Monod model) for all the cases. The 50/50 baobab-lemongrass probiotic beverage showed a good fit with the Logistic model and the model parameters were μ_{max} 0.0731 h^{-1} and X_m 13.9100. While the 75/25 baobab-lemongrass probiotic beverage fitted the substrate utilisation and product formation models. The substrate utilisation parameters were $Y_{x/s}$ 2.9675 g biomass/ g glucose and m_s 0.0003 g glucose h^{-1} g^{-1} biomass and the product formation parameters, α, 0.0118 g lactic acid g^{-1} biomass and β, 0.0038 g lactic acid g $biomass^{-1}$ h^{-1}.

The fermentation was dependent on the nutrient composition of the medium. The samples mixed with lemongrass extract (50/50 and 75/25 fermented baobab-lemongrass probiotic beverage) presented better results than the 100% fermented baobab beverage. The probiotic 50/50 and 75/25 baobab-lemongrass fermented beverage presented a *Lb. fermentum* count of 7.65-8,31 Log cfu/mL after 28 days of storage at 4 ^oC. The beverage developed unfavourable microbes upon storage after 2 weeks at room temperature. Thus, the probiotic-lemongrass probiotic beverage is a functional probiotic beverage, good for consumption and should be stored at 4 ^oC.

General conclusion and perspectives

This work had as general objective to produce a probiotic fermented beverage from baobab -lemongrass extracts using autochthonous LAB from baobab pulp.

The optimum pectin hydrolysis conditions determined were pectinase concentration, 0.2% v/v and time 120 min at temperature 50 ^{o}C. The partial hydrolysis of the baobab pectin increased the reducing sugar content of the baobab juice from 11.84 g/L to 25.23 g/L at the optimum conditions. The extracted juice served as carbon source during fermentation.

Lemongrass made up of high protein content (8.39±0.05 g/100g DW) and high amino acid content was extracted by decoction and the optimum conditions were lemongrass concentration, 5 g/100 mL, temperature, 93.8 ^{o}C and time, 11.3 min. The optimum yielded a TPC of 71.98 mg GAE/100 mL extract and DPPH activity 80.63 mg TE/100 mL.

The fermentation of baobab and lemongrass juice yielded a *Lb. fermentum* growth of 13.910, 11.878, and 9.559 log cfu/mL respectively for 50/50, 75/25 and 100/0 baobab-lemongrass mixtures after 72h of fermentation. The *Lb. fermentum* count was above the range 6-8 log cfu/mL of minimum probiotic concentration. This implies the baobab-lemongrass beverage can be considered a probiotic beverage. The 50/50 baobab probiotic fermented beverage presented best results with respect to maximum growth of *Lb fermentum*, followed by 75/25 and then 100%. The fermentation kinetic data fitted showed that; the Logistic model best fitted the growth model than the Monod model. The 50/50 baobab-lemongrass fermented probiotic beverage presented best results with maximum specific growth rate of 0.073 h^{-1} while the 75/25 probiotic beverage fitted the substrate utilisation and product formation models. It had as substrate utilisation parameters, $Y_{x/s}$ 2.967 g biomass/ g glucose and m_s 0.0003 g glucose h^{-1} g^{-1} biomass. The values of α of the beverages were higher than values of β, which means the formation of product (lactic acid) was growth associated.

The *Lb. fermentum* count at the end of 28 days of storage at 4 ^{o}C yielded 8.31, 7.65 and 4.79 log cfu/mL respectively for 50/50, 75/25 and 100/0 baobab-lemongrass fermented probiotic beverage. The *Lb. fermentum* count for both 50/50 and 75/25, fell within the range of minimum concentration (6-8 log cfu/mL) of biomass concentration of probiotics. Therefore, they can be successfully stored for 28 days.

In order to completely valorise this work, the following perspectives were envisaged:

- Produce highly pure autochthonous *Lb. fermentum* and test toxicity.
- Determine the fermentation kinetics of baobab-lemongrass mixture at higher lemongrass ratio.
- Fermentation of baobab (*Adansonia digitata* L.)-lemongrass (*Cymbopogon citratus*) was done in test tube. Scale up the fermentation process taking into account other engineering parameters.

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APPENDICES

Appendix 1: Procedure for measuring reducing sugar by DNS method

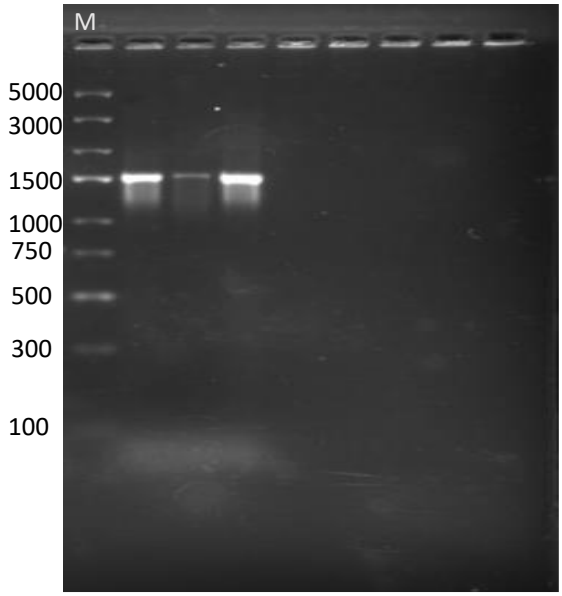
N° of tubes	1	2 S_1	3 S_2	4 S_3	5 S_4	6 S_5	Unknown
Standard maltose (ml)	0	0,25	0,25	0,25	0,25	0,25	
Solution à doser (ml)	/	/	/	/	/	/	0,25
Distilled water (ml)	1,25	1	1	1	1	1	1
DNS (ml)	0,25	0,25	0,25	0,25	0,25	0,25	0,25
Incubate tubes in water bath at 100°C for 5 min, and cool immediately in iced water							
Distilled water (ml)	4	4	4	4	4	4	4
DO at 540 nm							

Appendix 2: Measurement of total sugars by Phenol-sulfuric acid method

N° of tubes	1	2	3	4	5	6	Unknown
1mg/ml glucose standard (ml)	0	0.25	0.5	0.75	1	1.25	0
Titrating sample (ml)	/	/	/	/	/	/	0.5
Distilled water (ml)	4	3.75	3.5	3.25	3	2.75	3.5
5% aqueous phenol (ml)	1	1	1	1	1	1	1
sulfuric acid (d =1.83) (ml)	5	5	5	5	5	5	5
Allow to rest for 10 minutes in the water bath, agitate, then incubate in a water bath at 30°C for 20 minutes							
Quantity of glucose (mg) OD at 490 nm	0	0.25	0.5	0.75	1.0	1.25	0

Appendix 3: Central composite design coded values, real values and experimental responses

Run	Coded variables			Real variables			Experimental responses	
	Lemongrass powder concentration (w/v)	Decoction temperature (°C)	Extraction time (min)	Lemongrass powder concentration (w/v)	Decoction temperature (°C)	Extraction time (min)	DPPH (mg TE/100 mL extract)	TPC (mg GAE/100 mL extract)
19	0	0	0	3.5	90	7.5	58.73±0.18	52.62±0.24
10	1.52	0	0	5.78	90	7.5	85.26±0.15	65.92±0.03
2	1	-1	-1	5	85	5	77.13±0.49	59.39±0.16
8	1	1	1	5	95	10	77.01±0.01	68.09±0.07
1	-1	-1	-1	2	85	5	38.09±0.05	32.29±0.62
9	-1.52	0	0	1.22	90	7.5	19.10±0.06	23.32±0.41
13	0	0	-1.52	3.5	90	3.7	63.69±0.17	43.78±0.30
11	0	-1.52	0	3.5	82.4	7.5	51.09±0.51	44.15±0.11
14	0	0	1.52	3.5	90	11.3	55.93±0.01	55.04±0.05
18	0	0	0	3.5	90	7.5	59.08±0.04	56.17±0.10
3	-1	1	-1	2	95	5	36.92±0.03	29.57±0.11
17	0	0	0	3.5	90	7.5	61.01±0.01	55.08±0.55
12	0	1.52	0	3.5	97.6	7.5	54.04±0.53	54.33±0.51
4	1	1	-1	5	95	5	79.95±0.04	60.05±0.65
6	1	-1	1	5	85	10	73.40±0.38	64.96±0.23
5	-1	-1	1	2	85	10	25.50±0.33	28.80±0.49
7	-1	1	1	2	95	10	34.43±0.17	38.46±1.13
15	0	0	0	3.5	90	7.5	55.93±0.24	55.92±0.85
16	0	0	0	3.5	90	7.5	58.49±0.10	56.41±0.57
20	0	0	0	3.5	90	7.5	58.12±0.53	53.70±0.85



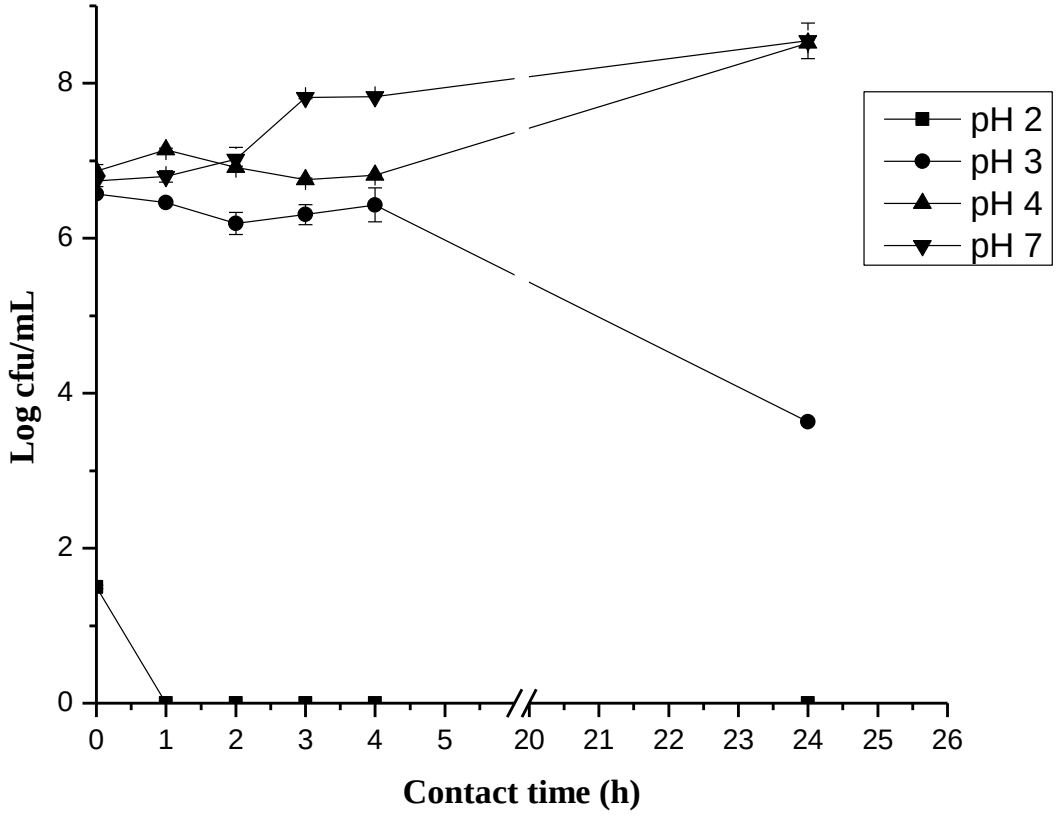
Appendix 4: Agarose gel electrophoresis-PCR amplification product of DNA of LAB isolated from baobab pulp

Appendix 5: Blast sequence by National Center for Biotechnology Information

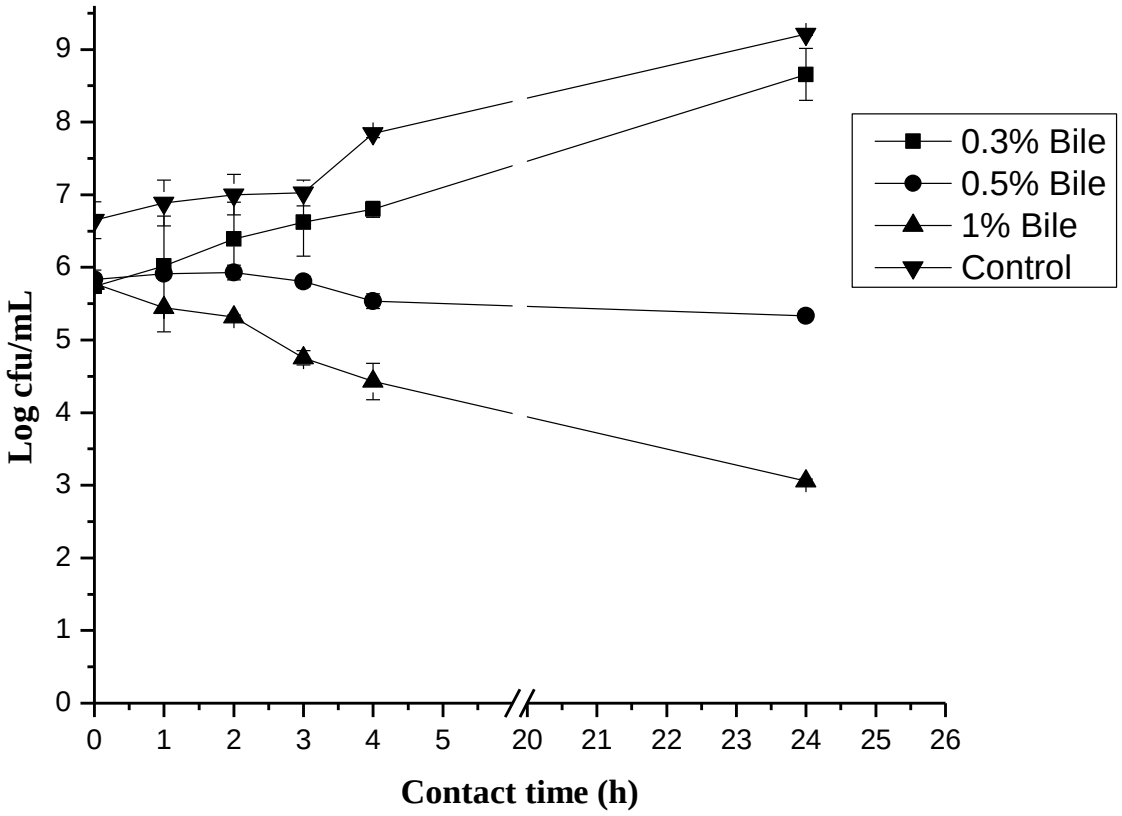
Alignments Download GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Lactobacillus fermentum strain CIP 102980 16S ribosomal RNA, partial sequence	294	294	100%	8e-80	100.00%	NR_104927.1
Lactobacillus fermentum strain NBRC 15885 16S ribosomal RNA, partial sequence	294	294	100%	8e-80	100.00%	NR_113335.1
Lactobacillus gorillae strain KZ01 16S ribosomal RNA, partial sequence	267	267	100%	2e-71	96.86%	NR_134066.1
Lactobacillus fermentum strain NCDO 1750 16S ribosomal RNA, partial sequence	255	255	89%	4e-68	98.59%	NR_118978.1
Lactobacillus ingluviei strain KR3 16S ribosomal RNA, partial sequence	195	195	96%	8e-50	89.68%	NR_028810.1
Lactobacillus mucosae strain S32 16S ribosomal RNA, partial sequence	189	189	96%	4e-48	89.10%	NR_024994.1
Lactobacillus alvi strain R54 16S ribosomal RNA, partial sequence	183	183	96%	2e-46	88.39%	NR_118032.1
Lactobacillus equigenerosi strain NRIC 0697 16S ribosomal RNA, partial sequence	178	178	96%	8e-45	87.74%	NR_041566.1
Lactobacillus coleohominis DSM 14060 16S ribosomal RNA, partial sequence	174	174	95%	1e-43	87.58%	NR_042436.1
Lactobacillus gastricus strain Kx156A7 16S ribosomal RNA, partial sequence	174	174	95%	1e-43	87.50%	NR_029084.1
Lactobacillus kimchiensis strain L133 16S ribosomal RNA, partial sequence	169	169	91%	5e-42	87.67%	NR_118101.1
Lactobacillus mudanjiangensis strain 11050 16S ribosomal RNA, partial sequence	163	163	93%	2e-40	86.75%	NR_125561.1
Pediococcus parvulus strain NBRC 100673 16S ribosomal RNA, partial sequence	163	163	91%	2e-40	86.99%	NR_113922.1
Pediococcus parvulus strain S-182 16S ribosomal RNA, partial sequence	163	163	91%	2e-40	86.99%	NR_029136.1
Lactobacillus amylotrophicus strain LMG 11400 16S ribosomal RNA, partial sequence	163	163	95%	2e-40	86.18%	NR_042511.1
Lactobacillus saerimneri strain GDA154 16S ribosomal RNA, partial sequence	163	163	93%	2e-40	86.67%	NR_029085.1
Pediococcus ethanolidurans strain Z-9 16S ribosomal RNA, partial sequence	158	158	91%	1e-38	86.30%	NR_043291.2
Lactobacillus modestisalitolerans strain NB446 16S ribosomal RNA, partial sequence	158	158	89%	1e-38	86.71%	NR_136786.1
Lactobacillus plajomi strain NB53 16S ribosomal RNA, partial sequence	158	158	89%	1e-38		

Questions/comme



Appendix 6: pH tolerance of *Lb. fermentum* in MRS broth at different pH (pH 2, 3, 4 and 7)



Appendix 7: Bile salt tolerance of *Lb. fermentum* in MRS broth supplemented with 0.3, 0.5, 1% oxgall and control (without oxgall)

Appendix 8: Resistance of *Lb, fermentum* to lysozyme

Lysozyme resistance				
Time (min)	**Log cfu/ml**	**Survival rate (%)**	**Control (Log cfu/ml)**	**Survival rate (%)**
0	6.85±0.01	/	6.87±0.01	/
30	6.79±0.01	99.06 ± 0.11^{aA}	6.84±0.01	99.49 ± 0.13^{aA}
60	5.61±0.07	81.92 ± 1.00^{aB}	6.80±0.00	98.97 ± 0.02^{bA}

Results are expressed as the mean ± standard deviation (n= 3). Different superscript uppercase letters in the same column within the same lysozyme concentration indicate significant difference ($p \leq 0.05$). Different lowercase letters in the same row within the same contact time indicate significant difference ($p \leq 0.05$).

Appendix 9: Viable count and survival rate of *Lb. fermentum* in simulated gastrointestinal juice at different pH

Simulated gastrointestinal juice						
Time (h)	**pH 2 (Log cfu/ mL)**	**Survival rate (%)**	**pH 3 (Log cfu/ mL)**	**Survival rate (%)**	**pH 7 (Log cfu/ mL)**	**Survival rate (%)**
0	3.30±0.08	/	5.59±0.20	/	7.64±0.04	/
1	2.77±0.01	$83.99±0.22^{aA}$	4.92±0.08	$88.09±1.39^{bA}$	6.88±0.04	$90.06±0.48^{bA}$
2	2.66±0.06	$80.65±1.93^{aA}$	4.83±0.07	$86.49±1.25^{abA}$	6.82±0.04	$89.30±0.51^{bA}$
3	2.43±0.06	$73.70±1.82^{aB}$	4.61±0.06	$82.54±1.07^{bA}$	6.39±0.01	$83.64±0.08^{bB}$

Results are expressed as the mean ± standard deviation (n= 3). Different superscript uppercase letters in the same column within the same pH indicate significant difference ($p \leq 0.05$). Different lowercase letters in the same row within the same contact time indicate significant difference ($p \leq 0.05$).

Appendix 10: Viable count and survival rate of *Lb. fermentum* in simulated intestinal juice at different pH

Time (h)	**pH 8**	**Survival rate (%)**	**pH 7**	**Survival rate (%)**
0	3.42±0.06	/	3.42±0.02	/
3	3.15±0.15	$92.24±4.41^{aA}$	3.37±0.05	$98.59±1.52^{aA}$
6	2.34±0.04	$68.61±1.24^{aA}$	3.28±0.05	$95.79±1.40^{bA}$
12	3.15±0.69	$92.33±20.24^{aA}$	3.32±0.02	$97.14±0.69^{aA}$
24	2.01±0.01	$58.80±0.37^{aA}$	4.00±0.00	$116.87±0.00^{bB}$

Results are expressed as the mean ± standard deviation (n= 3). Different superscript uppercase letters in the same column within the same pH indicate significant difference ($p \leq 0.05$). Different lowercase letters in the same row within the same contact time indicate significant difference ($p \leq 0.05$).

Appendix 11: Antibacterial activity of *Lb. fermentum* against pathogenic microorganisms

Pathogen	**Diameter (mm) (non-neutral free supernatant)**
Listeria monocytogenes	25.0 ± 1.0^{a}
Salmonella enterica	18.5 ± 0.5^{b}
Bacillus cereus	18.0 ± 0.0^{b}
Staphylococcus aureus	17.0 ± 0.0^{b}
Escherichia coli	/

Results are expressed as the mean ± standard deviation (n= 3). Values followed by different superscript lowercase letters mean statistically significant differences ($p \leq 0.05$).

Appendix 12: Antibiotic susceptibility assay of baobab isolate against antibiotics

Antibiotic	**Concentration (mcg)**	**Diameter (mm)**	**Antimicrobial susceptibility type**
Gentamicin (Gen)	10	17.25±0.25	S
Amikacin (Ak)	30	10.00±0.00	R
Cefoperazone (CPZ)	75	22.00±2.00	S
Penicillin-G (P)	10	23.00±0.00	S
Chloramphenicol (C)	30	20.00±0.00	S
Ampicillin/Cloxacillin (Ax)	10	22.00±1.00	S
Ampicillin/sulbactam (A/S)	10/10	41.00±0.00	S
Azithromycin (AZM)	15	15.50±0.50	S
Ciprofloxacin (CIP)	5	9.00±1.00	R
Cefuroxime (CXM)	30	20.50±0.50	S
Cephotaxime (CTX)	30	20.50±0.50	R
Netillin (Nitilmicin sulphate) (NET)	30	21.5±1.50	S
Co-trimoxazole (Sulpha/Trimethoprim) (COT)	25 (23.75/1.25)	0.00±0.00	R
Ceftazidime (CAZ)	30	15.50±1.50	R
Clarithromycin (CLR)	15	17.50±0.5	I
Vancomycin (VA)	30	0.00±0.00	/
Cloxacillin (COX)	1	0.00±0.00	/
Nitrofurantoin (NIT)	300	16.00±0.00	I
Amoxicillin (AMX)	10	34.00±1.00	S
Ampicillin (AMP)	10	13.00±0.00	R
Erythromycin (E)	15	22.00±2.00	S