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Development of probiotic fermented beverage from soursop (*Annona muricata Linn.)* fruit

A THESIS

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BY

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We accept that the thesis entitled: "**Development of a Probiotic Fermented Beverage from Soursop (*Annona muricata* Linn.) Fruit**" be publicly defended by **Ms MAKEBE Calister WINGANG** (Registration Number: **11I068EN**) at the National School of Agro-Industrial Sciences (ENSAI), of the University of Ngaoundere in view of obtaining a Doctorate/Ph.D Degree in Process Engineering.

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CERTIFICATION

We the undersigned, **Prof NSO Emmanuel JONG and Prof DESOBGO ZANGUE Steve Carly** supervisors of the thesis entittled "**Development of a Probiotic Fermented Beverage from Soursop (*Annona muricata* Linn) Fruit**" attest that it is the original work of Ms **MAKEBE Calister WINGANG** (Registration Number: **11I068EN**) 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 Agro Processing and Technology Division (APTD), CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Trivandrum, 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

Dedicated to

My beloved parents: Mr Ndekesoh John Makebe & Mrs Yembe Juvita Makebe & my sublings

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

AAD*:* Absolute average deviation

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

A_f*:* Exactitude factor

ANOVA*:* Analysis of variance

AOAC: Association of Official Analytical Chemists

AUA: Anhydrouronic Acid Content

B_f*:* Bias factor

CFU: Colony forming units

DD: Doehlert design

DE: Degree of esterification

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

FTIR: Fourier transform infrared

h*:* Parameter used to describe product inhibition

k_i*:* Substrate inhibition constant

k_s*:* Saturation constant or half-velocity constant

LA*:* *Lactobacillus acidophilus*

LAB: Lactic acid aacteria

LC*:* *Lactobacillus casei*

LCA*:* Consortium of LC and LA

m_s*:* Maintenance energy parameter (mg substrate/(log CFU/mL cell mass . h))

p: Probability level

P*:* Product concentration **(**organic acid concentration, mg/mL)

P_{max}*:* Finite production concentration (mg/mL)

RSM: Response surface methodology

RSME: Root mean square error

S*:* Substrate concentration (reducing sugar, mg/mL)

SCFA: Small chain fatty acid

SEM: Scanning electron microscopy

SSE: Sum of square error or residual sum of square

t*:* Time

TEAC: Trolox equivalent antioxidant capacity

TPC: Total polyphenol content

TSS*:* Total soluble solids

X*:* Biomass accumulation (log CFU/mL)

Y*:* Response

Y_{ps}*:* Yield of product based on utilised substrate

Y_{xs}*:* Yield of biomass based on utilised substrate

Symbols

μ*:* Specific growth rate (h^{-1})

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

β*:* Non growth-associated constant for product formation

α*: Growth-associated constant for product formation

β_o *:* Constant term of the mathematical model

β_i *:* Coefficient of linear term of the mathematical model

β_{ii} *:* Coefficient of quadratic term of the mathematical model

β_{ij} *:* Coefficient of interactions between two factors of the mathematical model

$\frac{dX}{dt}$**:** Biomass concentration rate

$\frac{dP}{dt}$**:** Product formation rate

$\frac{dS}{dt}$**:** Substrate utilisation rate

GENERAL ABSTRACT

Soursop (*Annona muricata L.*) is a fruit from the tropical and subtropical regions of the world with good nutritional and therapeutic benefits. In spite of its enormous potential, this fruit, like many others in the tropics and subtropics, suffers enormous post-harvest losses. The current study aimed at prolonging the shelf-life of its pulp by the production of a probiotic beverage, using *Lactobacillus* strains. Development of probiotic beverage involved the application of Doehlert design to optimise pectinase assisted-extraction of the juice from fruit pulp; the modelling of fermentation process of extracted juice; and the assessment of probiotic beverage shelf-life. For the optimisation of pectinase assisted-extraction, 3 factors including incubation time (30–180 min) and temperature (35-55 ℃), and enzyme concentration (0.01–0.1%, w/w) that can potentially influence juice yield, were assessed. At the optimum condition: an incubation time and temperature of 172 min and 42.2 ℃ respectively, and enzyme concentration of 0.04% (w/w), the results obtained showed extraction yield (75.20%), pH (3.74), clarity (87.06%T), TSS (7.35 °Brix), and titratable acidity (0.44% MAE). Rheological measurements demonstrated a drop in viscosity of the hydrolysed juice compared to the unhydrolysed juice and the *A. muricata* pulp. The pectinase-extracted juice was subsequently batch-fermented for 72 h at 37 ℃ using *Lactobacillus acidophilus* (LA) and *Lactobacillus casei* (LC) first independently, and then their consortium (*Lactobacillus acidophilus* and *Lactobacillus casei*, LCA). The biomass concentration, reducing sugar and lactic acid content were monitored throughout the fermentation process followed by the fitting of fermentation experimental data into the Monod, Luedeking and Piret and logistic models. The LCA obtained the highest maximum cell growth of 11.5 (log CFU/mL) after 36 h; followed by LA with 10.1 (log CFU/mL) after 36 h and 9.1 (log CFU/mL) for LC after 24 h. Furthermore, best fitting to the fermentation kinetic models was obtained with maximum specific growth rate of 0.28, 0.17 and 0.15 (h^{-1}) and saturation constant of 9.06, 9.93 and 9.07 (g/L) for LC, LA and LCA, respectively. The experimental data for LC, LA and LCA had best fitting with the logistic model for growth kinetics with maximum specific growth rate of 0.40, 0.22 and 0.16 (h^{-1}) and maximum biomass of 8.89, 8.92 and 9.93 (log CFU/mL) for LC, LCA and LA, respectively. The functional characteristics obtained showed that the microorganisms used in this study were able to survive passage through the gastrointestinal tract. The study of shelf-life of *A. muricata* probiotic juice at 4 °C for 28 days revealed that all three samples were microbiologically stable with a prebiotic load greater than 8.8 log CFU/mL which is greater than the stipulated minimum for probiotic products (6 log CFU/mL). In the light of the above results, *A. muricata* could be a suitable feedstock for the production of probiotic juice using LA and LCA.

Key words: *A. muricata* L., Pectinase, Optimisation, Juice, Fermentation, *Lactobacillus acidophilus*, *Lactobacillus casei*, Modelling, Shelf-life.

RÉSUMÉ GENERAL

Le corossol *(Annona muricata* L.) est un fruit des régions tropicales et subtropicales du globe avec de bienfaits nutritionnels et thérapeutiques. Malgré son énorme potentiel, ce fruit, comme beaucoup d'autres des régions tropicales et subtropicales, est sujet a d'énormes pertes post-récoltes. L'étude actuelle visait à prolonger la durée de conservation de sa pulpe par la production d'une boisson probiotique, en utilisant des souches de Lactobacillus. Le développement d'une boisson probiotique a impliqué l'application du plan de Doehlert pour optimiser l'extraction assistée par la pectinase du jus de la pulpe de fruit; la modélisation du procédé de fermentation du jus extrait; et l'évaluation de la durée de conservation des boissons probiotiques. Pour l'optimisation de l'extraction assistée par la pectinase, 3 facteurs, dont le temps d'incubation (30-180 min) et la température (35-55 ℃), et la concentration en enzymes (0,01-0,1%, w/w), qui peuvent potentiellement influencer le rendement en jus, ont été évalués. Dans les conditions optimales: un temps d'incubation et une température de 172 min et 42,2 °C respectivement, et une concentration en enzyme de 0,04 % (p/p), les résultats obtenus ont montré un rendement d'extraction (75,20 %), un pH (3,74), une clarte (87,06 % T), TSS (7,35 ° Brix) et acidité titrable (0,44 % MAE). Le jus extrait à la pectinase a été ensuite fermenté en batch pendant 72 h à 37 ° C en utilisant *Lactobacillus acidophilus* (LA) *et Lactobacillus casei* (LC) d'abord individuellement, ensuite avec leur mélange (*Lactobacillus acidophilus* et *Lactobacillus casei*, LCA). La concentration de la biomasse, la teneur en sucres réducteurs et en acide lactique ont été suivies tout au long du procédé de fermentation, suivies de l'ajustement des données expérimentales de fermentation dans les modèles Monod, Luedeking et Piret et logistiques. Le mélange LCA a obtenu la croissance cellulaire maximale la plus élevée de 11,5 (log CFU/mL) après 36 h; suivi par LA avec 10,1 (log CFU/mL) après 36 h et 9,1 (log CFU/mL) pour LC après 24 h. De plus, le meilleur ajustement aux modèles de cinétique de fermentation a été obtenu avec un taux de croissance spécifique maximal de 0,28, 0,17 et 0,15 (h^{-1}) et une constante de saturation de 9,06, 9,93 et 9,07 (g/L) pour LC, LA et LCA, respectivement. Les données expérimentales pour LC, LA et LCA correspondaient le mieux au modèle logistique pour la cinétique de croissance avec un taux de croissance spécifique maximal de 0,40, 0,22 et 0,16 (h^{-1}) et une biomasse maximale de 8,89, 8,92 et 9,93 (log CFU/mL) pour LC, LCA et LA, respectivement. Les caractéristiques fonctionnelles obtenues ont montré que les microorganismes utilisés dans cette étude étaient capables de survivre au passage au tractus gastro-intestinal. L'étude de la durée de conservation du jus de corossol probiotique à 4 °C pendant 28 jours a révélé que les trois échantillons étaient microbiologiquement stables avec une charge prébiotique supérieure à 8,8 log CFU/mL, ce qui est supérieur au minimum requis pour les produits probiotiques (6 log CFU /mL). A la lumière des résultats obtenus, *A. muricata* est une matière première appropriée pour la production de jus probiotique utilisant LA et LCA.

Mots clés: *A. muricata* L., Pectinase, Optimisation, Jus, Fermentation, Modélisation, Durée de conservation.

GENERAL INTRODUCTION

Annona muricata L. commonly called soursop is a delicious fruit widely grown in the tropics and subtropics regions of the world, including South America, Africa, Asia and Oceania (Kossouoh *et al*., 2007). Imbued with a peculiar flavour, this exotic fruit (Badrie and Schauss, 2010) has considerably been researched on during the last decade, as a result of growing information on its high nutritional profile and content of health protective phytochemicals (Sanusi and Abu Bakar, 2018). Literature reveals that *A. muricata* is a rich source of bioactive phenolic compounds with antidiabetic, antihypertensive, anticancerogenic, antiobesity, antihypercholesterolemic, hypoglycaemic and antibacterial properties (Adefegha *et al*., 2015; Moreau *et al*., 2018). The antioxidant properties and inhibitory activity against α-amylase, α-glucosidase, and angiotensin-I converting enzyme (ACE) of *A. muricata* L. pericarp extract, have also reported (Adefegha *et al*., 2015; Moreau *et al*., 2018).

Unfortunately, the highly perishable nature of this fruit, aggravated by lack of appropriate post-harvest techniques, leads to post-harvest losses of about 76% in the globe (Badrie and Schauss, 2010). As a means to curb post-harvest losses, a number of processing techniques such as spray drying of it's pulp (Lee Sin Chang *et al*., 2018), processing into products such as puree (Umme, 1999), nectar (Anaya-Esparza *et al*., 2017), jam (Quintana *et al*., 2018) and juice (Abbo *et al*., 2006; Dias *et al*., 2015; Ndife *et al*., 2014) have been reported.

A. muricata pulp is a suitable substrate for juice production. However, the processing of this fruit pulp into juice is fastidious due to its pectinaceous nature, which prevents the diffusion of solutes during the extraction process (Yusof and Ibrahim, 1994). These shortcomings of conventional juice extraction methods have generated the interest in innovative extraction methods. In this perspective, enzymatic-assisted extraction have been reported to improve depectinisation thereby the juice extraction yield by means of increase liquefaction and reduced viscosity (Sagu *et al*., 2014). Enzymatic-assisted extraction of fruit juice is a specific, straightforward and safe technique. This green extraction method favoured higher release of

phenolic and nutritional components (Kumar, 2015), reduced efforts to press (Lee Sin Chang *et al*., 2018), enhanced clarification (Kumar, 2015) and improved juice quality (Anuradha *et al*., 2016; Ninga *et al*., 2018; Sagu *et al*., 2014).

A. muricata L. juice is a complex system, rich in sugars, vitamins, minerals, proteins and bioactives. In addition, it's juice is rich in soluble fibres, especially pectin and hydrolysed pectin (pectic oligosaccharides). Pectic oligosaccharides (POS) are considered to be undigestible compounds that can reach the colon intact and have been recently catalogued as emerging prebiotics (Míguez *et al*., 2016). Prebiotics are englobed in the concept functional foods, and are non-digestible compounds, which, through their fermentation by gut microbiota, promote the growth and/or activity of probiotic bacteria, especially bifidobacteria and lactobacilli, thus conferring physiological positive effects on the host health (Bindels *et al*., 2015). *A. muricata* pectic oligosaccharides can therefore provide a good environment for growth and stability of probiotic strains (Fernandes Pereira and Rodrigues, 2018) and serve for production of probiotic juice. The use of fruit juices as vehicles for the delivery of probiotic species has also increased, especially as an alternative for those who do not consume dairy products (Fernandes Pereira and Rodrigues, 2018).

The fruit-based probiotic drinks market has shown an exceptional penetration in the global world food industry in recent years. The global fruit-based probiotic drinks market size is estimated to grow at a compound annual growth rate of 8.00% for 2021 to 2028 and is expected to account for a market value of USD 23.9 billion in 2028. The growth of the global market of fruit-based probiotic drinks is driven by increasing awareness about the various health benefits offered by probiotic drinks, rising demand among health-oriented consumers, increasing sales of functional ready-to-drink beverages, and surging demand for probiotic-fortified fruit juice globally.

Probiotics are “live microorganisms”, which, when administered in adequate amounts confers a health benefit on the host (FAO/WHO, 2002). Probiotics play an essential role in maintaining gut health and regular consumption improves digestive heath, boosts immunity, fights and prevents gastrointestinal problems. They also improve bowel movement regularity, reduce bloating, and combat gastrointestinal disorders such as diarrhea and irritable bowel syndrome. In addition, fermentation of fruit juice with probiotic, extend shelf-life of foods and offering unique organoleptic qualities (Leroy and De Vuyst, 2004). The most common probiotic microorganisms used and marketed in food worldwide belong to the genera *Lactobacillus* and *Bifidobacterium*. In fruits juices, the *Lactobacillus* have shown higher resistance to the acid as compared to *Bifidobacterium* (Champagne *et al*., 2011; Kumar, 2015). There are two ways of turning a fruit juice into a probiotic juice: the probiotic microorganism addition to the fruit juice and the fermentation with probiotic microorganisms. The first attempt at making probiotic juices was the addition of *Lactobacillus* in fruit juices. This technique is successful if the strain is acid tolerant. Fermentation on the other hand presents some advantages over the addition microorganisms because the growth of the probiotic strain in the juice results in a low-sugar product and a more adapted microbial strain, which might contribute to higher survival rates. Also, fermentation results in excellent competitiveness, production of metabolites that can help to increase product quality, such as bacteriocins, which preserves the juice from undesirable spoilage and pathogenic microflora. as well as the consumer wellbeing (Fernandes Pereira and Rodrigues, 2018). However, fermentation is complex process leading to production of inhibitor that can affect the growth patterns and stability of probiotic in juice during processing and storage. In addition, organic acids and polyphenols inherent to *A. muricata* L. juice can acts as inhibitors and may negatively affect the growth, viability and survival of probiotic strain during the fermentation process. In this context, it is important to acquire new knowledge on the different operations conditions of the fruit juice fermentation to help the engineer to design,

monitor, control, optimise, or scale up a lactic fermentation process. Important tools for the understanding, controlling, and optimisation of fermentation processes are kinetic models (Richard and Margaritis, 2004). Growth or non-growth related models are applied to describe the changes of other biochemical compounds and physical properties in these food systems. These changes include primary or secondary metabolites concentrations, volatile production as well as rheological and textural properties (Bouguettoucha *et al*., 2007). The aim of these models is to mathematically relate the biochemical properties (response variables) to environmental factors (controlling factors), such as temperature, pH, water activity and substrate composition.

There is however no information as to the production of fruit juice and probiotic potentials *A. muricata* substrate. The main objective of this work is therefore to valorise the pulp of this fruit in the production of a probiotic fermented beverage with specific objectives as;

1. To determine the optimum conditions (incubation time, temperature and enzyme concentration) for the enzymatic extraction of *A. muricata* juice.
2. To determine the fermentation kinetic parameters of *A. muricata* juice fermented either with *L. casei* or *L. acidophilus* and their consortium.
3. To evaluate the stability (microbial, and physicochemical) of the *A. muricata* probiotic juice.

These objectives are based on the following hypotheses;

1. Incubation time, temperature and enzyme concentration influence the yield and total soluble solids of *A. muricata* juice.
2. The maximum specific growth rate and saturation constant of the fermentation operation is dependent on the lactic acid bacteria used.
3. The shelf-life of probiotic depends on the lactic acid bacteria used and the storage conditions.

CHAPTER 1: LITERATURE REVIEW

1.1. Generalities on *Annona muricata*

1.1.1. Classification

Annonaceae is a family of the kingdom plantae with 50 genera and three of whose fruits are edible (*Annona*, *Rollinia*, and *Asimina*) with the first two being of commercial importance. It consist of 390 species and are tropically and subtropically distributed worldwide (Pinto *et al*., 2017). Four of these species are of great commercial importance: cherimoya, sugar apple, atemoya, and *A. muricata* L. (commonly known as soursop or graviola or guyabano) (George and Nissen, 2003). *A. muricata L*. is classified as follows;

Kingdom: Plantae

Division: Magnoliophyta (Angiosperms)

Class: Magnolids

Order: Magnoliales

Family: Annonaceae

Genus: Annona

Species: *Annona muricata*

Common name: Soursop

(a)

(b)

Figure 1: Mature A. muricata (soursop) (a) fruit and leaves and (b) fruits (Makebe, 2018)

1.1.2. Botanical description and ecology

A. muricata is widely distributed in the tropical regions of Central and South America (Brazil, Colombia, Costa Rica, Cuba, Jamaica, Mexico (main producer in the Americas), Panama, Peru, Puerto Rico, Venezuela), Western Africa and Southeast Asia (Padmanabhan and Paliyath, 2016; Pinto *et al*., 2005). The climatic conditions for its growth includes altitudes below 1200 m above sea level, with temperatures between 25 and 28 °C, relative humidity between 60 and 80%, and annual rainfall above 1500 mm. The height of the *A. muricata* tree is about 5–10 m and 15–83 cm in diameter with low branches (George and Nissen, 2003). *A. muricata* has the heaviest fruit in the genus, weighing from 0.9 to 10 kg with an average weight of 4 kg in some countries, but in Mexico, Venezuela and Nicaragua, it ranges between 0.4 kg and 1.0 kg (Gavamukulya *et al*., 2017; George and Nissen, 2003; Padmanabhan and Paliyath, 2016). The fruit is conical or heart-shaped, with inedible bitter skin from which protrude pliable "spines". At immaturity, *A. muricata* has a dark-green skin which turns slightly yellowish-green upon maturity (Coria-Téllez *et al*., 2018). The flesh (pulp) is creamy and acid-sweet fibrous juicy segments with a characteristic aroma and flavor (Gavamukulya *et al*., 2017; Padmanabhan and Paliyath, 2016). The pulp has pineapple-like aroma, musky, subacid to acid flavour is unique (Degnon *et al*., 2013) and the aroma volatiles consist mainly of esters (80%) (Padmanabhan and Paliyath, 2016).

1.1.3. Nutritional, physicochemical and functional properties

The proximate composition, nutritional, physicochemical and functional properties of the white edible pulp of *A. muricate* are presented in table 1. It contains water, protein, carbohydrate, vitamin and mineral salts. Phytochemical investigations also revealed the presence of alkaloids, tannins, coumarins, flavonoids, terpenoids, stearic acid, myristique acid and ellagic acid (Ngueguim *et al*., 2013).

Table 1: Composition of A. muricata fruit (wet basis)

Parameter	Values	References
Moisture content (%)	73.1 - 82.80	(Badrie and Schauss, 2010; Othman, 2014)
Ash content (%)	0.58 - 2.44	(Ndife *et al*., 2014; Othman, 2014)
Crude fat content (%)	0.30 - 2.60	(Abbo *et al*., 2006; Badrie and Schauss, 2010; Ndife *et al*., 2014)
Crude fibre content (%)	1.63 - 6.09	(Ndife *et al*., 2014; Othman, 2014)
Carbohydrate content (%)	14.6 - 18.23	(Pinto *et al*., 2005; Love and Paull, 2011)
Protein content (%)	3.32 - 5.35	(Bora *et al*., 2004; Ndife *et al*., 2014)
pH	3.68 - 4.56	(Onimawo, 2002; Lee Sin Chang *et al*., 2018)
Colour		
L*	74.44	(Lee Sin Chang *et al*., 2018)
a*	-0.94	(Lee Sin Chang *et al*., 2018)
b*	10.19	(Lee Sin Chang *et al*., 2018)
Titratable acidity (%)	0.19 - 3.43	(Ndife *et al*., 2014; Othman, 2014)
Reducing sugars (%)	7.8 - 18.9	(Orsi *et al*., 2012; Othman, 2014)
Total polyphenol content (µg GAE /100g)	34.63	(Dias *et al*., 2015)
Total soluble solids (°Brix)	11.0 - 15	(Umme, 1999; Onimawo, 2002)
Total sugars (%)	7.59 - 34.3	(Othman, 2014)
Energy (kJ)	61.3 - 71	(Peters *et al*., 2001; Love and Paull, 2011)
Vitamin A (IU)	192.50	(Onyechi *et al*., 2012)
Vitamin B_1 (mg)	0.07 - 2.10	(Onyechi *et al*., 2012)
Vitamin B_2 (mg)	0.05 - 0.20	(Love and Paull, 2011; Onyechi *et al*., 2012)
Vitamin B_3 (mg)	1.28 - 1.52	(Love and Paull, 2011; Onyechi *et al*., 2012)
Ascorbic acid (mg)	19.4 - 62.5	(Love and Paull, 2011; Othman, 2014)
Tannins (mg/100g)	53.96 - 85.3	(Onyechi *et al*., 2012)
Micronutrients (mg/100g-fw)		
Calcium	10.3 - 870	(Pinto *et al*., 2005; Othman, 2014)
Potassium	270 - 745.8	(Othman, 2014)
Sodium	14 - 895	(Love and Paull, 2011; Othman, 2014)
Phosphorus	27.7 - 29	(Love and Paull, 2011)
Heavy metal		
Iron	0.47 - 0.82	(Love and Paull, 2011; Othman, 2014)
Zinc	0.32	(Othman, 2014)
Copper	0.13	(Othman, 2014)
Lead	<0.0015 - 0.11	(Othman, 2014)

L*=lightness, a*= red/green coordinates, b*=yellow/blue coordinates

1.1.4. Virtues of *A. muricata*

Globally, all parts of the *A. muricata* tree (leaves, barks, roots, fruits and seeds) have been reported by many scientific researchers, to be used in phytomedicine in the tropics due to the presence of many bioactive compounds and phytochemicals (Gavamukulya *et al*., 2017).

- ***Fruits and fruit juices***

Elimination of worms and parasites, treat fever, increase mother's milk after child birth (lactagogue) could be effectuated by the consumption of *A. muricata* fruit or fruit juice as well as could serve as an astringent for diarrhea and dysentery (Pieme *et al*., 2014). Extract from ripe *A. muricata* pulp contains three prominent acetogenins: asimicin, bullatacin, and bullatalicin (Akomolafe and Ajayi, 2015). Apart from serving as food, it is used for the treatment of heart and liver diseases (Gavamukulya *et al*., 2017).

- ***Seeds***

Crushed seeds are used as anthelmintic and vermifuge to eliminate external parasites like head lice, and internal parasites as worms, respectively. They are also used as biopesticides, bioinsecticides and topical insect repellents (Coria-Téllez *et al*., 2018; Gavamukulya *et al*., 2017).

- ***Leaves, bark and roots***

Leaves, trunck barks and roots of *A. muricata* are used as tea to curb various disorders such as hypertension, hyperglycemia etc. The tea therefore serves as a sedative, antispasmodic, hypoglycemic, hypotensive, smooth musclerelaxant and nervine (Coria-Téllez *et al*., 2018; Florence *et al*., 2014). The leaves of *A. muricata* are used in Cameroon to manage diabetes and its complications (Ngueguim *et al*., 2013), as well as in Togo and Vietnam to treat malaria (Gavamukulya *et al*., 2017). The leaves of *A. muricata* are found to be rich in annonaceous acetogenins and the n-butanolic leaf extracts of this plant was also reported to protect normal

cells and selectively destroy cancer cells. Furthermore, an in vivo study has demonstrated the protective effects of aqueous extract of *A. muricata* in diabetic-induced rats (George *et al*., 2014). In India, the root, bark and leaves of this plant have being used for centuries, as antihelmintic and antiphlogistic agents, while its flowers and fruit pods are used as remedies for catarrh and the unripe fruit of the plant as an astringent, and used in the treatment of intestinal atony and for scurvy. In Uganda, all parts are used to treat malaria, stomachache, parasitic infections, diabetes, and cancer (Gavamukulya *et al*., 2017).

1.1.5. Other uses

A. muricata pulp because of its higher acidity, is the most suitable of all the *Annona* species for transformation. It is consumed as a delicious dessert like fruit or frozen concentrate. *A. muricata* has been exploited in the production of fruit jellies of fair flavour and quality with the addition of some gelatin, preparation of juice, semi dried fruit 'leathers', nectar, puree, jam, spray dried powder, ice creams (makes an excellent drink or ice cream after straining), syrups, and incorporated in yoghurt (Abbo *et al*., 2006; Anaya-Esparza *et al*., 2017; Lee S. Chang *et al*., 2018; Lee Sin Chang *et al*., 2018; Costa *et al*., 2014; Dias *et al*., 2015; Ndife *et al*., 2014; Quintana *et al*., 2018; Umme, 1999). The oil contained in the flat and hard seeds can be used for paint or insecticide (Rice *et al*., 1991). Flavouring of food products with the essential oil obtained from *A. muricata* species from Cameroon is reported by Jirovetz *et al*., (1998) to be able to play an important role in the future, because of the high value of fruity aromas in human nutrition (soft drinks, fruit products, flavoured teas, flavoured milk products, chewing gums, cakes, sweeties, arts, etc.). Also, in fine perfumery (class of fruity and green notes), this essential oil seems to be a valuable raw material for various applications.

1.2. Generalities on fruit juices

Fruit juice is defined as "the fermentable but unfermented product obtained from the edible part of fruit which is sound and ripe, fresh or preserved by chilling or freezing of one or more

kinds mixed together having the characteristic colour, flavour and taste typical of the juice of the fruit from which it comes," i.e., the juice obtained directly from fruit (Mihalev *et al*., 2018). The Fruit Juice Directive sets compositional requirements for six products, namely: fruit juice; fruit juice from concentrate; concentrated fruit juice; water extracted fruit juice; dehydrated/powdered fruit juice; fruit nectar. Fruit juices could also be classified according to their dispersion systems composition which include: clear/clarified (transparent) juice, opalescent (translucent) juice, cloudy (turbid) juice and pulp-enriched juice. On the other hand, fruit juices could be classified based on their preservation method which include: freshly squeezed juice ("Fresh"), chilled juice, frozen juice, pasteurised juice and concentrated juice (Mihalev *et al*., 2018). The classification of different fruit juices according to product composition is presented in figure 2.

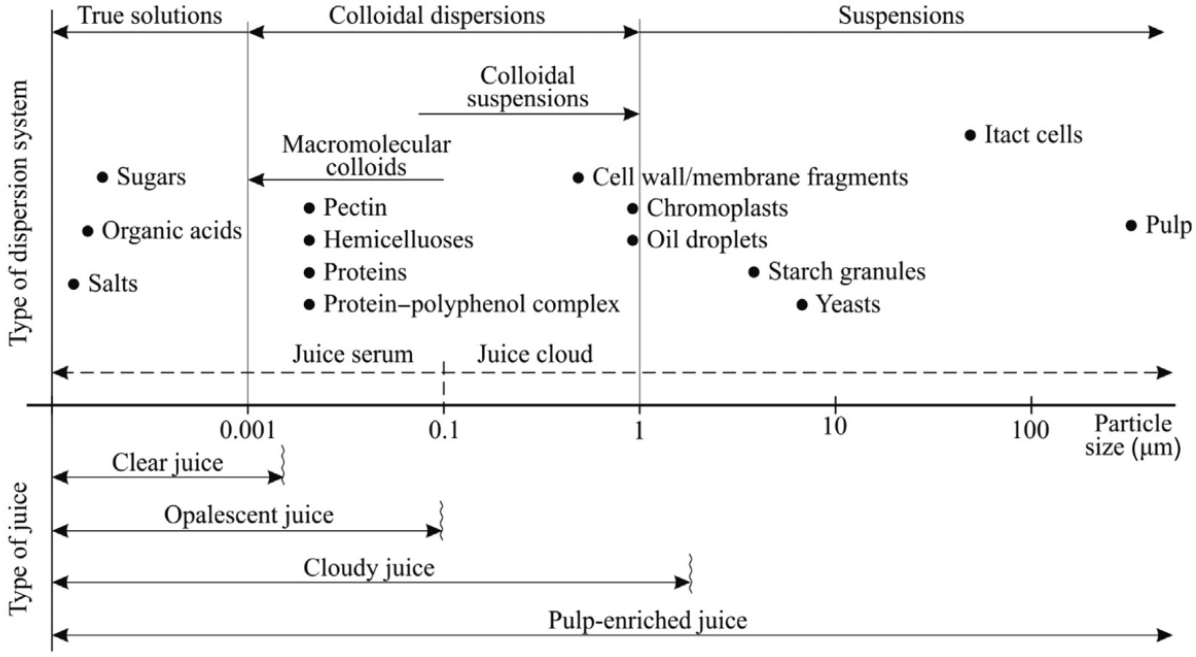


Figure 2: Dispersion system composition of different fruit juice types (Mihalev *et al*., 2018)

1.2.1. Methods of extraction of fruit juice

Several methods are used to extract fruit juices which include enzymatic, mechanical press, microwave, ultrasound, high pressure, membrane filtration, thermal treatment (cold and hot) and combined treatments.

- ***Enzyme extraction***

Many studies have reported the use of enzymatic extraction of fruit juice. Most often, this method serves as a pretreatment prior to mechanical pressing or diffusion extraction. Several advantages are associated to this method which include higher extraction yield, rapid and easy pressing and settling, reduced viscosity, higher release of phenolic and nutritional components, enhanced clarification, increase quality and sensory characteristics (Lee *et al*., 2018; Kumar, 2015; Mushtaq *et al*., 2018; Sagu *et al*., 2014). Pectolytic, cellulolytic, amylolytic enzymes or a combination of these could be used to obtain better extraction of juice with higher quality taking into account the interaction of the enzyme formulation, incubation time and temperature which vary with the fruit type, composition and stage of maturity (Mushtaq *et al*., 2018). Pectinases hydrolyse cell wall pectin (into monomers of galacturonic acid) while cellulases and hemicellulases degrade cell wall matrix made up of carbohydrate molecules such as cellulose and hemicellulose. This enhances the liberation of trapped juice in cell matrix which influences the titratable acidity and total soluble solids (Lee S. Chang *et al*., 2018; Sharma *et al*., 2017). Depectinisation has been reported for several fruit juices such as banana, carambola, carrot, monkey orange, litchi and mosambi (Lee *et al*., 2006; Liew Abdullah *et al*., 2007; Ngadze *et al*., 2018; Sagu *et al*., 2014; Sun *et al*., 2006). The use of a combination of enzymes resulted in an increased extraction yield compared to the application of an individual enzyme (Mushtaq *et al*., 2018; Sagu *et al*., 2014). The ability of enzymes to reduce industrial waste volume or reutilise or revalorize waste into value-added byproducts is a promising prospect as afar the use of enzymes in the juice industry is concerned, since most researches in the last decade have been focusing on how to reduce agricultural or food processing wastes (Mushtaq *et al*., 2018; Sharma *et al*., 2017).

The unique feature of enzymes is that they remain unaltered after the reaction is completed. Therefore, they can be used again and again if recovered. There are two main techniques of

applying enzymes: immobilised and soluble enzymes. Immobilised enzymes are advantageous in that they can be easily recovered of the product, product is free from enzyme hence no cost of purification of enzyme, enzyme can be used repeatedly and easily retained in a continuous-flow reactor. However, it presents limitations such as loss of catalytic properties and instability for some enzymes, additional cost of immobilisation and differential limitations (Brena *et al*., 2013). A main advantage of immobilised enzyme is that it can be reused since it can be easily separated from the reaction solution and can be easily retained in a continuous-flow reactor. Furthermore, immobilised enzyme may show selectively altered chemical or physical properties and it may sirnulate the realistic natural environment where the enzyme came from, the cell. Since most enzymes are globular protein, they are soluble in water. Therefore, it is very difficult or impractical to separate the enzyme for reuse in a batch process. Enzymes can be immobilised on the surface of or inside of an insoluble matrix either by chemical or physical methods. They can be also immobilised in their soluble forms by retaining them with a serniperrneable membrane.

Table 2: Techniques of fruit juice extraction from pulp

Method	**Advantages**	**Disadvantages**	**References**
Mechanical	Minimal processing (depletes or deteriorates not the nutritional sensory and antioxidant attributes of the products)	Energy challenges as well as the unchecked discharge of residues and pollutants	Alvarez-Parrilla *et al*., 2010; Mushtaq *et al*., 2018
Microwave	New, green (environmentally friendly) and efficient technology Shorter time, consumes less energy and less organic solvents to produce higher extraction yield and rapid and uniform heating	High temperature	Gerard and Roberts, 2004; Zhao *et al*., 2018
Ultrasound	Elimination of enzymes, pathogenic and spoilage microorganisms Improve juice shelf life and physical properties without altering the original organoleptic characteristics	More input of energy Quality impairment of food products Formation of radicals	Dias *et al*., 2015; Rojas *et al*., 2016; Mushtaq *et al*., 2018
Ohmic heat treatment	Shorter processing times and higher yields potentials to improve juice shelf life and physical properties	Viscosity of juice affected, electrical conductivity, and fouling deposits. Difficult to monitor and control and narrow frequency band. Lack of generalized information	Kaur *et al*., 2016

Pulse electric field (PEF)	Safe foods with a fresh-like quality and longer shelflife Destroys or inactivates microbes while maintaining the organoleptic characteristics and nutritional profile	Complexity and high cost of pulses Enzymes and spores not affected	Agcam *et al*., 2016; Mushtaq *et al*., 2018
Thermal processing	Preservation of food products, enzymatic and microbial decontamination or deterioration.	Flavor and nutrient loss, physicochemical properties affected.	Petruzzi *et al*., 2017; Mushtaq *et al*., 2018
High pressure processing (HPP)	Its immediate and uniform effect throughout different media, avoiding difficulties such as nonstationary conditions typical for convection and conduction processes.	Complexity	Muñoz *et al*., 2007
Filtration	Remove impurities and suspended solids Recover enzymes, reduce the labor and production costs	Fouling	Echavarría *et al*., 2012; Bhattacharjee *et al*., 2017
Combined methods	In shelf life extension, enzymatic activity reduction and microorganism inactivation	Time consuming	Rajauria and Tiwari, 2018
Enzymatic extraction	Improves the yield of extraction via increase liquefaction, reduced viscosity, higher release of phenolic and nutritional components, reduce efforts to press and increases quality		Sagu *et al*., 2014; Kumar, 2015; Lee S. Chang *et al*., 2018

1.3. Fermentation

Fermentation is a process in which a biological system (microorganisms) derives energy via a breakdown mechanism of substrate (organic compounds) producing end-products such as carbon dioxide, organic acids, alcohols, bacteriocins, alkanes etc (Soccol *et al*., 2013). The type of end-product formed is dependent on the species of microorganism, type of substrate (organic compound) metabolised, fermentation conditions (temperature, time, with or without agitation and aeration) etc. Fermenting microorganisms are of two main genera; fungi (molds and yeast) which are multicellular and unicellular Eumycetes (pure bacteria). The principal fermenting bacteria are functionally classified as lactic acid bacteria (LAB) because they metabolise carbohydrates to produce lactic acid as the main end-product. These microorganisms are often genetically modified in order to optimise the expression of the desired product (Stanbury *et al*., 1995). Industrially vital fermentations could be grouped based on end-products derived which include production of microbial cells (biomass), microbial enzymes, microbial metabolites, recombinant products, and modification of compounds added to fermentation (transformation process) (Stanbury *et al*., 1995).

For several centuries, fermented foods have been produced with aim to increase the storage stability of the processed foods as well as improving the organoleptic, textural and sensorial properties of raw materials. A high portion of the foods consumed on daily basis are fermented food such as dairy products (cheese, yogurt, buttermilk and sour milk, kefir, yakult and koumiss), alcoholic drinks (beer, wine, cider and vinegar, spirits, mead, sake and others), fermented vegetables (cucumber, cabbage, olives, pickles and sauerkraut), fermented meat (salami and sausages) (Soccol *et al*., 2013). Fermentation is advantageous in that it transforms agricultural substrates into new (processed) foods with increased storage stability and modified organoleptic and textural characteristics with enhanced health benefits at low cost (low energy expenditure) (Stanbury *et al*., 1995). There are two main classes of fermented beverages which

include alcoholic and non-alcoholic beverages. These fermented beverages vary with respect to the type of food preparation, fermentation duration and the application of microbes addition.

1.3.1. Types of fermentation

1.3.1.1. Batch

Batch fermentations are the simplest to operate, where all carbon source and media components are added in bulk at the start of the fermentation, and the batch then runs until carbon source is depleted. Here no addition or removal of medium occurs during fermentation. The conditions are highly dynamic, with the substrate concentration decreasing over time, and the biomass and product concentrations increasing. This operation has as advantage that it is simple to operate, and the risk of contamination is greatly reduced by the closed operation. However, the method requires a long downtime for batch turnaround due to the sterilisation requirements. It is also inefficient with changing substrate concentrations, and does not allow for control of the growth rate or the product formation rates (Mears, 2016)**.**

In batch fermentation, four major phases of the growth curve are observed by the microorganisms which include; lag, logarithmic, stationary and death phases. Slow growth of microorganisms is observed in the lag phase which is attributed to the adaptation time of the cells to a new environment as well as new metabolic pathways or enzyme synthesis. In the logarithmic phase, microbial growth is at its maximum rate meanwhile microbial growth rate equals death rate for stationary phase and secondary metabolic products could be produced by the cells. In the death phase, death rate of microorganisms is higher than the growth rate which is as a result of nutrient depletion and/or accumulation of primary or secondary inhibitory metabolites (Shuler and Kargı, 2002).

1.3.1.2. Fed Batch

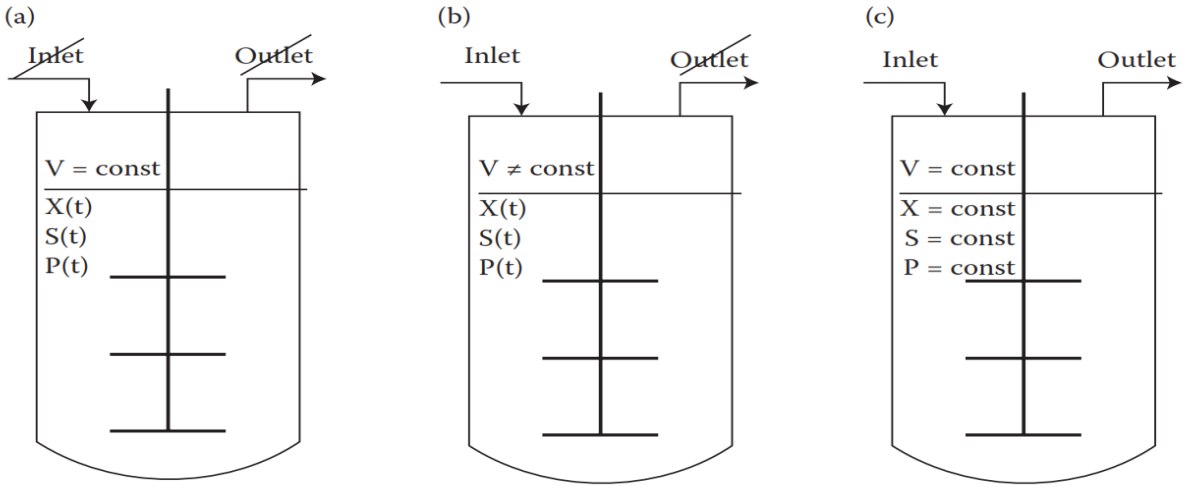
A majority of industrial fermentation processes employ a fed-batch operating mode in a stirred tank (Birol *et al*., 2002; Bodizs *et al*., 2007). The first stage of the fermentation is operated in

batch mode with a bulk of carbon source to promote biomass accumulation. Once this bulk is depleted, feeding begins during which more carbon sources are supplied to the system for both product formation and biomass growth as well as biomass maintenance. This results in significantly greater biomass and product concentrations compared to batch fermentation. In addition, processes can be operated in fed batch fermentation for significantly reduced down-time compared to the process time hence increase equipment utilisation. With fed batch, there is a need for improved monitoring and control of the process in order to supply the feed at a suitable rate, and to monitor the tank fill which is continuously increasing over the process time. In as much as fed batch can reduce the inconveniencies of batch fermentation, it is also beneficial in that, in the case where cell growth is a limitation for the process, microbial growth is not affected due to sufficient nutrient supply and little or no substrate inhibition (Sánchez and Cardona, 2008).

1.3.1.3. Continuous

Continuous operation (chemostat, continuous-flow, or stirred-tank fermentation), is where feed is added, and the product stream removed, at an equal rate. The aim is to maintain the system at a steady state with high product formation. This can result in a highly productive process, with a comparably low operational cost, as well as production of products which could be catabolised at high concentrations (Villadsen *et al*., 2011), or may be inhibitory at high concentrations, low construction costs of bioreactors, lower maintenance and operational requirements, higher yield, and a better control of the process (Sánchez and Cardona, 2008). However, there are operational challenges, especially at industrial scale, as it requires tightly controlled conditions and robust monitoring methods, yield can be decreased by the slightest change in parameters (temperature, dilution rate, substrate concentration of feed). There may also be scheduling challenges as the downstream operations cannot always be operated continuously. In addition, this long operation demands a genetically stable production host

system (microbial cultures instability), and there is also a higher risk of contamination (Sánchez and Cardona, 2008).



X=biomass concentration, **S**=substrate conconcentration, **P**=product formed, **V**=total volume of media and **t**=time

Figure 3: Simplified representation of (a) batch, (b) fed-batch, and (c) continuous fermentation (Paulova *et al*., 2013)

1.3.2. Factors Influencing Fermentation

The type of microorganisms present, process and environmental conditions can dictate the nature of the fermentation and the end product. Factor such as pH, moisture, temperature, nature and composition of medium, dissolved carbon dioxide, dissolved oxygen, precursors feeding and mixing shear rates (speed of agitation) in the fermenter. The fermentation operation system (type of fermentation: batch, fed batch, or continues) influence the fermentation process. The extent or effect of these factors can be impacted on the rate of fermentation, organoleptic properties of the product (which includes taste, aroma, texture, and appearance), the product spectrum and yield, generation of toxins nutritional quality as well as other physicochemical properties (Erkmen and Bozoğlu, 2016).

Fermentation broth (medium) formulation also has a significant impact on the yield, rate, and product profile. Requirements such as carbon, nitrogen, trace elements and micronutrients (like vitamins) for microbial growth must be presented/provided by the fermentation medium.

Specific types of carbon and nitrogen sources may be required and the carbon:nitrogen ratio may have to be controlled. Some trace elements may have to be avoided; for instance, minute amounts of iron reduce yields in citric acid production by *A. niger*. Additional factors, such as cost, availability, and batch-to-batch variability also affect the choice of medium (Erkmen and Bozoglu, 2016).

1.3.3. Types of microorganisms used for fermentation

Bacteria, yeast and mould are the most common group of microorganisms involved in food fermentation as well as microbial enzymes which also play a great role. To obtain the desire product with specific properties, a single or multiple microbial species can be used simultaneously and/or sequentially. In vinegar processing for instance, yeast is used for the production of alcohol by the conversion of sugars, the alcohol which then serves a substrate for acetic acid bacteria (*Acetobacter*) to produce acetic acid. Since bacteria are the smallest group of microorganisms amongst those stated, they generally initiate growth then yeast and lastly moulds which happen to be the largest. Smaller microorganisms multiply and take up nutrients from the surrounding area most rapidly (Erkmen and Bozoglu, 2016).

- **Bacteria**

Several researchers determined various fermentative bacterial species in different food types especially those involved in food spoilage. Amongst these, lactic acid bacteria (LAB) is of topmost importance since it is involved in a desirable food fermentation due to its ability to produce lactic acid from carbohydrates. Acetic acid producing bacteria (Acetobacter spp.) are also importantly involved in the oxidation of foods (Erkmen and Bozoglu, 2016).

- ***Lactic Acid Bacteria (LAB)***

LAB group is generally defined as ‘all members of fermenting bacteria that produce lactic acid from hexoses and lack functional heme-linked electron transport systems or cytochromes and have no Krebs cycle’. LAB convert carbohydrate (source of energy) to lactic acid, carbon

dioxide, and other organic acids without the need for oxygen. The main genera of LAB include *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus*, and *Streptococcus.* Other microorganisms which could be considered as LAB include *Aerococcus*, *Corynebacterium*, *Enterococcus, Erysipelothrix*, *Eubacterium*, *Mycobacterium*, *Oenococcus*, *Peptostreptococcus*, *Propionibacterium*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Erkmen and Bozoglu, 2016). LAB are Gram-positive, non-motile, non-respiring, non-spore forming, catalase- and oxidase-negative rods or cocci. Most lactic acid producers are aerotolerant anaerobes and they grow in the presence of small amounts of oxygen. LAB have need for amino acids, B-vitamins, and nucleic acid bases (purine and pyrimidine); some grow at high temperatures (as high as 45 °C); and some grow at variable pH ranges, but mostly well at 4.0–4.5 (some at as low as 3.2 and some at as high as 9.6). The time of generation for LAB is between 30 and 90 min. LAB can be divided into different groups depending on glucose metabolism, growth temperature, and other characteristics (Erkmen and Bozoglu, 2016).

- **LAB based on carbohydrate (glucose) metabolism**

Based on the different pathways which determines the end products, LAB can be classified as homofermentative (homolactic), facultative heterofermentative and heterofermentative (heterolactic). The heterolactics are more important than homolactics in the production of distinctive flavor and aroma compounds, such as acetaldehyde and diacetyl.

- ***Homofermentative LAB***

Homofermentative LAB breakdown glucose to produce mainly lactic acid (70–90%) as end product. The Embden–Meyerhof–Parness (EMP) pathway (glycolysis) is used to ferment sugar. These LAB lack phosphoketolase but have aldolase and hexose isomerase as their key enzymes. In this pathway, six molecules of carbon in glucose is phosphorylated and cleaved by the enzyme aldolase to glyceraldehyde-3-phosphate. This is then converted to pyruvate alongside the production of two ATP molecules by substrate-level phosphorylation. Pyruvate

reduction to lactate is done via oxidization of NADH to NAD+ and maintenance of an oxidation–reduction balance. The fermentation of 1 mole of glucose yields 2 moles of lactic acid

- ***Facultative Heterofermentative LAB***

Hexoses are almost exclusively fermented by facultative heterofermentative LAB to lactic acid by the EMP pathway. They can additionally ferment pentoses (such as gluconate) by an inducible pentose phosphoketolase and produce acetic acid, formic acid, and ethanol besides lactic acid under glucose limitation. The bacteria possess both aldolase and phosphoketolase. In the presence of glucose, the enzymes of the pentose pathway are repressed.

- ***Heterofermentative LAB***

Heterofermentative LAB produce equal amounts of lactate (50%), ethanol, acetic acid, CO2, and others from glucose and other simple sugars. They possess phosphoketolase but lack aldolase and hexose isomerase. They use pentose pathway in the conversion of hexose and glucose, to pentose and ribose by oxidative decarboxylation. The pentose is cleaved into glyceraldehyde-3-phosphate and acetyl phosphate by phosphoketolase. The triose phosphate is converted into lactate with two ATP molecules and acetyl phosphate is reduced to ethanol. The fermentation of 1 mole of glucose yields 1 mole each of lactic acid and other end products including CO2. The heterofermenters and homofermenters can be easily distinguished in the laboratory by the ability of heterofermenters to produce CO2 in the media containing glucose.

Table 3: LAB based on carbohydrate (glucose) metabolism (Erkmen and Bozoglu, 2016)

Homofermentative	**Facultative heterofermentative**	**Heterofermentative**
Lactococcus spp.	*Lactobacillus spp.*	*Lactobacillus spp*
Lac. lactic subsp. lactis	*Lb. animalis*	*Lb. brevis*
Lac. lactic subsp. cremoris	*Lb. bifermentans*	*Lb. buchneri*
Lactobacillus spp.	*Lb. casei*	*Lb. cellobiosus*
Lb. acidophilus	*Lb. curvatus*	*Lb. confusus*
Lb. lactis	*Lb. homuhiochii*	*Lb. coprophilus*
Lb. bulgaricus	*Lb. murinus*	*Lb. fermentum*
Lb. leichmannii	*Lb. plantarum*	*Lb. kefir*
Lb. salivarius	*Lb. pentosus*	*Lb. reuteri*
Pediococcus spp.	*Lb. rhamnosus*	*Lb. sanfrancisco*
Ped. acidilactici	*Lb. sake*	*Lb. viridescens*
Ped. damnosus		*Leuconostoc spp.*
Ped. pentosaceus		*Leu. dextranicum*
Streptococcus spp.		*Leu. mesenteroides*
Str. bovis		*Leu. paramesenteroides*
Str. thermophilus		*Leu. carnosum*
Enterococcus spp.		*Leu. gelidium*
Ent. faecalis		
Ent. faecium		

- **LAB based on growth temperature**

There are two main groups of LAB based on temperature of growth which includes mesophilic and thermophilic LAB.

- ***Mesophilic LAB***

Mesophilic LAB are mostly exploited in the production dairy products (such as semi hard cheese, fresh cheese, butter and sucuk) since their optimum temperature is between 20 to 30 ℃ meanwhile their growth temperature ranges between 5 and 38 ℃. They produce diacetyl and CO^2 at growth temperatures ranging from 18 to 25 °C. examples include; *Lac. lactis* subsp. *lactis* and *Lac. lactis* subsp. *Cremoris* etc

- ***Thermophilic LAB***

Thermophilic starter cultures have optimum growth temperature ranging from 40 to 45 °C with temperature ranging from 20 to 52 °C. Thermophilic starter cultures are mainly used for the processing of yogurt, hard cheese, and soft cheese. Examples of thermophilic cultures include *Str. thermophilus, Lb. bulgaricus, Lb. helveticus, Lb. lactis, Lb. casei subsp. casei, and* Lb. acidophilus.

- **Other bacteria**

Other LAB include *Bifidobacterium longum, Bifidobacterium infantis,* and *Propionibacterium shermanii* with optimum growth temperature ranging from 37 to 41 °C. *Bifidobacterium spp.* are used as probiotic cultures and *Propionibacterium* in the processing of some type of cheese.

- **Forms of Commercial LAB**

Lactic cultures used in fermentation may include single or mixed strains based on the food producers' specifications and always include bacteria that can convert lactose to lactic acid.

For instance, a culture producer can produce cultures with specific product characteristics, such as texture, flavour, acid and viscosity. Commercial LAB can thus be sold in different forms:

- ***Liquid**:* for propagation of mother culture.
- ***Deep-frozen***: for propagation of bulk starter.
- ***Freeze-dried***: concentrated cultures in powder form, for propagation of bulk starter.
- ***Deep-superfrozen***: superconcentrated cultures in readily soluble form, for direct inoculation of the product (Erkmen and Bozoglu, 2016).

1.3.4. Modelling of fermentation processes

Modelling is an essential step in the development of a process under consideration by predicting the behaviour of the system and usually the output variable and 'state of art' of the system can be predicted from the input variables (Nielsen *et al*., 2003). A mathematical model is a set of relationships between the variables of interest in the system being studied. Input variables could be parameters that impact the process such rate of agitation, feed rate, pH of medium, temperature, and substrate concentration meanwhile output variables are metabolic product concentration, substrate concentration and state of biomass (Nielsen *et al*., 2003). Mathematical models are mainly used for defining the biological, chemical, or physical basis of the process, planning the experimental conditions and evaluating the experimental results (Sinclair and Kristiansen, 1993). The purpose of fermentation modelling is to design large-scale fermentation processes using data obtained from small-scale fermentations. Modelling is generally carried out in two stages; primary and secondary stages. In the first stage, the primary models are applied to the experimental data describing the change of a response variable over time meanwhile in the second stage, the secondary models are developed expressing the bio-

kinetic parameters derived from the primary models as a function of a single environmental factor (Charalampopoulos *et al*., 2009). Different kinds of models are used in fermentation engineering which includes:

- ***Kinetic models***

Kinetic models are important tools for understanding, designing controlling, and optimizing fermentation processes (Bouguettoucha *et al*., 2011). Microbial processes are inherently complex, and it is of critical importance in practical applications to develop models that provide an accurate description of the process without unnecessary complexity (Richard and Margaritis, 2004). Kinetic models predict how fast the microorganisms can grow and use substrates or make products. It is dependent on several factors such as environmental conditions, intraparticle processes and morphological characteristics (Viccini *et al*., 2001).

- ***Stoichiometric models***

Stoichiometric models predict how much substrate is needed or product is produced given a known amount of biomass or vice versa (Viccini *et al*., 2001).

- ***Transport models***

Transport models predict how fast for example oxygen can be transported to the cells or how fast heat can be removed.

These different models can be put together into a process model, in order to predict the combined biological and physical effects in a fermenter. The overriding factor that propels biotechnology is profit. The maximisation of profits is closely linked to optimising product formation by cellular catalysts; i.e. producing the maximum amount of product in the shortest time at the lowest cost. Setting up a mathematical model involves specifying the model complexity, which involves defining the number of reactions to be considered in the model and specification of the stoichiometery for these reactions.

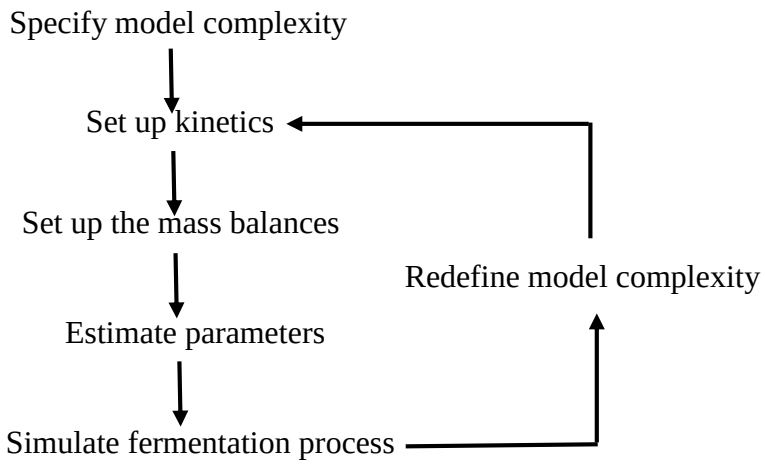


Figure 4: Different steps in quantitative description of fermentation *(Viccini* et al*., 2001)*

1.3.4.1. Types of models

Different types of kinetic models do exist; it could be mechanistic vs empirical or it could be structured vs unstructured. Structured and unstructured models are mostly exploited/developed in the description of microbial processes that is simulation of bioprocesses (Bouguettoucha *et al*., 2011; Charalampopoulos *et al*., 2009).

- **Mechanistic vs empirical models**

In process modelling, mechanistic and empirical models complement each other. In simulation models, processes are usually represented by a combination of empirical and mechanistic models, where mechanistic models gradually replace empirical models when more knowledge about a process or a unit operation becomes available (Zhou *et al*., 2015a)

- ***Mechanistic model***

Mechanistic models are models that take into account the mechanisms through which the microbial changes or operational changes occur. Here a model uses huge amount of theoretical information describing what happens during each level of the process hierarchically. These process models for fermentation and biocatalytic processes are developed based on mass, heat and momentum balances, supplemented with appropriate mathematical formulation of

mechanisms (e.g. kinetic expressions to reflect process dynamics) (Charalampopoulos *et al*., 2009; Gernaey *et al*., 2010).

- ***Empirical model***

Unlike mechanistic models, empirical models do not take into account the mechanisms through which the changes in the system occur. Rather it is noted that such changes occur and the model quantitatively accounts for the changes related with different conditions (Charalampopoulos *et al*., 2009). In the case of the description of bio-based processes, the kinetic expressions themselves are often empirical, providing a simplified and idealised view of a complex biological mechanism; the most well-known example is the use of the Monod expression for microbial growth kinetics (Gernaey *et al*., 2010). Empirical models are useful in a process control context, where software sensors often rely on these models for the prediction of variables that are not measured directly owing to on-line measurement difficulty or excessive sensor cost (Gernaey *et al*., 2010).

❖ ***Structured vs unstructured models***

Models may be structured and segregated, structured and unsegregated, unstructured and segregated, unstructured and unsegregated. Models containing both structure and segregation are the most realistic, but they are also computationally complex. The degree of realism and complexity required in a model depends on what is being described; the researcher should always choose the simplest model that can adequately describe the system (Shuler and Kargı, 2002).

- ***Structured model***

Although structured models seem complex, they provide a better understanding of the modelled system since the take into account basic aspects of cell structure, their function, composition (such as the RNA content, enzymes, reactants) and products as well as have been demonstrated

to accurately describe lactic acid fermentation (Bouguettoucha *et al*., 2011). The major setback of structured model is the complexity of the substrates and the difficulties in obtaining large sets of experimental data for the intracellular components (Charalampopoulos *et al*., 2009).

- ***Unstructured model***

In unstructured models the biomass is considered as one entity which is described only by its concentration (total cellular concentration). Here a fixed cell composition is assumed, which is equivalent to assuming 'balanced growth' hence mainly used to describe bacterial kinetics in complex natural substrates. They are frequently used because of their simplicity and adequacy for technical purposes (Charalampopoulos *et al*., 2009; Bouguettoucha *et al*., 2011). This model does not take into account any changes that could take place in the inner cells nor any physiological characterisation of the cells. However, they have proven to accurately describe lactic acid fermentation in a wide range of experimental conditions and media (Bouguettoucha *et al*., 2011).

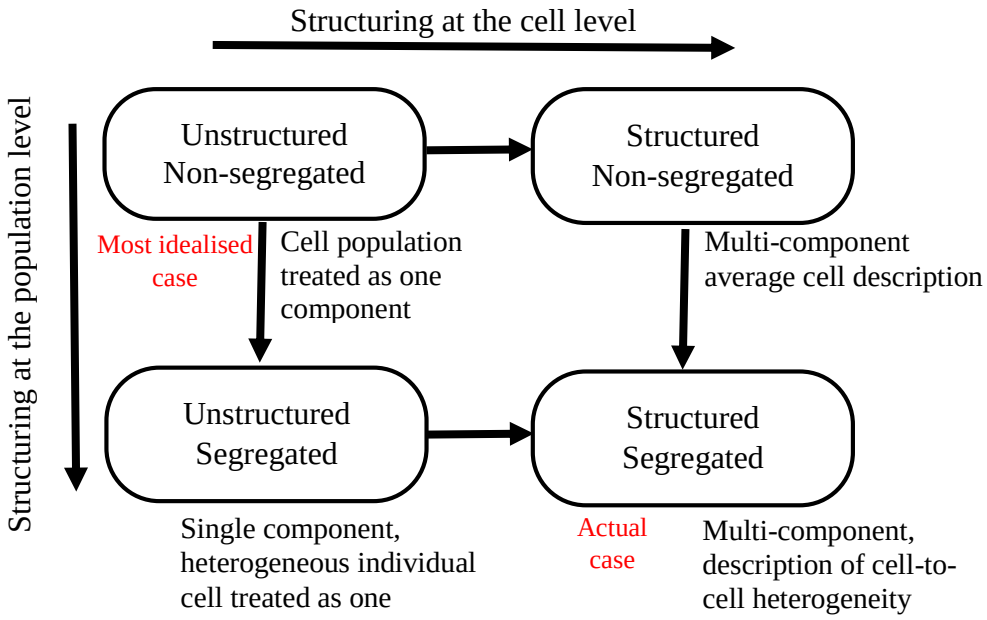


Figure 5: Model classification for mathematical of cell population (Gernaey *et al.*, 2010; Zhou *et al*., 2015b)

1.3.4.2. Model application

Kinetics are very useful in describing changes (desired and undesired) occurring during food processing and storage (van Boekel and Tijskens, 2001). Modelling has been applied in the development of bioprocess, biological detoxification of agro-industrial residues, biotransformation of crops and crop residues for nutritional enrichment, biopulping, and production of value-added products such as biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids (Pandey, 2003).

1.3.4.3. Advantages and limitation of kinetics modelling

Kinetic modelling has several advantages such as:

- Extrapolation of existing models (generic models) in the domains out of the testing areas given that the processes are governed by the same or similar mechanisms. Hence model parameters can be validated on separate data sets, obtained for example in favourable laboratory conditions, and applied in practical situations. Thus easy transfer of parameter values.
- Regular application of kinetic modelling reduces the scaling up challenges
- A clear demarcation between kinetic parameters for fundamental processes and batch parameters can be made.
- There is so much available literature on modelling and models that can be regularly applied/exploited with prevailing and accepted theories.
- The rules for building kinetic models are well rooted in the theories on chemical kinetics and thermodynamics. Consistent application of these rules leads to fundamental and generic models (Labuza, 1984; Van Boekel and Tijskens, 2001).

A few challenges involved in kinetic modelling include:

- It is often difficult, if not impossible, to detect and deduce the mechanism at work. Problem decomposition is a major assisting technique to overcome this disadvantage.

- Simplifying the mechanism, without including unnecessary processes and without excluding necessary processes is often very difficult, which is probably the reason that the 'engineering approach' is still widely popular.
- Correct application of kinetic modelling in foods requires insight in chemical kinetics, biochemistry, physics, mathematics, statistics and engineering, as well as knowledge of the food matrix. It may be difficult to unite all this knowledge in one researcher (Labuza, 1984; Van Boekel and Tijskens, 2001).

1.3.5. Microbial Growth Curve and Microbial Products

The most essential response of microorganisms to their physiochemical environment is via growth. It is a result of both replication and change in cell size. Microorganisms can grow under different physical and chemical conditions. They require substrates mainly:

- ✓ To synthesize new cell material
- ✓ To synthesize extracellular products
- ✓ To provide the energy necessary to maintain concentrations of materials within the cells which differ from those in the environment and in synthetic reactions.

Hence growth, substrate utilisation, maintenance and product formation are all closely related (Sinclair and Kristiansen, 1993). When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass. In a typical batch process, the cell number varies with time and the following phases occur: lag phase, logarithmic or exponential growth phase, deceleration phase, stationary phase, and death phase.

The *lag phas*e is an adaptation period of cells to a new environment which occurs immediately after inoculation. During this adaptation period, new enzymes are synthesized while synthesis

of some other enzymes is suppressed. Cell mass may increase, while cell number density remains constant. The lag period is affected by the age and size of the inoculum culture and the nutrient medium and usually, the lag period increases with the age of the inoculum. To minimise the duration of the lag phase, cells should be young and active, and the inoculum size should be large. The nutrient medium may need to be optimised and certain growth factors can be included in order to minimise the lag phase.

The *exponential* or *logarithmic growth phase* comes immediately after the cells have adapted to their new environment, start to multiply rapidly and consequently cell mass and cell number density increase exponentially with time.

The *deceleration growth phase* follows the exponential phase. In this phase, growth decelerates due to either depletion of one or more essential nutrients or the accumulation of toxic by-products of growth. For a typical bacterial culture, these changes occur over a very short period of time.

The *stationary phase* starts at the end of the deceleration phase, when the net growth rate is zero (no cell division) or when the growth rate is equal to the death rate. Even though the net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. *Primary metabolites* are growth associated products such as lactic acid, ethanol and *secondary metabolites* are non-growth associated products such as antibiotics. During the stationary phase, the cell catabolises cellular reserves for new building blocks and for energy-producing monomers. This is called *endogenous metabolism.* The cell must always spend energy to maintain an energised membrane and transport of nutrients and for essential metabolic functions such as motility and repair of damage to cellular structures. This energy expenditure is called *maintenance energy.*

The *death phase* follows the stationary phase. Often, death cells lyse, and a cellular nutrient released into the medium is used by the living organisms during stationary phase. At the end

of the stationary phase, because of either nutrient depletion or toxic product accumulation the death phase begins.

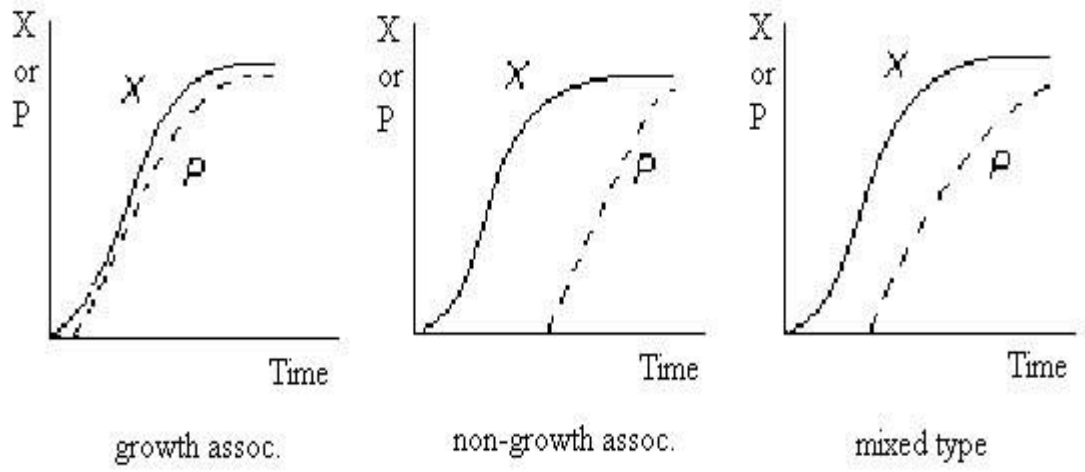
Microbial products can be classified in three major categories (Figure 6):

- ***Growth associated products formation***

Growth associated products are produced simultaneously with microbial growth. The specific rate of product formation is proportional to the specific rate of growth. The production of a constitutive enzyme is an example of a growth-associated product.

- ***Non-growth associated product formation***

Non-growth associated product formation takes place during the stationary phase when the growth rate is zero. The specific rate of product formation is constant. Many secondary metabolites, such as antibiotics (for example, penicillin), are non-growth associated products.



(a) (b) (c)

X= biomass concentration, P= product formed

Figure 6: Kinetic patterns of product formation in batch fermentations: (a) growth associated product formation, (b) non-growth associated product formation, (c) mixed growth associated product formation

- ***Mixed-growth-associated product formation***

Mixed-growth-associated product formation takes place during the slow growth and stationary phases. Lactic acid fermentation, xanthan gum, and some secondary metabolites from cell culture are examples of mixed-growth-associated products (Shuler and Kargı, 2002).

1.4. Probiotics

The word ‘probiotic’ is derived from the Greek word ‘pro-bios’ meaning ‘for life’ opposed to antibiotics which mean ‘against life’. This concept evolved from the theory proposed by Nobel Prize winner Ellie Metchnikoff, who suggested that the long life of Bulgarians resulted from their consumption of fermented milk products which consists of rod-shaped bacteria. It was first used by (Lilly and Stillwell, 1965) to describe substances secreted by one microorganism which stimulated the growth of another. It was not until Parker, (1974) defined it as ‘organisms and substances which contribute to intestinal microbial balance.’ In an attempt to improve this definition, Afrc, (1989) redefined probiotics as ‘a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’. However, among the scientific community the term ‘probiotic’ is much more complex and diverse. An expert panel commissioned by Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined it as: “live microorganisms”, which, when administered in adequate amounts confers a health benefit on the host (FAO/WHO, 2002). In a nutshell, probiotics also known as "friendly bacteria", are a microbial cell preparation or non-pathogenic microorganisms which upon consumption confer a favourable outcome on the health and well-being of the host by improving its intestinal microbial balance (Allen *et al*., 2010; FAO/WHO, 2002). To observe a positive health benefit from consumption, a minimum level of microorganisms is required: this level depends on the strain used and the required health

benefit. The dose recommended is usually between 10^6 - 10^{11} CFU/ day (Mombelli and Gismondo, 2000). Examples of foods containing probiotics in markets are yoghurt, fermented and unfermented milk, miso, tempeh, kefir, aged cheese, dark chocolate, pickled vegetables, sausages, sauerkraut, some juices and soy beverages.

Majority of microbes used as probiotics belong to the genus Lactobacillus and Bifidobacterium which are summarised in Table 4.

Table 4: Different species of probiotic microorganisms (Gupta and Garg, 2009; Kechagia et al., 2013)

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Others spp.**
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*
L. casei	*B. animalis*	*E. faecium*
L. crispatus	*B. bifidum*	*Pediococcus acidilactici*
L. gasseri	*B. infantis*	*Bacillus cereus*
L. johnsonii	*B. lactis*	*B. subtilis*
L. reuteri	*B. breve*	*Saccharomyces boulardii*
L. rhamnosus	*B. longum*	*S. cerevisiae*
L. lactis	*B. essensis*	*Leuconostoc*
L. delbrueckii subsp. bulgaricus	*B. laterosporus*	*Propionibacterium freudenreichii*
L. helveticus		*Streptococcus thermophilus*
L. fermentum		*Escherichia coli Nissle*
L. paracasei		*Streptococcus lactis*
L. sporogenes		*Pediococcus pentosaceus*
L. bulgaricus		*Streptococcus equinus*
L. brevis		*Streptococcus faecium*
L. bifidus		

1.4.1. Properties of probiotic

An effective probiotic is required to operate under a variety of environmental conditions and to survive in many different forms. There are many parameters used for screening probiotics

and the choice is dependent on the intended application of a probiotic in a specific target population (Saarela *et al*., 2000). Several evaluations/assessments have been employed to support a few properties; tolerance to acid and bile salts, adhesion to mucosal and epithelial linings, exhibition of antimicrobial activity towards pathogens, should not cause lysis of RBCs *in vivo*, and production of lactic acid. Probiotic assessment may identify the potential capability to influence local metabolic activity: for example, ability to stimulate intestinal mucosa lactase activity which can prevent some types of diarrhoea, stimulation of the immune system and capable of anti-carcinogenic activity (Gupta and Garg, 2009; Kechagia *et al*., 2013; Vimala and Dileep, 2006). Below is a list of the ideal criteria for probiotic bacteria.

- High cell viability, they must be resistant to low pH and acids.
- Ability to persist in the intestine even if the probiotic strain cannot colonise the gut.
- Adhesion to the gut epithelium to cancel the flushing effects of peristalsis.
- They should be able to interact or send signals to the immune cells associated with the gut.
- They should be a normal inhabitant of the species targeted
- Should be non-pathogenic.
- Resistance to processing.
- Probiotics must be safe
- They should be genetically stable.
- Must have capacity to influence local metabolic activity.
- Efficacy is proven in well- designed, placebo-controlled clinical trials.
- Ease of large-scale commercial production and distribution.

The desired selection criteria of choice for a particular strain of microorganisms is presented in Figure 7.

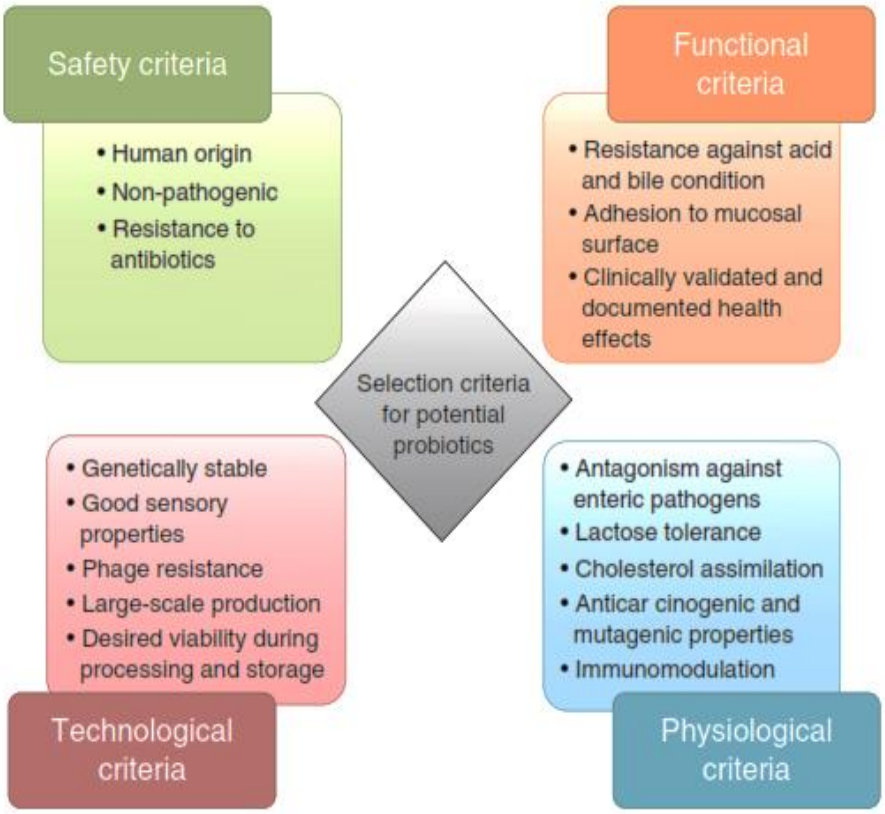


Figure 7: Criteria for the selection of probiotic microorganisms (Saarela *et al*., 2000)

1.4.2. Mechanisms of probiotics

Probiotics could be beneficial through a range of mechanisms these mechanisms cannot be generalised for all the strains due to the variation of host response to each lactobacillus strain (Suvarna and Boby, 2005). They include;

- Inhibition of bacterial growth (pathogen) through the production of inhibitory substances such as acids or bacteriocins and restoration of microbial homeostasis
- Inhibition of bacterial colonisation through competitive exclusion of binding sites via enhancement of epithelial barrier function
- Inhibition of pathogenic virulence factors
- Modulation of host innate immune responses
- Antioxidant activity.

1.4.3. Benefits of probiotics

Probiotics have been found effective in many clinical disorders such as infantile diarrhoea, antibiotic-related diarrhoea, necrotising enterocolitis, relapsing colitis due to Clostridium difficle, irritable bowel syndrome, *Helicobacter pylori* infection, urogenital infections, surgical infections, gingivitis, oral candidiasis and colon cancers (Pal and Jadhav, 2013; Senok *et al*., 2005). Has been exploited in the decrease of cholesterol, triglycerides in blood and antiobesity effect, increase calcium absorption, digestion of lactose, and inactivation of pathogen microorganisms (Saarela *et al*., 2000; Senok *et al*., 2005).

1.4.4. Prebiotics and synbiotics

Prebiotics are dietary substances (mostly consist of nonstarch polysaccharides and oligosaccharides poorly digested by human enzymes) that nurture a selected group of microorganisms living in the gut. They favour the growth of beneficial bacteria over that of harmful ones. Unlike probiotics, most prebiotics are used as food ingredients in biscuits, cereals, chocolate, spreads, and dairy products, for example. Commonly known prebiotics are oligofructose, inulin, galacto-oligosaccharides, lactulose and breast milk oligosaccharides. Fermentation of prebiotics such oligofructose in the colon results in a large number of physiologic effects, including:

- Increasing the numbers of bifidobacteria in the colon
- Increasing calcium absorption
- Increasing fecal weight
- Shortening gastrointestinal transit time
- Possibly, lowering blood lipid levels

The increase in colonic bifidobacteria has been assumed to benefit human health by producing compounds to inhibit potential pathogens, by reducing blood ammonia levels, and by producing vitamins and digestive enzymes.

Synbiotics, on the other hand, are appropriate combinations of prebiotics and probiotics. A symbiotic product exerts both a prebiotic and probiotic effect (Oelschlaeger, 2009; Saarela *et al*., 2000; Senok *et al*., 2005).

CHAPTER 2: OPTIMIZATION OF PECTINASE-ASSISTED EXTRACTION CONDITIONS OF *Annona muricata* L. JUICE AND INVESTIGATION OF THE IMPACT OF LIQUEFACTION ON THE STRUCTURE OF ITS PECTIN

2.1. Abstract

A. muricata L. is a very perishable fruit thus requires transformation into other products. This work aim to optimise the conditions of pectinase-assisted extraction of *A. muricata* L. juice by applying the Doehlert design and to study the effect of pectinase on its pectin structure. The results show that the models generated from the Doehert design were valid for all the responses (yield, pH, clarity, total soluble solid and titratable acidity) studied and the coefficients of determination were in the range 0.905 to 0.987 ($p \leq 0.05$) for soursop juice extraction. An incubation time and temperature respectively 172 min and 42.9 °C and enzyme concentration 0.04% (w/w) were found to be the optimum condition for *A. muricata* L. juice extraction, with a resultant respective 75.20%, 3.74, 87.06%T, 7.35 °Brix, and 0.44% MAE of *A. muricata* L. juice yield, pH, clarity, TSS, and titratable acidity. Under the optimum conditions, the numerical predictions for extraction were very close to the experimental results obtained, thus confirming the validity of the models. Morphologically, untreated *A. muricata* L. pulp presented a non-uniform spherical surface while the enzyme treated exhibited ruptured and wrinkled surface; meanwhile for the different pectins obtained, untreated *A. muricata* L. pectin depicted porous surface and enzyme treated pectin showed whirling rough surface. FTIR confirmed the presence of similar chemical groups (C-O, C-H and C=O) in commercial pectin and soursop pectin. Application of enzyme treatment caused the breakdown of pectin structure as illustrated by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) analyses.

Keywords: *Annona muricata* L., Juice, Pectinase, Optimisation, Liquefaction, Pectin

2.2. Introduction

The *Annona muricata* L. fruit is processed into products which give it an added value coupled to an increased shelf life. The white, juicy and highly aromatic pulp can be eaten fresh or processed into jams, custards, syrups, ice cream or high-quality nectars and juices (Peters *et al*., 2001). The fibrous nature of the fruit pulp renders the juice extraction process laborious. Several researchers have used enzymatic hydrolysis to extract juice from fruits with similar fibrous content. The extraction conditions reported by Sagu *et al*., (2014) were: 30–60 ℃ and 20–120 min *as* incubation temperature and time respectively, at a 0.01–0.05% v/w pectinase concentration using banana fruit pulp. While Sin *et al*., (2006) varied conditions from 30–50 ℃, 30–120 min and 0.03–0.10% pectinase concentration using sapodilla fruit pulp. *A. muricata* pulp has been enzymatically treated to obtain juice (Yusof and Ibrahim, 1994), puree (Lee Sin Chang *et al*., 2018a) and spray-dried powder (Lee S. Chang *et al*., 2018b). Liquefied *A. muricata* puree with reduced viscosity of up to 50% was observed by Lee Sin Chang *et al*. (2018) where the impact of enzyme concentration (pectinase, α-amylase, and cellulase) and incubation time on puree extraction was studied.

Another study carried out by Yusof and Ibrahim, (1994), in which they evaluated the quality of soursop juice after treatment using various concentrations of pectinase and for different time intervals at (37 ℃) fixed temperature. The optimum conditions were: 0.075% (w/w) for pectinase concentration and 2 h as incubation time, this improved the juice yield by 41%. However, the above studies did not consider the impact of liquefaction temperature on the extraction yield and the quality of *A. muricata* L. juice. As extraction temperature is an important parameter with respect to enzyme activity and the extraction yield, it is important to optimise the extraction conditions with respect to enzyme concentration, incubation time and temperature.

Response surface methodology (RSM) is modelling and analysis of scientific problems by grouping mathematical and statistical systems where a given outcome/response is impacted by many variables and having as objective, to optimise this outcome/response (Montgomery, 2017). Optimisation is the improvement of a process performance so as to maximise the outcome. This can be obtained through RSM and other approaches by the use of experimental design (Montgomery, 2017). Designs such as Box-Behnken (three level factorial arrangement) and central composite (full or factorial arrangement) have been highly exploited by researchers though it presents some limitations (Sagu *et al*. 2014). In 1970, Doehlert proposed a plan having a uniform distribution of the experimental points in the experimental space. All of the points are equidistant from the center of the experimental domain and are regularly distributed on the trigonometric circle of the unity radius (Goupy and Creighton, 2006). Compared to the others especially second-order experimental matrices, this design is practical, economical, increase uniformity, has few experimental application points, and higher efficiency (Sagu *et al*., 2014). The objectives of this chapter were to determine the optimal conditions for the extraction of soursop juice using pectinase assisted low-temperature extraction process and the extent of liquefaction by visualising the action of pectinase on the soursop pectin structure and morphology

2.3. Materials and methods

The methodology of work done in this chapter is outlined in the figure below;

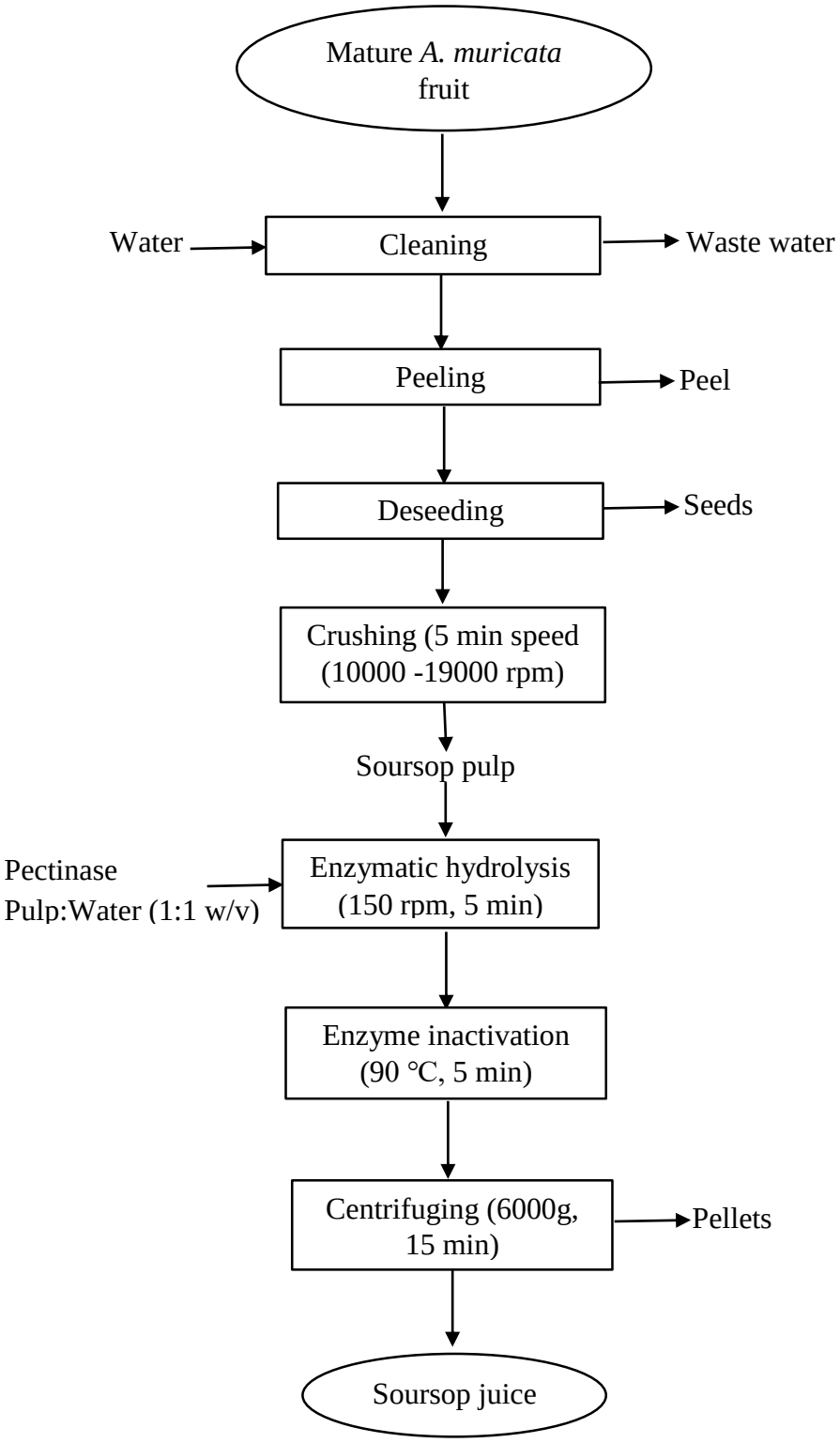


Figure 8: Process diagram of the extraction of A. muricata juice

2.3.1. Raw material

Fully fresh mature *A. muricata* fruits, yellowish green in colour, were purchased directly from a farmer in Penja, 4.6377° N, 9.6870° E, Littoral region of Cameroon. The fruits were washed under a running tap then immersed in 2% hypochloric acid which serves as a sanitising agent, drained and allowed to ripen at ambient temperature (25 ± 2 °C) for about 72 hours. After ripening, the fruits were peeled, seeds removed, then pulp crushed for 5 min at the interval of 2.5 min using an electric mixer (MG 218; Zodiac Preethi, Chennai, India) at the speed II (motor characteristics: universal 750 Watt high power motor, no load speed approx. 19000 rpm and with load speed approx. 10000 rpm). The pulp was stored in sealed plastic bags at −20 °C until use.

2.3.2. Enzyme and chemical reagents

Pectinase from *Aspergillus niger* with enzymatic activity of 1.11 unit/mg was purchased from Sigma-Aldrich, Denmark.

Sodium hydroxide, Folin Ciocalteu's phenol reagent and gallic acid standard, carbazole, D-(+)-galacturonic acid monohydrate, commercial citrus pectin, sodium potassium tartrate, sulfuric acid, potassium ferrocyanide, sodium carbonate and zinc acetate. All chemicals were of analytical grade.

2.3.3. Modelling and optimization

The Doehlert design (DD) was used as a RSM to model as factors; incubation time (min) and temperature (°C) and enzyme concentration (%, w/w) to obtain optimum condition for *A. muricata* juice extraction. The experimental domain of independent variables was chosen based on literature and preliminary studies. The independent variables were studied at three levels (-1, 0, +1) with a total of 17 trials with 5 center points. The dependent variables (responses) were: Yield (%), pH, TSS (°Brix), clarity (%T) and titratable acidity (% malic acid equivalent). The different domains of the independent variables are represented in Table 4.

Table 5: Selected parameters for the optimisation of A. muricata juice extraction

Parameter	Units	Abbreviations	Domain
Incubation time	min	X_1	30 - 180
Enzyme concentration	% w/v	X_2	0.01 – 0.1
Incubation temperature	°C	X_3	35 – 55

All experiments were carried out in triplicate and the mean values with the standard deviations were noted. Once the optimal conditions were obtained using DD, *A. muricata* juice extraction was carried out under these conditions to validate the accuracy of the model and the extract was further analyzed.

The mathematical model terms from DD, chosen for the different responses were linear, quadratic and interaction, linked to the dependent variables by a second order polynomial (equation 1).

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad [1]$$

Where, Y_i is the response, X_i and X_j are the variables, β_0 a constant, β_i the coefficient of the linear term ($\beta_1, \beta_2, \beta_3$), β_{ii} the coefficient of the quadratic terms ($\beta_{11}, \beta_{22}, \beta_{33}$) and β_{ij} coefficient of the interaction term ($\beta_{12}, \beta_{23}, \beta_{13}$).

To this effect, the model equations were analyzed by ANOVA (Analysis of Variance) using Minitab 19.1.1 (Minitab, LLC, *State* College, Pennsylvania, USA) software meanwhile response surface curves obtained using OriginPro 9.0.0 (OriginLab Corporation, Northampton, Massachusetts, USA) to describe single and interactive effects of the factors on the responses. Model validation is valuable for the prediction of responses in a given domain studied hence experimental and theoretical values given by the models were compared. Furthermore, the linear regression coefficient, the Absolute Average Deviation (AAD), Bias factor (B_f) and Exactitude factor (A_f) were used to validate the models (Makebe *et al*. 2017). All trials were done in triplicates.

2.3.4. *A. muricata* juice extraction

The *A. muricata* juice was extracted using commercial pectinase. Crushed *A. muricata* pulp (150 g) was weighed into a 250 mL Erlenmeyer flask followed by addition of water to give a final water-to-substrate ratio of 1:1 v/w, which was aimed to create a medium for easy homogenisation of pectinase enzyme. The Erlenmeyer flask was further pre-incubated in a temperature-controlled water bath at the appropriate temperatures as per the DD. Pectinase was added to the pre-incubated sample to give a final enzyme-to-substrate (w/w) ratio as mentioned for each trial of experimental designs. The enzyme-to-substrate contact time was neglected. Hydrolysis was runned with continuous agitation at an interval of 5 min with a speed of 150 g using a Remi Motor agitator (RQ 122; Elektrotechnik Ltd, Kolkata, India).

After liquefaction, the inactivation of pectinase was realised by heating the sample at 90 ℃ in a water bath for 5 min. Sample was cooled at room temperature and centrifuged at 6000 g for 15 min at 25 ℃ using a centrifuge (7780; Kubota, Bunkyo-ku, Tokyo, Japan). The juice extract (supernatant) was carefully separated from the pellet and utilised for further analysis.

2.3.5. Proximate composition analysis

Moisture, crude fibre, crude fat, crude protein, ash and carbohydrate contents of the soursop pulp were determined by the official methods of the Association of Analytical Chemist (AOAC, 2005).

2.3.5.1.Moisture content

The water content of *A. muricata* (crushed) was determined according to the AOAC 925.10 (1990) method. An empty crucible was cleaned, dried and weighed (Mo). Approximately 5 g of crushed sample was added into the container and a new mass taken (M1). The crucible containing the sample was then placed in an oven at 105 ℃ for 24 h. After drying, the crucible was weighed (M2) and the moisture content calculated (equation 2).

$$Moisture\ content\ (\%) = \frac{M_1 - M_2}{M_1 - M_0} \times 100 \quad [2]$$

This method was adapted to the freeze-dried samples. They were put in glasswares and the masses before and after freeze drying obtained.

2.3.5.2. Ash content

Ash content refers to the total mineral residue left after incineration of organic matter. It is a measure of food mineral content expressed as g ash per 100 g sample The ash content of *A. muricata* was analyzed using AOAC 920.87 (1990) method. A crucible (silica dish) was dried at 105 ± 1℃ for 3 h, cooled in desiccator and weighted (W_0). Five grams of sample were weighted into the crucible and the total weight of sample and crucible was recorded (W_1). It was placed in an oven at 105 ± 1℃ for 4h then transferred to a muffle furnace at 550 ℃ for 12 h of incineration. After incineration, the temperature of the furnace was decreased to 180ºC and the crucibles containing residue of incineration transferred into a desiccator, cooled and weighted (W_2). The ash content of sample was calculated (equation 3).

$$Ash\ (\%) = \frac{W_2 - W_0}{W_1 - W_0} \times 100 \quad [3]$$

2.3.5.3.Crude fats content

Extraction of lipids into hexane is easily achievable, provided that the moisture content of the food sample does not exceed 10%. This method may be used for quantitation of lipids in both low-fat and high-fat source materials (AOAC, 2005). The Soxhlet extraction procedure is a semicontinuous process, which allows the buildup of the solvent in the extraction chamber for 5 to 20 min. The solvent surrounds the sample and is then siphoned back into the boiling flask. The procedure provides a soaking effect and does not permit channeling.

Cellulose extraction thimble was dried, cooled in a desiccator and weighted (W_0) then 10 g of freeze-dried sample was filled in the thimble and the total weight recorded (W_1). The cellulose extraction thimble containing the sample was placed into the Soxhlet extractor and the extraction was carried out for 10 h at the boiling point of hexane (≈ 69 °C) using a heating

mantle. After the extraction, the cellulose extraction thimbles containing samples were removed from extractor, cooled down and dried in a vacuum oven at full vacuum for 10mins then weighted (W_2). The lipid content was expressed as g of lipid per 100 g of sample

$$Lipid\ content\ = \frac{W_1 - W_2}{W_1 - W_0} \times 100 \qquad [4]$$

2.3.5.4.Crude fibre content

The dietary fibre is the edible part of plant or their extracts, or analogous carbohydrates that are not easily digested or absorbed in the human small intestine, but are partly or completely fermented in the large intestine. It includes polysaccharides, oligosaccharides (cellulose, hemicellulose), lignin and associated plant substances. The crude fibres content of *A. muricata* was analysed by the Wolff (1968) method where 5 g of defatted sample (W) was mixed with 50 mL of 0.25 N sulfuric acid in a 100 mL conical flask, boiled (95 °C) in a water bath for 30 min and filtered through Watman paper N°1. The residue was mixed with 50 mL of 0.31 N sodium hydroxide and boiled (95 °C) for 30 min and filtered with ashless filter paper. The residue is thoroughly washed with boiling water then washed with approximately 15 mL alcohol. The final residue was dried in a ventilated oven at 105°C to constant weight (after 4h), weighted (W1) and incinerated at 550 °C for 3 h. The weight of ash obtained (W2) was calculated as Wf-Wi (Wi, weight of empty crucible before incineration; Wf, weight of crucible ash after incineration). The total fibres content was calculated (equation 5) and given in g/100g DM.

$$Crude\ fiber\ = \frac{100 \times (W_1 - W_2)}{W \times DM} \qquad [5]$$

2.3.5.5.Total carbohydrate content

Total carbohydrate content was calculated by difference

Total carbohydrate (g/ 100g) = 100 - (L + P + A +Cf + MC) [6]

With L: lipids content in g/100 g; P: protein content in g/100 g; A: ash content in g/100 g, Cf: crude fibre content in g/100g and MC: moisture content in %.

2.3.5.6.Protein content

One gram of dried sample was mixed with 3 g of digestion mixture ($CuSO_4$ + $NaSO_4$ in ratio 1:5) in a butter paper, while for the blank just 3 g of digestion mixture was weighed into a butter paper then 10 mL of nitrogen free H_2SO_4 (98%) was added into a 500 mL Kjeldahl digestion flask. The digestion tube was placed in the digestor and the sample digested for around 6 h at a final temperature of 420 ℃ then the digestion was stopped automatically and allowed to cool to room temperature (25 ± 2 ℃). The tubes were transferred to the distillation unit then double distilled water, 40 mL of 40% sodium hydroxide, 25 mL of 4% boric acid with 4 drops of mixed indicator (methyl red and bromocresol green) were automatically taken up to the digests and the distillation lasted for 6 mins. After distillation approximately 150 mL distillate was obtained and the distillate was titrated against standardized 0.1 N hydrochloric acid until the first appearance of the pink colour (KDIGB 8M: KjelTRON, Tulin equipment, Chennai, Tamil Nadu, India).

$$Nitrogen\ content\ (g\ \%) = \frac{(ml\ 0.1N\ HCL\ sample - ml\ 0.1N\ blank) \times 0.0014 \times N\ HCL \times 100}{g\ Weight\ of\ sample} \quad [7]$$

$$Protein\ (g/100g) = Nitrogen\ content\ (g\ \%) \times 6.25 \quad [8]$$

2.3.6. Physicochemical analysis

Different physicochemical analyses were determined.

2.3.6.1.Yield

The juice yield (% v/w) was evaluated as a percentage of the volume of juice extract (supernatant) obtained after centrifugation by the initial mass of the pulp, as presented below:

$$Yield\ (\%) = \frac{Volume\ of\ extract\ (supernatant)}{mass\ of\ pulp} \times 100 \quad [9]$$

2.3.6.2. pH

The pH value of *A. muricata* juice was read using of a pH-meter (pH700; Eutech, Ayer Rajah Cresent, Singapore). The electrode was immersed into the *A. muricata* juice, and the value read after about 30 s to 1 min when the reading was stable. The electrode was rinsed with distilled water prior each reading.

2.3.6.3.Total soluble solids (TSS)

The soluble solid content of *A. muricata* juice was obtained using a hand refractometer (0-32% Brix; ERMA, Nashik, Maharashtra, India) which measures from 0 – 32 °Brix. The refractometer was calibrated using distilled water, and *A. muricata* °Brix was then measured.

2.3.6.4.Titratable acidity

Titratable acidity was measured according to the standard method (AOAC, 2005) by titrating 10 mL of the juice against 0.1 N NaOH, utilizing phenolphthalein as indicator and, results were expressed in % malic acid equivalent.

2.3.6.5.Clarity

Clarity of the *A. muricata* juice was measured at 600 nm by noting the percentage transmittance (%, T) using a spectrophotometer (UV-2600; Shimadzu, Kyoto, Japan).

2.3.6.6.Colour

Instrumental colour readings for LSDSF were obtained using a ColourFlez EZ HunterLab colourimeter. Konica Minolta CM5 Spectrophotometer Instrument (Konica Minolta Optics, Inc, Japan) control with SpectraMagic NX software and equipped with a D65 circumferential optical sensor. The samples were filled in the transparent Petri dish and the CIE LAB colour coordinate system L*, a* and b* (L* = whiteness, a* = redness, b* = yellowness) values were recorded.

2.3.6.7. Isolation of dietary fibre

The total dietary fibres of the sample were isolated according to Bureau of Indian Standard Method (IS: 11062, 1984) with slight modifications. Briefly, 3g of defatted, moisture free

sample was mixed with 50mL water and autoclaved at 120 ℃ for 20 min. it was then cooled and the pH was adjusted to 1.5 with 5 M HCL followed by the addition of 50 mg pepsin and 200 mL of chloroform. It was incubated at 37 ℃ for 20 h while stirring at 100 rpm using a magnetic stirrer (SGMlab Solutions, Delhi, India). After incubation, the pH was adjusted to 6 with 3 N NaOH and 25 mL phosphate buffer, 100 mg pancreatin, 20 mg glucoamylase and few crystals of thymol were added. This mixture was incubated for 18 h at 37 ℃ while stirring at 100 rpm. After incubation, the content was centrifuged at 3000 g for 30 min, the residue was collected and washed with acetone and diethyl ether and lyophilised to constant weight to obtain insoluble dietary fibre. To the supernatant (soluble fibre), ethanol was added in the ratio 1:4 and centrifuged for 30 min at 3000 g. The residue (insoluble fibre) was collected and washed with alcohol and diethyl ether then lyophilised to constant weight in order to obtain soluble dietary fibre

2.3.6.8.Total polyphenol content

The phenolics compounds were extracted using 70% ethanol and Folin - Ciocalteu reagent (Marigo, 1973). Sample (5 g) was mixed with 25 mL of 70% ethanol and magnetically stirred for 30 min at 500 rpm. The mixture was filtered through the Watman paper N°1. Four other extractions were carried out, the filtrates were put together and made up to 100 mL with 70% ethanol. The standard curve was prepared from 0.2 g/L Gallic acid according to the protocol described in appendix 1. The total phenolic compounds content (Q) (in mg equivalent of gallic acid for 100 g of dried material) was calculated using standard curve regression equation.

2.3.6.9.Reducing sugars

Reducing sugars are measured by a colourimetric method with the reagent 3,5-Dinitrosalycilique acid (DNS). It is a non-stoichiometric redox reaction for quantifying reducing sugars. In this reaction, the aldehyde function of the free sugar (reducing) is

transformed into carboxylic function by the DNS (oxidant). The absorbance of the oxidised DNS is read at 540 nm.

A. muricata (0.5 g) was dissolved in 20 mL distilled water, allowed for an hour then boil for 15 min in a water bath at 100 ℃. It was removed, cooled immediately then 10 mL of 70% ethanol added, 0.5 mL of zinc sulphate (2 g/100 mL) and 0.5 mL of potassium ferricynate (10.6 g/100 mL). The solution was allowed to rest for 15 min then centrifuged and the supernatant made up to 50 mL with distilled water. Mixing was done for both sample and standard. At the optical density - 540 nm, all solutions were read by the use of a UV / Visible spectrophotometer (UV 2600, Shimadzu, Kyoto, Japan). The standard curve was obtained using glucose solution at different concentrations (Miller, 1959).

2.3.6.10. Determination of some minerals

Calcium, magnesium and phosphorus contents were determined using inductively coupled plasma mass spectrophotometer (ICP-MS) (ICAP RO, KRUSS GmbH, Hamburg, Germany). To 0.1g of sample, 3 mL of nitric acid (HNO_3) and 2 mL of hydrogen peroxide (H_2O_2) was added to the sample then micro digestion was done. After micro digestion, filtrate was diluted with 50mL distilled water then injected for analysis. Values were determined from standard curves in ppm.

2.3.6.11.Determination of Antioxidant

The different antioxidant compounds may act through different mechanisms; consequently, one method alone cannot be sufficient to fully evaluate the antioxidant capacity of foods and does not reflect the antioxidant capacity of pure compounds (Gülçin *et al*., 2011). Moreover, during extraction, solvents diffuse into the solid material and solubilize the compound with similar polarity. The nature of the solvent used will determine the type of chemicals likely extracted from plant materials, thus the antioxidant capacity of the extract (Mijanur *et al.*,

2013). It is recommended to use more than one extraction system for better assessment of the antioxidant activity of natural products.

DPPH• (2.2-diphenyl-1-picrylhydrazyl) scavenging method was used to evaluate the radical scavenging activity of compounds due to its simple, rapid, sensitive, and reproducible procedures. This was done following the protocol of Gülçin *et al*. (2011) with slight modification. Ferrous ion chelating activity was measured according to the modified method of Thammanna *et al*. (2010).

2.3.6.12.Polyphenol determination

The HPLC analysis was performed according to the method of Rodriguez-Delgado *et al*., 2001 with some modifications. The extracts of *A. muricata* obtained as described above (subsection 2.3.6.11) and the reference compounds (1 mg/mL) were filtered through 0.45µm PTFE filter; 20µl was injected into the HPLC system. The analysis was performed on a Prominence UFLC system (HPLC, Shimadzu, Japan) containing LC-20AD system controller, Phenomenex Gemini C18 column (250 _ 4.6mm, 5µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20µL volume and a diode array detector (SPD-M20A).

The mobile phase used was, solvent A: methanol – acetic acid – water (10:2:88, v/v) and solvent, B: methanol – acetic acid – water (90:2:8, v/v) with the gradient program 0–15 min 15% B, 16–20 min 50% B, 21–35 min 70% B, 36–50 min 100% B and finally the column was regenerated in 10 min. The injection volume was 20µL, and the flow rate was kept at 1 mL/min. The column was maintained at room temperature and the eluted fractions were monitored at 280 nm. Sample peaks were identified by comparing with retention times of standard peaks. LC LabSolutions software was used for data acquisition and analysis.

Thirteen standards of polyphenolic compounds (1 mg/mL concentration) were analyzed (retention times): gallic acid (7.724), catechol (12.773), chlorogenic acid (23.807), caffeic acid (25.349), syringic acid (26.298), p-coumaric acid (27.567), ferulic acid (28.629), ellagic acid

(31.155), myricetin (31.814), cinnamic acid (33.329), quercetin (34.531), kaempferol (37.586) and apigenin (38.322)

2.3.6.13.Pectin analysis

2.3.6.13.1. Galacturonic acid (GA) determination

Pectin quantification in terms of galacturonic acid content in *A. muricata* before and after enzymatic hydrolysis was obtained using the method described by Ninga *et al*., (2018) with slight modifications. Sample (1 g) was extracted with 0.05 N NaOH for 30 min then 6 mL of concentrated sulfuric acid was added. The mix was homogenised and heated at 95 °C for 10 min in a water bath then cooled in an ice bath. The mixture was further added with 0.5 mL of 0.15% of carbazole and incubated for 15 min at 25 °C to observe a colour change. Absorbance was read at 520 nm using a spectrophotometer (UV-2600; Shimadzu, Kyoto, Japan). After enzymatic action, pure ethanol was added to the hydrolysate at the ratio 1:3 (w/w) while agitating and the mixture was kept at 4 °C overnight then centrifuged at 10000 rpm for 15 min at 4 °C, which was aimed at precipitating the pectin. The supernatant was discarded and the pellet washed successively with 50%, 75% and pure (96%) ethanol to eliminate any monosaccharides and disaccharides that could be present in the pellet then analyzed. The values of GA were determined and expressed as mg /g of sample.

2.3.6.13.2. Pectin extraction

Pectin was extracted as described by Ranganna, (2007) with some modifications. *A. muricata* sample was dissolved in distilled water and the pH dropped to 2.0 using 0.5 M HCl and was allowed to boil for 1 h at 90 °C while stirring every 15 min. Sample was rapidly filtered using a muslin cloth, and absolute ethanol was added to the liquid phase at a ratio 1:3 (v/v) and centrifuged at 5000 rpm for 10 min at 4 °C. The pellet was rinsed with acetone to remove impurities then freeze dried. The pectin yield was estimated as follows:

$$Pectin\ yield = \frac{p}{B_i} \times 100 \qquad [10]$$

Where p is the extracted pectin in grams and *B*i the weight of alcohol insoluble residue in grams

2.3.6.13.3. Equivalent weight (EqW)

After extraction, on 0.5 g of the pectin was added with 5 mL ethanol, 1 g NaCl and 100 mL of distilled water. The mixture was used for equivalent weight (EqW) analysis after haven been tested for the absence of amide. This mixture was stirred till the complete dissolution of pectin then 6 drops of phenol red indicator were added and the mix was titrated against 0.1 N NaOH. Calculations were performed following the formula:

$$EqW = \frac{Weight\ of\ pectin\ sample \times 1000}{Volume\ of\ \ alkali \times normality\ of\ alkali} \quad [11]$$

2.3.6.13.4. Methoxyl content (MeO)

The methoxyl content (MeO) was obtained by introducing 25 mL of 0.25 N NaOH to the neutral solution of EqW and kept at ambient temperature for 30 min after thorough shaking. To this, 25 mL of 0.25 N HCl was introduced and the titration was executed utilizing 0.1 N NaOH to attain a purple colour. The MeO was then derived from the formula:

$$MeO\ (\%) = \frac{Volume\ of\ \ alkali \times normality\ of\ alkali \times 3.1}{Weight\ of\ sample} \quad [12]$$

2.3.6.13.5. Total anhydrouronic acid content (AUA)

The total anhydrouronic acid content (AUA) was calculated thus

$$AUA\ (\%) = \frac{176 \times 0.1 \times (z + y) \times 100}{1000 \times w} \quad [13]$$

Where 176 is the molecular weight of 1 unit of AUA, 31 is the molecular weight of 1unit of methoxyl group, z (mL) is the titre of NaOH from EqW determination, y (mL) is the titre of NaOH from MeO determination and W (g) is the weight of sample.

2.3.6.13.6. Degree of esterification (DE)

The degree of esterification was expressed as

$$DE = \frac{176 \times MeO}{31 \times AUA} \times 100 \quad [14]$$

2.3.7. Scanning Electron Microscopy (SEM)

A scanning electron microscope (EVO 18; Zeiss, Oberkochen, Germany) was used to observe the morphological change of treated sample. Unhydrolysed and hydrolysed *A. muricata* L. residue, *A. muricata* pectin and the hydrolysed pectin were lyophilised and cut/spread on the metallic plate and followed by the coating operation (with thin layer of gold) for about an hour. Micrographs were taken at 20000 magnification with 15kV of acceleration.

2.3.8. Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infrared (FTIR) spectra of commercial pectin, pectin from unhydrolysed and hydrolysed *A. muricata* were analysed using an attenuated total reflectance-Fourier transform infrared (ATR- FT-IR) spectrometer (IR spectrophotometer; Bruker Optik Gmbh, Ettlingen, Germany). The sample was deposited on the surface of a diamond crystal and pressed with a system press tip flap. Spectra were registered in transmission mode within the wavenumber range 4000–500 cm^{-1} with 32 scans per spectrum at a resolution of 8 cm^{-1}. All experiments in this work were done in triplicates.

2.4. Results and discussion

2.4.1. Proximate composition, physicochemical and phytochemical characteristics of *A. muricata*

The proximate composition of *A. muricata* is presented in Table 6. The *A. muricata* fruit investigated in the current study has higher crude fat value (1.5 ± 0.001%) than 0.97% reported by Sanusi and Abu Bakar, (2018). Similarly, the values of ash (2.44 ± 0.001%) and crude fiber (3.99 ± 0.001%) were higher than those reported by Ndife *et al*., (2014) respectively 1.83% and 2.26%. This fiber content is an indication of the need for enzymatic hydrolysis. Meanwhile, the carbohydrate, protein, and moisture contents showed similarities to the findings of Badrie and Schauss, (2010), Ndife *et al*., (2014) and Pinto *et al*., (2005). Some researchers termed this fruit to be a high caloric value fruit because of the high carbohydrate content (Pinto *et al*., 2005). These discrepancies in the proximate composition of this fruit pulp compared to other authors could be due to the dissimilarities in climatic conditions, agronomical practices, fruit variety and maturity.

Table 6: Proximate composition of A. muricata pulp

Parameter	**Values** (%)
Moisture content	82.27 ± 0.510
Ash content	2.44 ± 0.001
Crude fat content	1.50 ± 0.001
Crude fibre content	3.99 ± 0.001
Carbohydrate content	17.33 ± 0.940
Protein content	5.08 ± 0.250

The physicochemical analysis of *A. muricata* fruit presented in Table 7 revealed that the pH (3.66 ± 0.005), colour (82.08 ± 0.005 L*; 82.08 ± 0.005, a*; 0.09 ± 0.005 and b*; 16.64 ± 0.005) and reducing sugars (13.11 ± 0.3 g/100 g of sample) were similar to previous reports

(Abbo *et al*., 2006; Lee Sin Chang *et al*., 2018; Dias *et al*., 2015). Nevertheless, there were dissimilarities between titratable acidity and total soluble solids (4.13 ± 0.63 % MAE and 11.12 ± 0.3 g/100 g, respectively) and those from previous studies (Ndife *et al*., 2014; Umme, 1999). The variation in the physicochemical properties of this pulp is attributed to fruit varietal differences, horticultural practices, and climatic conditions (Pinto *et al*., 2005), meanwhile other findings associated these discrepancies to the ripening processing typical of *Annonaceae* fruits due to the decomposition of complex carbohydrates and organic acids (Orsi *et al*., 2012). The physicochemical properties present attributes that would render the juice fermentable.

The main minerals present in *A. muricata* pulp used in this study were calcium, magnesium, iron and manganese respectively 3.238, 57.624, 5.681 and 0.389 (mg/100g). Badrie and Schauss, (2010) reported the presence of calcium (10.3 mg), iron (0.64 mg) and phosphorus (27.7 mg).

Table 7: Physicochemical properties of A. muricata pulp

Parameters	Values
pH	3.660 ± 0.010
Colour	
L*	82.080 ± 0.005
a*	0.090 ± 0.005
b*	16.640 ± 0.005
Titratable acidity (% MAE)	4.130 ± 0.630
Total soluble solids	11.120 ± 0.300
Reducing sugars (g /100 g sample)	13.110 ± 0.300
Total polyphenol content (g GAE /100 g sample)	0.080 ± 0.004
Total flavonoid content (g quercetin /100 g sample)	0.001 ± 0.000
Total dietary fibres(g/100g)	20.900 ± 0.02

Insoluble fibre	6.460 ± 0.00
Soluble fibre	2.230 ± 0.08
Acid detergent fibre (%)	0.073 ± 0.000
Neutral detergent fibre (%)	0.081 ± 0.002
Antioxidant capacity	
DPPH (µM TE/g sample)	52.653 ± 9.722
FRAP (µM TE/g sample)	188.285 ± 22.475
Vitamin C (mg/100g)	19.987
Calcium (mg/100g)	3.238
Magnesium (mg/100g)	57.624
Iron (mg/100g)	5.681
Manganese (mg/100g)	0.389

*ND: Not detected

The phytochemical content of *A. muricata* is presented in Tables 8 and 9. Phenolic compounds occur in most fruits and often contribute to colour and taste. They can form metal complexes during fruit processing, resulting in discolouration of fruit pulp. These compounds may be classified into different groups as a function of the number of phenol rings they contain as well as the structural elements that bind these rings to one another. About 22 phenolic compounds were detected in *A. muricata* pulp used, with the highest been ferulic acid, Shikkimic acid, *p*-Coumaric acid, catechin, syringic acid, cinnamic acid and epicatechin. The findings of Jiménez *et al*., (2014) demonstrated that this fruit pulp is mainly made of *p*-coumaric acid, caffeic acid and ferulic phenolic acids. Meanwhile, Belitz and Grosch, (1999) reported *p*-coumaric, ferulic, caffeic and sinapic acids to be the most widespread phenolic acids in fruits and vegetables.

Table 8: Phenolic acid content of A. muricata fruit pulp

	***A. muricata* pulp (ppb)**
Catechin	35.306 ± 4.49
Quinine	1.501 ± 0.19
Narigenin	26.626 ± 0.72
Tocopherol	3.725 ± 0.91
Gallic acid	10.95 ± 0.30
Chlorogenic acid	1.190 ± 0.07
Epicatechin	26.68 ± 3.63
Syringic acid	33.846 ± 1.32
Vanilic acid	18.031 ± 0.64
Caffeic acid	4.924 ± 0.45
Epigallo catechin	3.126 ± 0.12
Ferulic acid	181.222 ± 9.39
Mycertin	1.148 ± 0.09
p-Coumaric acid	105.635 ± 1.15
Luteolin	0.005 ± 0
Apigenin	0.537 ± 0
Kampferol	0.571 ± 0.01
Hesperitin	0.951 ± 0.04
Shikkimic acid	158.711 ± 4.06
Elagic	ND
Genstein	0.558 ± 0
Cinnamic acid	39.044 ± 0.73

Chrysine	0.881 ± 0.00
Catechol	ND
Morin	ND
Quercetin	ND
Rutin	ND
Diadzein	ND

*ND: Not detected

Free amino acids are not only important active biological compounds but also serve as building blocks of protein and polypeptides which are essential in maintenance of metabolism, growth, reproduction amongst others. Free amino acids in plant foods are generally divided into two classes, essential and nonessential. Essential amino acids are amino acid that cannot be synthesised in vivo by humans hence fruits, vegetables, nuts amongst others (plants in general) are a very important source of amino acids meanwhile nonessential amino acids are those synthesised in the human body (Egydio *et al*., 2013).

In this study, a total of 20 free amino acids detected in *A. muricata* fruit pulp were tryptophan, leucine, histidine, methionine, cysteine, threonine, hydroxyl proline, aspargine, lysine, arginine, valine, glutamine, aspartic acid, glutamic acid, alanine, phenylalanine, isoleucine, tyrosine, proline and serine. Amongst all, the highest of the amino acids were proline asparagine, lysine, arginine and gluthamine found in this fruit pulp. Methionine, lysine and tryptophan were reported by Badrie and Schauss, (2010) and Egydio *et al*., (2013) to be the major amino acids present in *A. muricata* fruit meanwhile Agu and Okolie, (2017) reported the arginine and cysteine as the main amino acids. As earlier mention, several factors could be attributed to these compositional variations. Functional amino acids which are mainly arginine, cysteine, glutamine, leucine, proline, and tryptophan were present in our sample, and these

functional amino acids contribute to several health benefits (Egydio *et al.*, 2013). Free amino acid profiles could be a very important device for the identification of the occurrence of adulteration or falsification of Annona derivatives (Belitz and Grosch, 1999).

Table 9: Amino acid content of A. muricata fruit pulp

Amino acids	**Values (in ppb)**
Tryptophan	16.109 ± 2.14
Serine	167.639 ± 2.26
Leucine	22.601 ± 0.43
Histidine	51.279 ± 5.55
Methionine	1.117 ± 0.03
Asparagine	1682.31 ± 50.55
Cystine	ND
Threonine	60.037 ± 2.53
Valine	56.869 ± 1.46
Phenylalanine	2.074 ± 0.06
Glutamic acid	216.309 ± 1.48
Proline	8109.76 ± 57.65
Aspartic acid	269.12 ± 6.88
Glutamine	407.361 ± 22.83
Lysine	342.829 ± 2.79
Tyrosine	21.478 ± 0.59
Isoleucine	22.627 ± 0.19
Arginine	340.244 ± 59.05
Hydroxyl proline	21.272 ± 0.15
Cysteine	6.177 ± 0.13
Alanine	14.391 ± 1.70
Glycine	ND

Pectin yield, EqW, MeO, AUA, DE and GalA were 9.6%, 559.67, 2.86%, 48.0%, 33.95% and 5.96 ± 0.37 mg / g of sample, respectively (Table 9). The colour was light brownish. The pectin yield is in accordance with other findings, which proposed that the pectin in *A. muricata* could

be applied as an important by-product (Pinto *et al*., 2005). The EqW represents an index of pectin's jelly-forming potential under suitable conditions. *A. muricata* pectin was of the low methoxyl type since the value of DE was below 50%. Hence, it exhibited a very slow gel set and the possible formation of thermo-reversible gels at low pH and calcium ions. However, *A. muricata* pectin has the capacity to form gel even in the presence of lower sugar concentration. The DE obtained in this study was similar to that reported by Liew Abdullah *et al*., (2007), who obtained a DE of 41.67% for passion fruit peel pectin extracted at pH 3.3 for 120 min. The AUA value of pectin was below 65%, which showed that it contained some impurities like proteins, starches, and sugars. This AUA result is in agreement with those obtained from banana peel pectin that displayed AUA ranging from 55.61 to 58.77% (Kamble *et al*., 2017). The presence of pectin in *A. muricata* pulp brings out the need for pectinase-assisted extraction of this pulp to obtain juice with a higher content of fermentable sugars since the juice will later be fermented.

Table 10: A. muricata pectin extraction and characterisation

Parameter	Values
Yield (%)	9.60 ± 0.001
Galacturonic acid before pulp hydrolysis (mg / g sample)	5.96 ± 0.370
Galacturonic acid after hydrolysis (mg /g sample)	15.42 ± 0.978
Amide test	Negative
Equivalent weight	559.67 ± 57.240
Methoxyl content (%)	2.86 ± 0.540
Total anhydrouronic acid content (%)	48.0 ± 1.310
Degree of esterification (%)	33.95 ± 7.320
Surface tension (mN/m)	48.644 ± 0.172
Molecular weight (kDa)	16.599 ± 5.70

Colour of pectin	Light brown

2.4.2. Statistical analysis and model fitting of Doehlert design (DD)

The following models were obtained by modelling using RSM which linked the responses to factors (incubation time and temperature and enzyme concentration) (Table 11):

Table 11: Doehlert design of coded, real variables and experimental responses

Coded values			Real values			Responses				
Incubation time	Enzyme concentration	Incubation temperature	Incubation time (min)	Enzyme concentration (%)	Incubation temperature (°C)	Yield	pH	Clarity	Total soluble solids (TSS)	Titratable acidity
x_1	x_2	x_3	X_1	X_2	X_3	(%)		(% T)	(°Brix)	(% MAE)
0	0	0	105	0.055	45.0	73.67 ± 0	3.76 ± 0	69.8 ± 4.95	7.4 ± 0.14	0.447 ± 0
0	0	0	105	0.055	45.0	73 ± 0.47	3.775 ± 0.01	71.51 ± 4.30	7.5 ± 0.28	0.447 ± 0
0	0	0	105	0.055	45.0	73.33 ± 0	3.77 ± 0.01	67.68 ± 2.47	7.4 ± 0.14	0.447 ± 0
0	0	0	105	0.055	45.0	72.66 ± 0	3.77 ± 0.01	70.68 ± 1.67	7.4 ± 0.14	0.467 ± 0
0	0	0	105	0.055	45.0	74 ± 1.64	3.76 ± 0.01	68.98 ± 3.70	7.5 ± 0.18	0.447 ± 0.03
-1	0	0	30	0.055	45.0	71.5 ± 1.41	3.825 ± 0.01	62.8 ± 2.78	7.1 ± 0	0.424 ± 0.03
1	0	0	180	0.055	45.0	75 ± 0	3.74 ± 0.01	78.82 ± 2.30	7.4 ± 0	0.469 ± 0
-0.5	-0.866	0	67.5	0.01513	45.0	71.67 ± 0	3.77 ± 0	48.69 ± 3.67	7.1 ± 0.14	0.411 ± 0.03
0.5	-0.866	0	142.5	0.01513	45.0	74 ± 0.94	3.74 ± 0.01	77.47 ± 1.06	7.3 ± 0.28	0.467 ± 0.06
-0.5	0.866	0	67.5	0.09487	45.0	74.67 ± 0	3.775 ± 0.01	83.21 ± 3.52	7.4 ± 0	0.447 ± 0
0.5	0.866	0	142.5	0.09487	45.0	75 ± 1.77	3.72 ± 0.04	84.43 ± 0.41	7.5 ± 0.14	0.409 ± 0
-0.5	-0.289	0.816	67.5	0.041995	53.16	72.63 ± 0.47	3.77 ± 0.01	64.19 ± 3.25	7.3 ± 0	0.500 ± 0.03
0	0.577	0.816	105	0.080965	53.16	74.5 ± 0	3.755 ± 0.01	77.15 ± 4.64	7.2 ± 0.28	0.438 ± 0.31
0.5	-0.289	0.816	142.5	0.041995	53.16	72.5 ± 1.89	3.75 ± 0	63.95 ± 5.34	7.4 ± 0	0.469 ± 0.03
-0.5	0.289	-0.816	67.5	0.068005	36.84	71.67 ± 0.94	3.78 ± 0	70.15 ± 1.92	7.1 ± 0	0.424 ± 0.03
0	-0.577	-0.816	105	0.029035	36.84	73 ± 0.94	3.77 ± 0	72.52 ± 2.49	7.1 ± 0.14	0.433 ± 0.06
0.5	0.289	-0.816	142.5	0.068005	36.84	74.33 ± 1.88	3.735 ± 0.01	87.94 ± 6.14	7.3 ± 0.14	0.502 ± 0

$$Y_1 = 73.332 + 1.524x_1 + 1.126x_2 + 0.667x_2^2 - 1.129x_1x_2 - 2.109x_1x_3 + 1.105x_2x_3 \quad [15]$$

$$Y_2 = 3.767 - 0.04x_1 - 0.025x_2^2 \quad [16]$$

$$Y_3 = 69.73 + 9.95x_1 + 11.61x_2 - 5.17x_3 - 15.55x_1x_2 - 16.56x_1x_3 \quad [17]$$

$$Y_4 = 7.44 + 0.15x_1 + 0.1x_2 - 0.19x_1^2 - 0.2414x_3^2 \quad [18]$$

$$Y_5 = 0.451 + 0.019x_1 - 0.007x_2 + 0.009x_3 - 0.020x_2^2 + 0.002x_3^2 - 0.053x_1x_2 - 0.085x_1x_3 - 0.048x_2x_3 \quad [19]$$

Where, Y_1 = yield (%), Y_2 = pH, Y_3 = Clarity (%T), Y_4 = Total soluble solids (^{o}Brix), Y_5 = Titratable acidity (% MAE), x_1 = incubation time, x_2 = enzyme concentration and x_3 = incubation temperature.

All the models were valid upon consideration of R^2, R^2 adjusted, AAD, Af and Bf as presented in Table 12, and in agreement with the previous reports (Makebe *et al*., 2017) A factor or interaction was considered as having a significant effect on response if its probability was less than 0.05.

Table 12: Coefficients of the second order polynomial models for the responses (Yield, pH, Clarity, TSS, Titratable acidity), p values and validation tools (R2, adjusted R2, AAD, Af, and Bf)

Source	Yield (%)		pH		Clarity (% T)		TSS (°Brix)		Titratable acidity (% MAE)	
	Coefficient	*p values*	Coefficient	*p values*	Coefficient	*p values*	Coefficient	*p values*	Coefficient	*p values*
x_0	73.332	NA	3.767	NA	69.73	NA	7.44	NA	0.451	NA
x_1	1.524	0.0001	-0.04	0.0001	9.95	0.0010	0.15	0.0107	0.019	0.0001
x_2	1.126	0.0006	-0.005	0.1420	11.61	0.0004	0.10	0.0432	-0.007	0.0036
x_3	0.129	0.4198	-0.002	0.5629	-5.17	0.0161	0.0817	0.0831	0.0098	0.0148
x_{11}	-0.082	0.7685	0.0155	0.0655	1.08	0.6824	-0.19	0.0451	-0.0045	0.0580
x_{22}	0.667	0.0484	-0.025	0.0060	4.39	0.1376	-0.086	0.2812	-0.0208	0.0023
x_{33}	-0.487	0.1145	-0.008	0.1599	3.02	0.2754	-0.24	0.0161	0.0213	0.0056
x_{12}	-1.129	0.0192	-0.014	0.1168	-15.55	0.0051	-0.0564	0.5376	-0.053	0.0003
x_{13}	-2.109	0.0026	0.010	0.2792	-16.56	0.0068	-0.081	0.4423	-0.0856	0.0001
x_{23}	1.105	0.0331	0.019	0.0695	3.47	0.3950	-0.213	0.0798	-0.0484	0.0003
R^2	0. 938		0.974		0.969		0.901		0.959	
R^2_{adj}	0.941		0,928		0.930		0.775		0.906	
AAD	0.003		0.001		0.023		0.007		0.021	
A_f	1.003		1.001		1.023		1.007		1.022	
B_f	1.000		1.000		1.001		1.000		0.988	

x is the coefficient of the equations for each mathematical model; x_0 is the constant term, x_1, x_2, and x_3 are the linear effects (1, 2, 3 for incubation time, enzyme concentration and incubation temperature respectively), x_{11}, x_{22}, and x_{33} are quadratic effects and x_{12}, x_{13}, and x_{23} are the interactions.

NA: Not available

2.4.2.1.Effect of linear and quadratic factors on responses

2.4.2.1.1. Effect of linear factors on responses

- **Effect of incubation time (x_1)**

Table 2b shows that incubation time had a significant impact on all the responses. An increase in incubation time resulted in a significant increase in the yield, clarity and titratable acidity ($p < 0.05$). In contrast, an increase in incubation time caused a significant decrease in pH value (Figure 9, 10, and 11).

Results shown in Figure 9 were obtained upon fixing enzyme concentration (x_2) at 0.015 % and the incubation temperature (x_3) at 36.84 °C while varying the incubation time (x_1). An increase of incubation time from 30 to 180 min led to a significant increase from 68.90 to 77.35% for yield, 35.76 to 100% for clarity and 0.27 to 0.55% MAE for titratable acidity meanwhile a decrease in pH value from 3.81 to 3.74 was observed. As for TSS, an increase in incubation time from 30 min to 52.66 min, contributed to an increase in the TSS value from 6.45 °Brix to 7.00 °Brix. The increase of incubation time from 52.66 min to 180 min led to a slight decline in TSS to 6.98 °Brix (Figure 9).

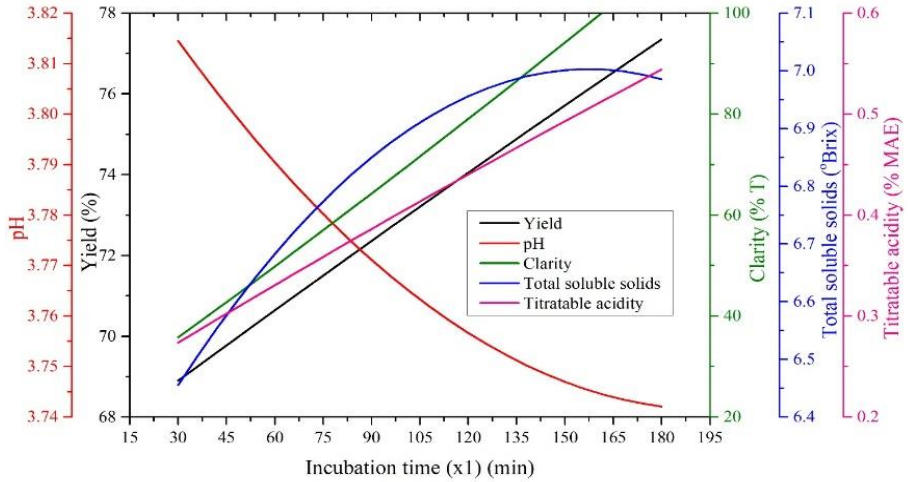


Figure 9: Evolution of responses as a function of factors: (a) Incubation time (enzyme concentration and incubation temperature fixed respectively at 0.015% and 36.84 °C).

Juice yield during the increase of the incubation time was paired with a more pronounced action of the pectinase. Indeed, pectinase acts by cleavage of the pectin constituting the cell wall of the *A. muricata* pulp at the α-1,4-glucosidic bonds into monomers of galacturonic acid, resulting in the breakdown of cell walls and a release of intracellular liquids containing solutes that migrate from intracellular environment to extracellular environment and hence increased extraction yield of juice (Lee *et al*., 2006). It has been reported that prolonged incubation resulted in a higher extraction yield of fruit juice (Lee *et al*., 2006; Yusof and Ibrahim, 1994).

On the other hand, during enzymatic treatment, pectinases hydrolyse pectin molecules over time, facilitating the formation of protein-pectin complexes and the elimination of these colloidal particles in juice contributes to an increase of juice clarity (Sin *et al*., 2006; Yusof and Ibrahim, 1994). This result is similar to that reported (Rai *et al*., 2004; Sagu *et al*., 2014) whereby the effect of pectinase on the extraction yield of banana juice was studied.

Commercial pectinase is a mixture of several enzymes, including polygalacturonase and pectin methyl esterase. Hydrolysis of pectin at high incubation time leads to the release of

galacturonic acids and other organic acids due to the action of these two enzymes, thus increasing the titratable acidity of the juice. Several authors have observed this phenomenon (Makebe *et al*., 2017; Nguyen and Nguyen, 2018; Yusof and Ibrahim, 1994). Organic acids play a relevant role in the processing of fruit juice. It serves as a mild preservative, contributes to flavour development via a balanced sugar: acid ratio and stimulates saliva secretion through a thirst-quenching effect (Lee Sin Chang *et al*., 2018; Ndife *et al*., 2014; Umme *et al*., 1997).

The model of the total soluble solids exhibits first order and zero order phases with increase in incubation time. The increase in total soluble solids with increasing incubation time is explained by extensive hydrolysis of cellular pectin, resulting in increased release of compounds such as sugars (Bitange *et al*., 2009) and other components that are soluble solids. The stationary phase of the total soluble solids after a long incubation time was explained by the fact that in that zone the maximum total soluble solids could have been extracted (Nguyen and Nguyen, 2018). From this curve, it is therefore technologically advisable to extract juice with maximum TSS at 120 min.

The decrease in pH value with the increase of the incubation time could be due to the liberation of the organic acids following the enzymatic hydrolysis of the pectin.

- **Effect of enzyme concentration (x_2)**

Impact of enzyme concentration was significant only on yield, clarity and TSS (Table 10). An increase in enzyme concentration indeed contributed to increase of these responses (Figure 10).

The following results were achieved by fixing incubation time (x_1) at 30 min and incubation temperature (x_3) at 36.84 °C. Increase of enzyme concentration from 0.015% to 0.095%, induced a significant increase (Figure 10) in yield (from 68.90% to 71.25%), clarity (from 35.76%T to 77.90%T) and TSS (from 6.45 °Brix to 7.03 °Brix).

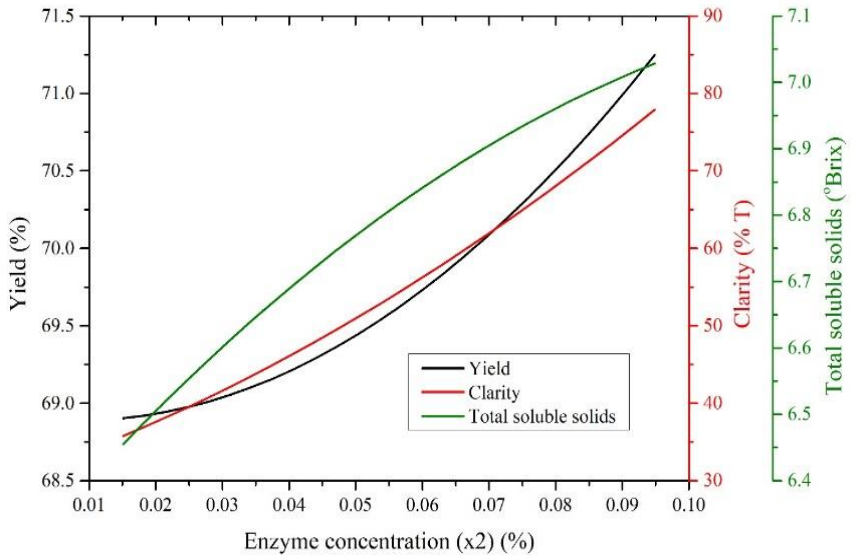


Figure 10: Evolution of responses as a function of factors: (b) Enzyme concentration (incubation time and incubation temperature fixed respectively at 30 min and 36.84 °C)

Since the concentration of enzyme is very low compared to that of the substrate, the reaction rate is directly proportional to the concentration of the enzyme. That is, the reaction rate increased as enzyme concentration increased. In other words, the significant increase in yield could be related to the rate of hydrolysis of the pectin following the increase in pectinase concentration. A similar observation has been reported (Sin *et al*., 2006; Yusof and Ibrahim, 1994) where a pectin based pulp was treated with pectinase.

Increasing the concentration of pectinase could increase the clarity of juice suggesting the formation of larger aggregates as earlier explained and thus settling (Sin *et al*., 2006), hence increasing the clarity of the juice.

Increase in TSS per incubation time could be due to the faster hydrolysis of pectin linked to the increase of enzyme concentration. This contributes to the release of significant soluble solids components into the medium.

- **Effect of incubation temperature (x_3)**

The effect of incubation temperature was significant on clarity and titratable acidity (Table 10). An increase in incubation temperature contributed to increase the responses (Figure 11).

The following results (Figure 11) were obtained by fixing the incubation time (x_1) at 30 min and enzyme concentration (x_2) at 0.015%. Increase in incubation temperature from 36.84 to 53.16 °C, contributed to a significant increase in clarity (from 35.77% T to 49.52% T) and titratable acidity (from 0.274 % MAE to 0.498 % MAE).

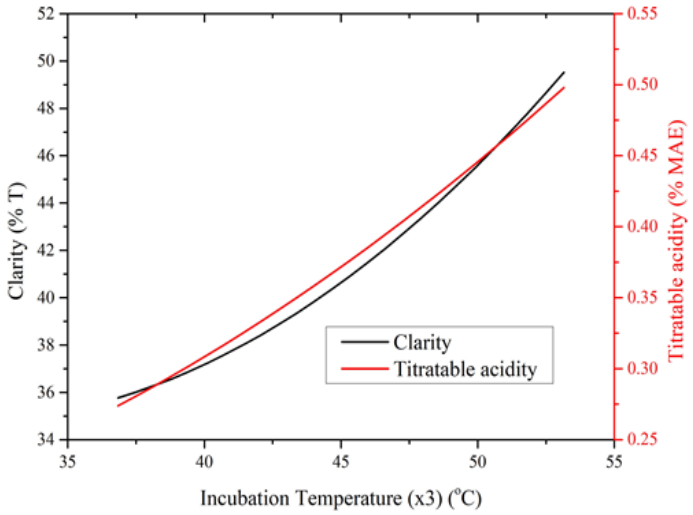


Figure 11: Evolution of responses as a function of factors: (c) Incubation temperature (incubation time and enzyme concentration fixed respectively at 30 min and 0.015%)

The increase in juice clarity with incubation temperature is explained by the fact that optimal temperature of pectinases is between 40 and 55 °C (Karangwa *et al*., 2010). An increase in the temperature in this range allows for a more efficient pectinases action upon hydrolysis of pectin, and thus an increasingly clear juice hence the increase in clarity.

Increase in titratable acidity is justified by the fact that further hydrolysis of pectin by pectinases with increasing incubation temperature allows for a greater release of galacturonic acids

and other organic acids in the juice, hence, an increase in the titratable acidity of this juice at the same time.

2.4.2.1.2. Effect of interactions on responses

Interaction of factors had a significant impact on responses (Table 10) as follows: Clarity and titratable acidity for interaction x_1x_2; Yield, clarity and titratable acidity for interaction x_1x_3; pH, total soluble solids and titratable acidity for interaction x_2x_3.

❖ **Effect of interaction x_1x_2**

The significant impact of interaction x_1x_2 (incubation time/enzyme concentration) is presented in Figure 12. It was observed that, the simultaneous increase in incubation time and enzyme concentration contributed to significant increase in clarity (from 35.77 %T to 97.89 %T) and titratable acidity (from 0.273 % MAE to 0.508 % MAE) indicating a synergistic effect between both factors. This is quite normal because incubation time and pectinase concentration are considered as key parameters for pectin hydrolysis. Knowing the product of the reaction of pectinase on pectin and the consequences on the juice clarity, it is therefore normal to obtain an increase of both responses. This was obtained at the same incubation temperature of 36.84 °C.

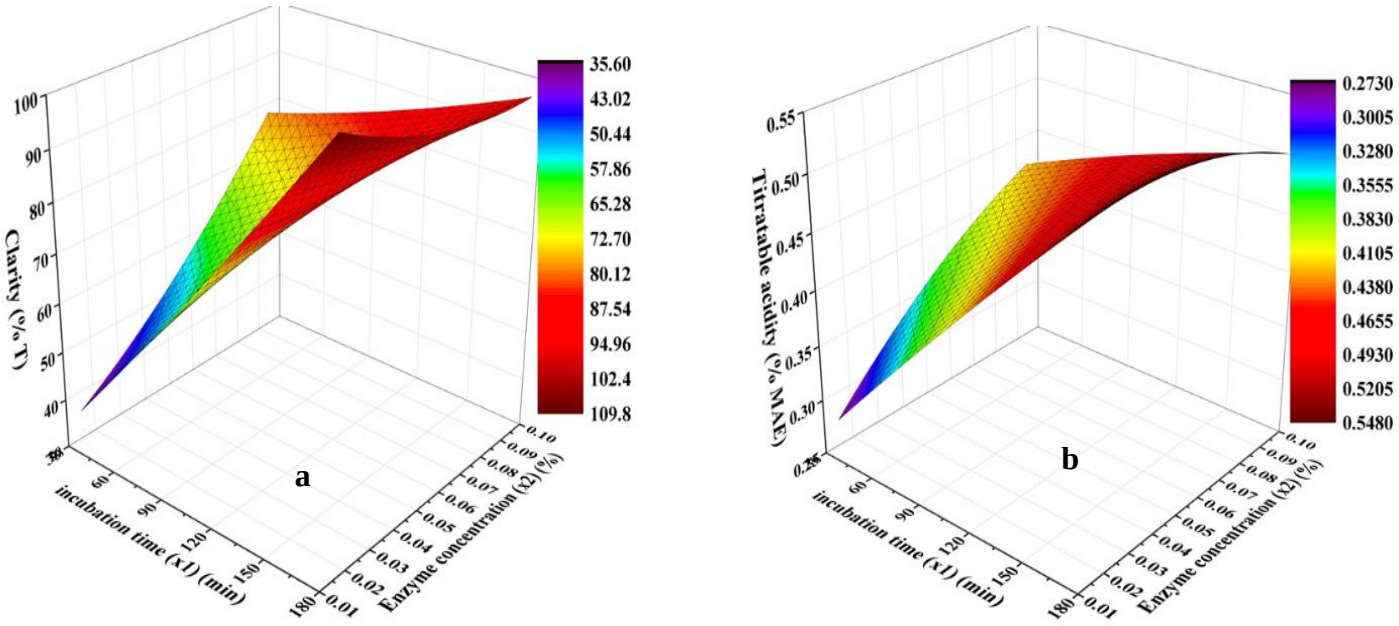


Figure 12: Mesh plot of clarity (a) and titratable acidity (b) as a function of incubation time (x1) and enzyme concentration (x2) (incubation temperature fixed at 36.84 °C)

❖ **Effect of interaction x_1x_3**

The impact of interaction x_1x_3 (incubation time/incubation temperature) is shown in Figure 13. It was observed that the factors concerned with the interactions increased all the responses (yield, clarity and titratable acidity) simultaneously. As mentioned previously, the higher the incubation time and incubation temperature within the experimental range, the better and faster the pulp hydrolysis resulting in an increase in juice extraction yield (because of pulp degradation by pectinase and then juice release (Yusof and Ibrahim, 1994)), clarity (because of better decantation of trub) and titratable acidity (because of galacturonic and other organic acids release (Bitange *et al*., 2009)).

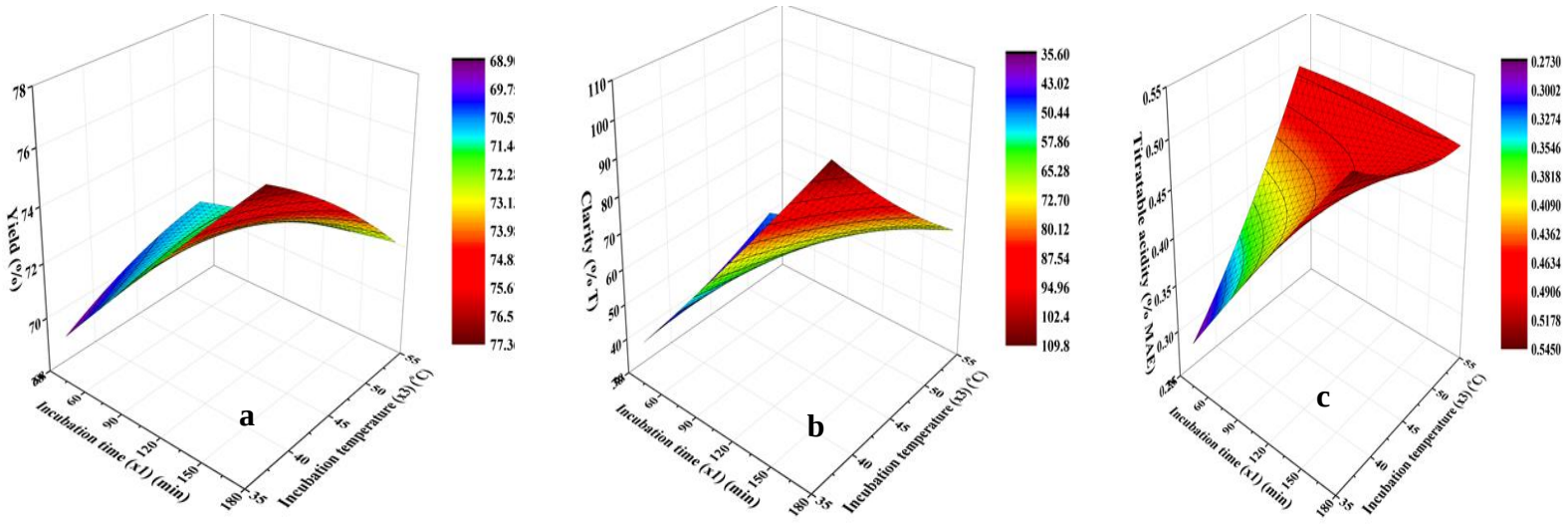


Figure 13: Mesh plot yield (a), clarity (b) and titratable acidity (c) as a function of incubation time (x1) and incubation temperature (x3) (enzyme concentration fixed at 0.015 %)

❖ **Effect of interaction x_2x_3**

It is observed from Figure 14 that the impact of the interaction x_2x_3 (enzyme concentration/incubation temperature) was significant on pH, TSS and titratable acidity. The simultaneous increase in value of both factors contributed to increase in TSS and titratable acidity while decreasing in pH value. In fact, an efficient enzyme hydrolysis is related to its concentration and also its incubation temperature. In this case, an increase in TSS and titratable acidity was due to accumulation the products formed (soluble solids and galacturonic acid) upon hydrolysis (Bitange *et al*., 2009). Decrease in pH is obviously due to the release of galacturonic and other organic acids.

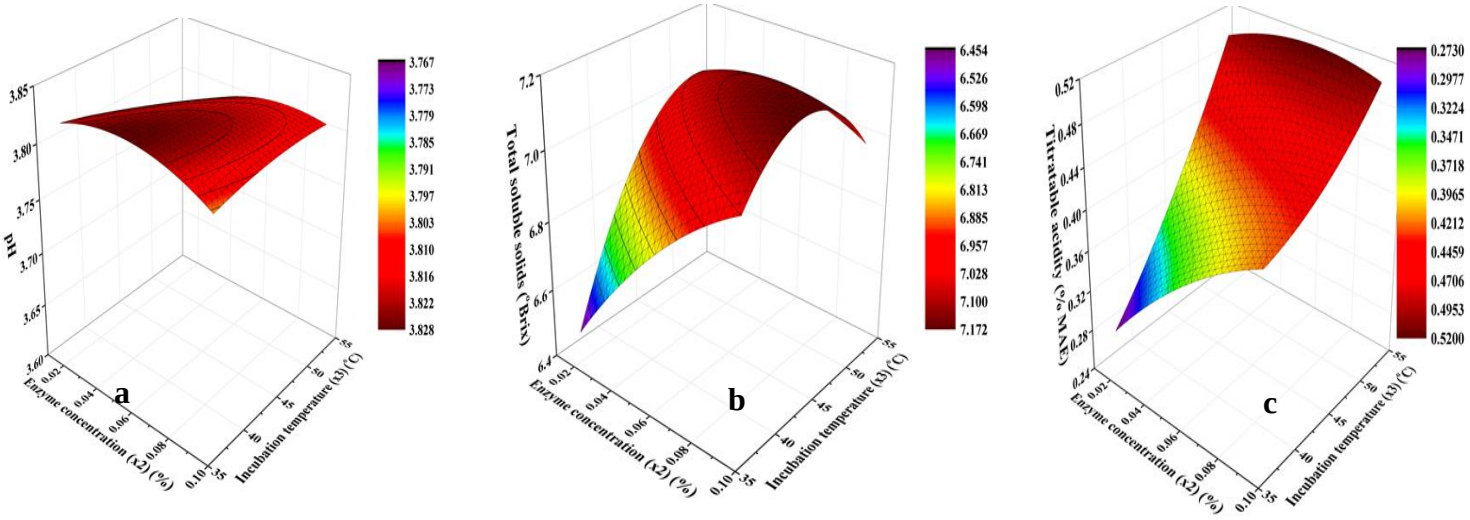


Figure 14: Mesh plot of pH (a), total soluble solids (b) and titratable acidity (c) as a function of enzyme concentration (x2) and incubation temperature (x3) (incubation time fixed at 30 min)

2.4.2.2.Optimization and verification of the predicted optimum

The optimum conditions for juice maximum yield, maximum pH, maximum clarity, maximum TSS and minimum titratable acidity were found to be different. Therefore, there was need to find a predicted composite optimum from the models via the desirability approach, which should take into account all responses (yield, pH, clarity, TSS and titratable acidity). The model simulation allowed to obtain the theoretical optimum conditions for extraction of *A. muricata* juice with deisirability of 0.94 as follows: 172.22 min, 0.0398%, and 42.15 °C for incubation time, enzyme concentration and temperature, respectively. Under theoretical conditions, the predictive responses were 75.23 %, 3.746, 87.06 %T, 7.35 °Brix, and 0.44 % MAE for juice yield, pH, clarity, TSS and titratable acidity, respectively. Using the optimum conditions, the following experimental results obtained were 77.33 %, 3.64, 94.95 %T, 7.5 °Brix, and 0.46 % MAE for juice yield, pH, clarity, TSS and titratable acidity, respectively. These results were close to that predicted by the model

simulation, which implies that each model was quite precise in its prediction and confirmed once more the validation done using statistical tools.

2.4.3. Rheological changes during enzymatic hydrolysis

The rheological behavoiur before and after enzymatic hydrolysis was studied and is represented in Figure 15. The viscosity of the unhydrolysed and hydrolysed *A. muricata* were evaluated and the unhydrolysed was seen to have a greater viscosity. At shear rate of 0 – 50 s^{-1} the samples exhibited a pseudoplastic behaviour with yield stress, meanwhile after 100 s^{-1} the samples presented a Newtonian flow behaviour. This implies there is structural change that is why there is a sudden drop after 50 s^{-1}then the fluids transit to Newtonian fluids. Technologically, handling these fluids without any stress to pump will imply after the shear rate of 150 s^{-1}. From the shapes of the curves, we can predict a hysteresis loop which means it is a time dependent rheological fluid. The pseudoplastic pattern is similar to what was obtained by Quek *et al*., (2013) using soursop juice concentrates with greater effect seen on samples with more than 40 ^{0}Brix. Gratão *et al*., (2007) also experienced same flow behaviour.

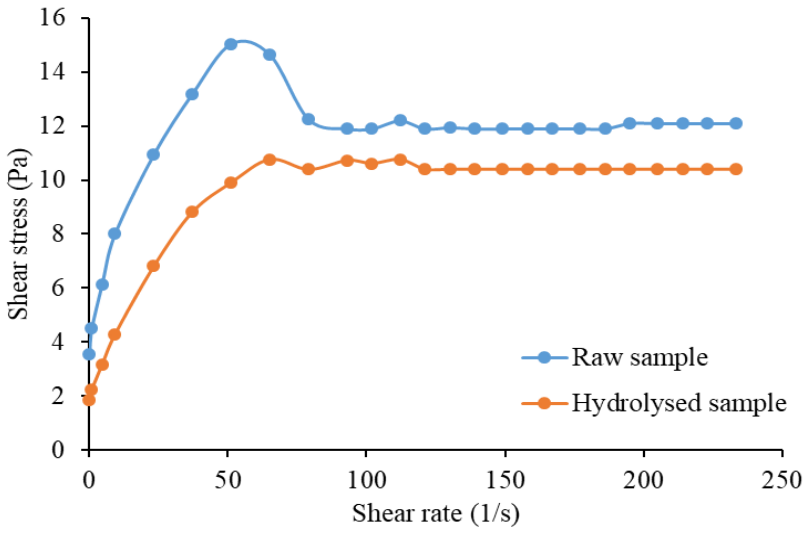


Figure 15: Representation of the shear-stress plots for the unhydrolysed and hydrolysed samples

2.4.4. Morphological analysis

In order to better understand the effect of pectinase on the structure of the pectin during *A. muricata* juice extraction, structural changes in unhydrolysed and hydrolysed *A. muricata* powder on the one hand, and pectin from unhydrolysed and hydrolysed fruit pulp, on the other hand, were visualised using SEM (Figure 16-19). The respective observed physical structures are shown in Figure 16, 17, 18 and 19. In Figure 16, unhydrolysed fruit powder presented a non-uniform spherical surface, and this was ascribed to the entrapmeent of ice in intermolecular spaces of the *A. muricata* during lyophilisation at the point of freezing of the free water, limiting the molecular movement of polysaccharides linked to bound water. This observation was also reported for guava pulp structure (Osorio *et al*., 2011).

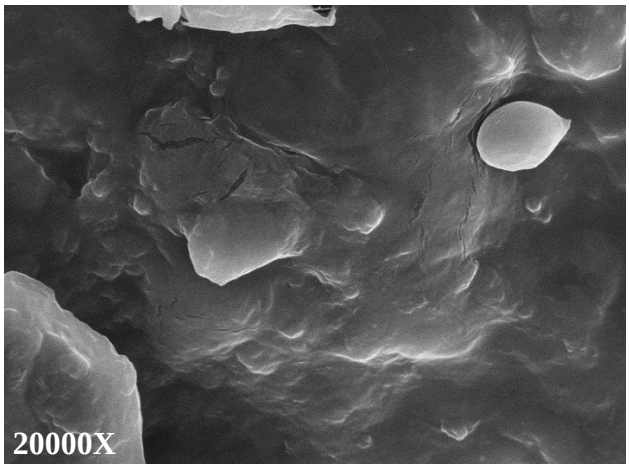


Figure 16: Micrograph of A. muricata

In contrast, hydrolysed fruit powder (Figure 17) presented a ruptured, coarse and wrinkled surface compared to the unhydrolysed (Figure 16), which still maintained all its fibrous structures. The damaged structure is linked to pectinase hydrolysis of pectic substances like polygalacturans which make up the middle lamella and are bound by α-1-4-glucosidic bonds of galacturonic acid units (Platt-Aloia *et al*., 1980). The hydrolysis of pectic substances caused the collapse of the

middle lamella and the loss of structural matrix of the cell walls resulting in the release of more solutes from *A. muricata* and increase of yield of *A. muricata* juice extraction. Therefore, the destruction of *A. muricata* cell walls with the help of pectinase was beneficial for releasing solutes, which were previously detained in the plant cell structure.

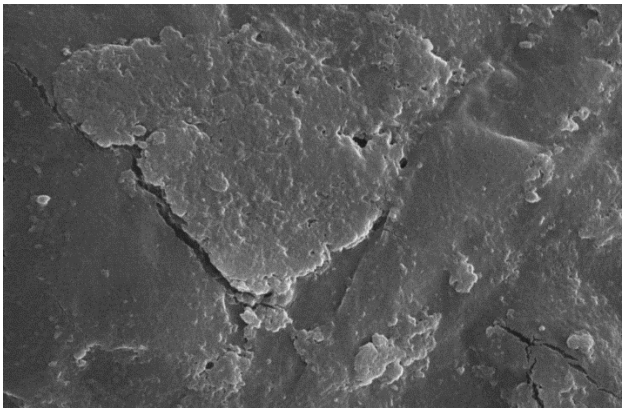


Figure 17: Micrograph of hydrolysed A. muricata

The image of pectin extracted from *A. muricata* pulp (Figure 18) was found to be porous. This is presumed to be as consequence of the high incubation temperature (90 °C) which generates a disintegration in the structure leading to a thinner surface (Zhongdong *et al*., 2006).

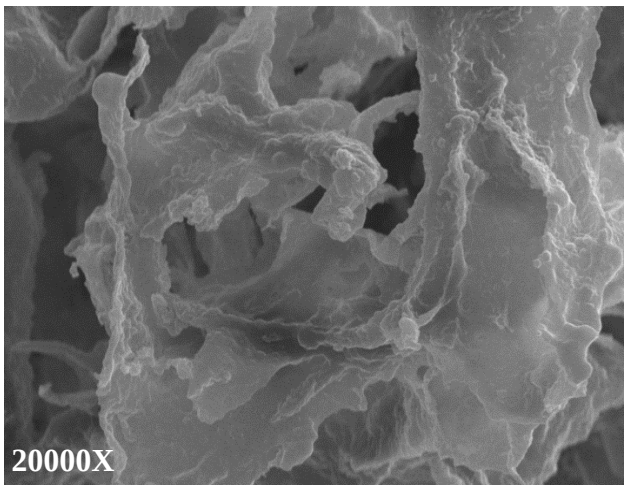


Figure 18: Micrograph of A. muricata pectin

Pectin from *A. muricata* is low methoxyl and its destruction is responsible for the whirling rough surface presented by hydrolysed *A. muricata* pectin in Figure 19 which is predicted to be as a result of the removal of methyl esters leading to degradation of the galacturonic acid linkages and calcium-mediated cross-links between pectins hence total structural rupture. This corroborates with observations reported by Devaux *et al*., (2005).

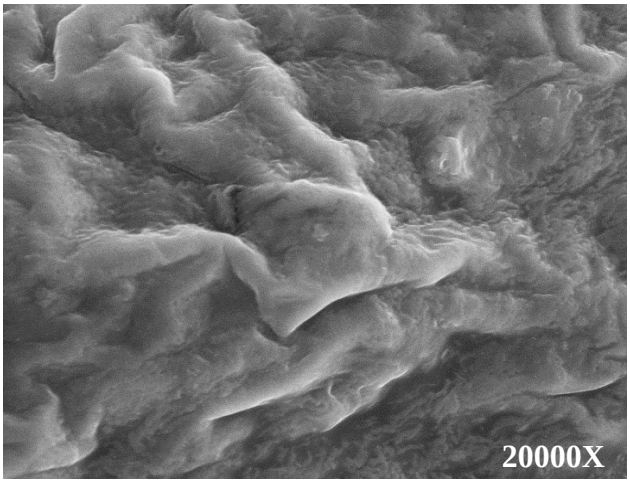


Figure 19: Micrograph of hydrolysed A. muricata pectin

2.4.5. Structural analysis

The FTIR spectra of commercial pectin, pectin from unhydrolysed and hydrolysed *A. muricata A. muricata* pulp are shown in Figure 20. FTIR spectra of different pectin samples have characteristic peaks at 3390.6, 2939.0, 1749.0, and 1052.1 cm^{-1} corresponding to –OH, –CH, C=O of ester and acid, and –COC– stretching of galacturonic acid (Kalapathy and Proctor, 2001; Karaca *et al*., 2012). The esters are not on the backbone but rather on the side chains because there is the presence of glucose molecule with groups on it which esterifies the pectin group hence hydrolysis can easily be observed with the change in acidity than viscosity. The intensity of bands between 3400 – 2900 cm^{-1} was higher in pectin from hydrolysed pulp, confirming the hydrolysis of glycosidic bonds and exposition of free –OH to a greater extent. This is in agreement with the findings of Xu *et al*.,

(2018), who observed broad absorption peaks at 3410 cm^{-1} (hydroxyl groups) and weak bands at 2920 cm^{-1} (C-H stretching) for jackfruit pectin. Likewise, Manrique and Lajolo, (2002) also reported the same stretching at 3400 cm^{-1} and 2930 cm^{-1} bands for pectin isolated from ripe papaya fruit. The results obtained in this work for commercial pectin and unhydrolysed *A. muricata* pectin are in accordance with the findings of Xu *et al*., (2018) where their FTIR data confirmed that methyl-esterified forms existed predominantly in pectin samples. Other studies revealed the assignment of C=O stretching vibration of the methyl-esterified carboxyl groups of bands at 1750 cm^{-1} for soy hull pectin (Kalapathy and Proctor, 2001), at 1746 cm^{-1} for jackfruit pectin (Xu *et al*., 2018) and 1737 cm^{-1} for the cell wall pectin fraction of ripe strawberry fruit (Posé *et al*., 2012). FTIR spectra of both samples showed a good match with the spectrum of commercial pectin. The relatively weak intensity of the Raman bands at 1470, 1183, and 1165 cm^{-1} evidenced that pectins from hydrolysed and unhydrolysed fruit were acetylated (Figure 3). The region of 1200–1000 cm^{-1} contained skeletal C–O and C–C vibration bands of glycosidic bonds and pyranoid ring, and this is in agreement with the works of Kalapathy and Proctor, (2001). The band intensity of ring vibrations and C-O stretching was accentuated in pectin from hydrolysed *A. muricata*. In this zone the signal for polysaccharides are observed which implies that we are dealing with pectins and the intensity is greater for the unhydrolysed than the hydrolysed pectin confirming pectin degradation. Moreover, the intensity of band of glycosidic bonds was higher in commercial and unhydrolyded fruit pectin, evidencing the breakdown of glycosidic bonds by pectinase during liquefaction. The results obtained in this work for commercial and unhydrolysed *A. muricata* pectin are in accordance with the findings of Xu *et al*., (2018), whose FTIR data confirmed that methyl-esterified forms existed predominantly in pectin samples. Therefore, FTIR spectra revealed evidence of the breakdown of *A. muricata* pulp pectin during liquefaction.

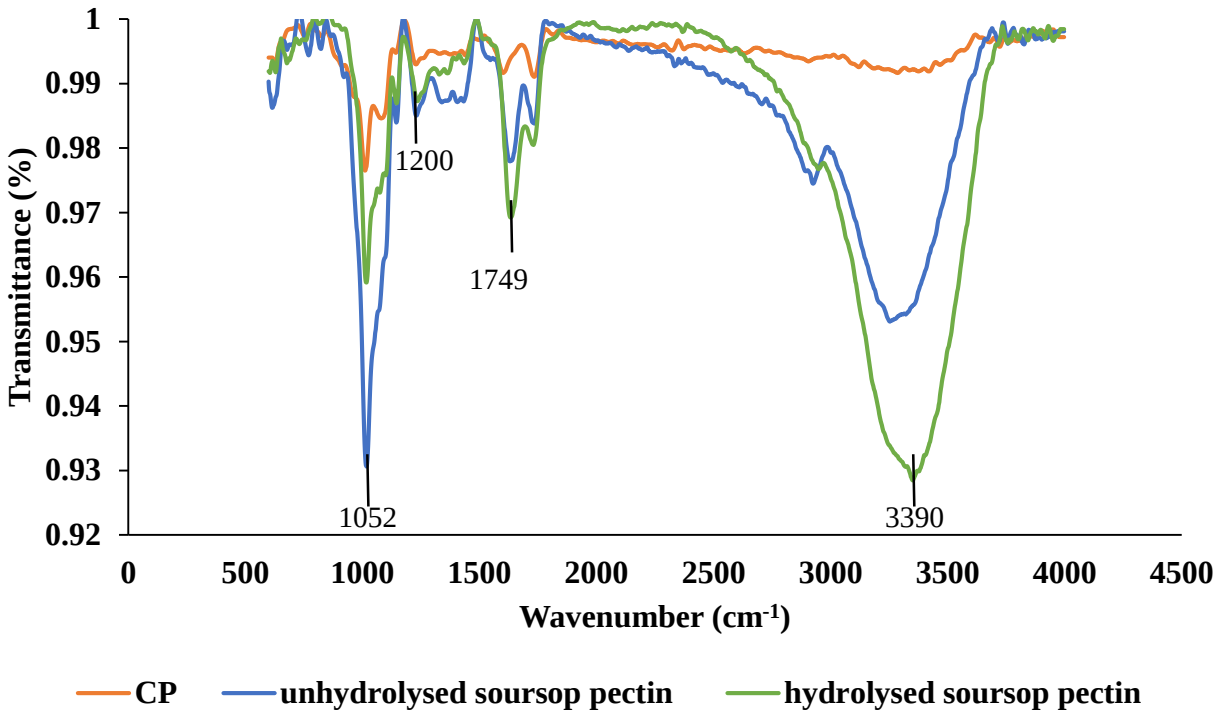


Figure 20: FTIR spectra for unhydrolysed A. muricata pectin, hydrolysed A. muricata pectin and commercial citrus pectin

Table 13 presents the different major functional groups of the pectin structure obtained at various wavelength in comparison with other research findings.

Table 13: Transmittance of FTIR spectra for pectin from A. muricata pulp, hydrolysed A. muricata pulp and commercial pectin of major groups

Wavenumber (cm^{-1})	**Major functional groups**	**Reference**
3390.6	-OH stretching vibration	Xu *et al*. (2018)
2939.0	C-H stretching	Xu *et al*. (2018)
1749	C=O stretching vibration of alkyl ester	Manrique and Lajolo, (2002)
1470	COO^- symmetric stretching	Szymanska-Chargot and Zdunek, (2013)
1200	C-O-C ring	Kalapathy and Proctor, (2001)
1183	C-O stretching vibration	Posé *et al*. (2012)
1165	O-C-O asymmetric stretching	Szymanska-Chargot and Zdunek, (2013)
1052.1	C-C stretching	Szymanska-Chargot and Zdunek, (2013)
1000	C-C vibration of pyranoid ring	Kalapathy and Proctor, (2001)

2.5. Conclusion

The current study optimised the pectinase-assisted extraction of *A. muricata* juice. The optimum conditions of the *A. muricata* juice extraction process obtained were 172 min of incubation time, 0.04% (w/w) of enzyme concentration, and incubation temperature at 42.9 °C. The combination of these optimal conditions resulted in 75.2% yield, pH of 3.75, TSS of 7.35 °Brix, 87.06% T clarity, and titratable acidity of 0.46% MAE. With the optimal conditions, the numerical predictions were similar to the experimental data obtained, which were in the range 0.9047 to 0.9874, thus confirming the validity of the models. The morphological analysis using SEM revealed that pectinase hydrolysed the pectin in *A. muricata* and improved the juice extraction process. The unhydrolysed and hydrolysed fruit pulp contain galacturonic acid with a higher level in the hydrolysed fruit pectin. This structural variation was attributed to the hydrolysis of the fruit pectin. This study provided the optimised conditions for pectinase-assisted extraction of *A. muricata* juice, which could be one of the promising methods for the value addition of *A. muricata*.

CHAPTER 3: MODELLING OF THE FERMENTATION PROCESS OF ENZYMATICALLY EXTRACTED *ANNONA MURICATA* L. JUICE

3.1.Abstract

Traditional liquid-state fermentation processes of *Annona muricata* L. juice can give fluctuating product quality and quantity due to difficulties in control and scale up. This chapter describes a laboratory-scale batch fermentation process for the production of a probiotic *Annona muricata* L. enzymatically extracted juice. It aimed at better understanding of the traditional process, as an initial step for future optimisation. *Annona muricata* L. juice was fermented with *L. acidophilus* (NCDC 291) (LA), *L. casei* (NCDC 17) (LC) and then with a mixture (LCA) for 72 h at 37 ℃. Experimental data were fitted into mathematical models to describe biomass growth, sugar utilisation and organic acid production. Optimum fermentation time was realised based on cell viability which was 24 h for LC and 36 h for LA and LCA. The model was particularly effective in estimating the biomass growth, reducing sugar consumption, and lactic acid production. With the kinetic model proposed by Luedeking and Piret for lactic acid production rate, the growth associated and non-growth associated coefficients were determined as 1.0028 and 0.0109 respectively. The model was demonstrated for batch growth of *L. acidophilus*, *L. casei* and then the consortium in *A. muricata* juice. The present investigation validates the potential of *A. muricata* based medium for heightened economical production of a probiotic medium.

Key words: *L. acidophilus***,** *L. casei*, Fermentation, Modelling, Kinetics.

3.2.Introduction

Consumption of functional foods has gained a growing interest since it provides health benefits beyond the basic nutritional features of fresh foods. Nowadays, fruit-based fermented juices are by far one of the most active functional food category (Corbo *et al*., 2014), especially due to the current tendency to veganism and vegetarianism among consumers and also to lactose intolerance caused by dairy products. Fermented fruit juices are traditionally produced by using LAB. LAB have traditionally and extensively contributed to biologically preserve foods due to their acidification ability through the synthesis of organic acids, thus preventing the development of future contaminants and harmful microorganisms. This extends the shelf life of food products. The use of carefully selected LAB strains as starter cultures for fruit and vegetable fermentation not only preserve food quality but also contributes to initiate in situ expression of desired sensorial attributes which can enhance their nutritional properties and promote human health care. Liquid fermentation technique is the main traditional process that has been used for millennia, for production of fermented juice. The yield and quality of liquor however fluctuates, and the process is time and labor consuming (Di Cagno *et al*., 2013). Therefore, process engineering studies are needed to understand and improve process design, monitoring and control. In this perspective, mathematical modelling can pave the way for design, monitoring and scale up of lactic fermentation process of *A. muricata* fruit.

In the last 10 years, there has been an increasing interest in modelling the kinetics of beneficial microorganisms in food systems. Mathematical modelling has been used more often to predict growth or inactivation of spoilage bacteria and pathogens. Recently attention has however been paid to biokinetics of beneficial food grade microorganisms, such as lactic acid bacteria. Growth or non-growth-related models are also applied to describe the changes of other biochemical compounds and physical properties in these food systems (Charalampopoulos *et al*., 2009). These

changes include primary or secondary metabolites concentrations, volatile production as well as rheological and textural properties (Bouguettoucha *et al*., 2007). The aim of these models is to mathematically relate the biochemical properties (response variables) to environmental factors (controlling factors), such as substrate composition and pH. This contributes to a better understanding and control of the fermentation process. LAB research has so far focused on modelling the dependence of the growth rate on temperature and pH at pH-controlled conditions. Very little research has been done in the modelling of growth when pH is not controlled, or taking into account other bio-kinetic parameters, such as lactic acid and bacteriocins production (Vázquez and Murado, 2008). Therefore, the aim of this study was to determine mathematical models that best fit the experimental data that would be able to simulate the kinetics of cell growth, lactic acid production and reducing sugar consumption in *A. muricata*-based fermentations using LAB.

3.3.Materials and Methods

The extracted *A. muricata* juice was fermented to obtain a probiotic beverage based on the following work plan

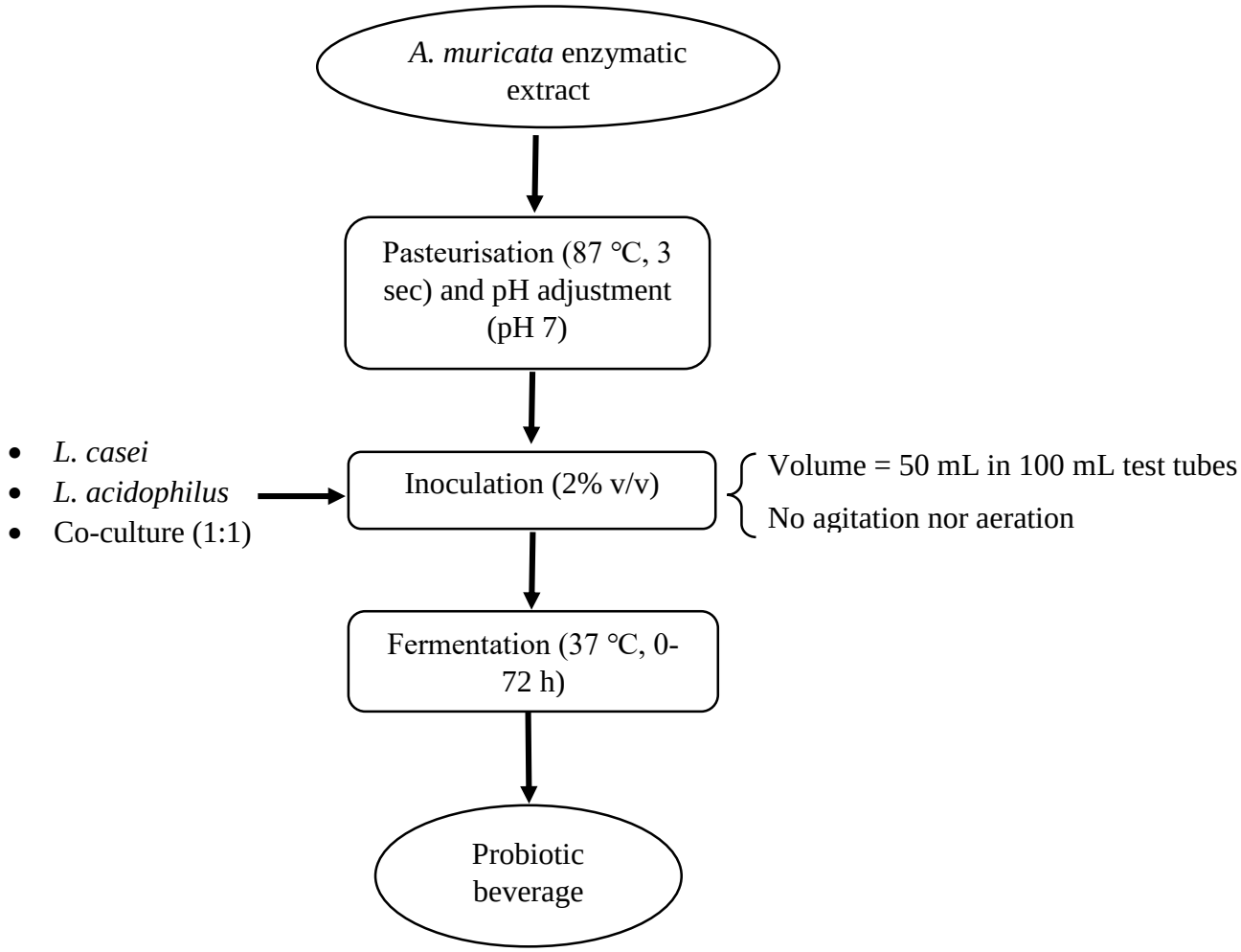


Figure 21: Block diagram of the fermentation process

3.3.1. Microorganisms and chemical reagents

Freeze dried *L. acidophilus* (NCDC 291) and *L. casei* (NCDC 17) strains were purchased from the National Collection of Diary Cultures (NCDC), Karnal, India. These microorganisms were chosen because *L. acidophilus* and *L. casei* can grow in fruit matrices as they survive in acidic

environments (pH ranging 4.3 to 3.7) as well as the fact that *L. casei* complements the action of *L. acidophilus* (Peres *et al*., 2012; Tripathi and Giri, 2014).

Man Rogosa and Sharp (MRS) broth, nutrient agar, sodium chloride, sodium hydroxide, sodium bicarbonate, Folin-ciocalteu and DNS were purchased from Himedia (Bengaluru, Karnataka, India).

Sterile PTFE filters (0.22 µm), sterile syringes and pH paper were procured (Bengaluru, Karnataka, India).

3.3.2. Fermentation process

3.3.2.1. Strain and inoculum preparation

Freeze dried bacterial strains were reactivated by sub-culturing in MRS broth for 48 h, cells were collected by centrifugation (5000 g, 10 min, 4 ℃), washed twice with sterile saline water (0.85% NaCl) then re-subcultured in MRS broth for another 48 h, collected, washed and resuspended in saline water as described by Bao et al., 2010 without any modifications. The bacterial suspensions were plate counted using MRS agar then used as inocula for the fermentation studies where 2% v/v of the bacterial stock with a microbial load of 10^9 CFU/mL was obtained from an 18 h pre-cultured cells.

3.3.2.2. Fermentation

Fruit juice sample extracted using the optimal conditions of the extraction process was used for fermentation process. Fermentation was done by inoculating two lactic acid bacteria strains (separately then a consortium) into the pasteurised fruit juice (87 ℃, 3 sec) with stabilised pH at 7 using a pH-meter (pH700; Eutech, Ayer Rajah Cresent, Singapore) by addition of sodium bicarbonate and readings taken at various times from 0 to 72 h. The bioreactors or fermentors used were 100 mL test tubes with 50 mL working volume of *A. muricata* juice. The bioreactors were

sealed with rubber stopper caps. The fermentors were mixed on a New Brunswick G10 Gyrotory shaker at 120 revolutions/min. All bioreactors were placed slated (in order to increase the surface area for the action of the microorganisms) in a CO_2 incubator (MCO-20AIC, ThermoFisher Scientific, Tokyo, Japan) maintained at 37 ℃ with 5% CO_2 as well as there was neither agitation nor aeration hence there was neither transfer of heat nor mass. The main objectives of agitation in fermentation are to disperse the air bubbles, to suspend the microorganisms, and to enhance heat and mass transfer in the medium. The samples were analysed for viable counts, pH, total soluble solids, reducing sugars, organic acid, short chain fatty acids, titratable acidity and total polyphenol content.

3.3.2.3. Chemical and biological analyses

3.3.2.3.1. Viable counts determination

Viable counts of the LAB were determined by pour plate count method using MRS agar after serial dilution to maximum recovery and also by taking the optical density at 600 nm. The MRS agar plates were incubated anaerobically for *L. acidophilus* and aerobically for *L. casei* at 37 ℃ for 48 h in a CO_2 incubator (MCO-20AIC, ThermoFisher Scientific, Tokyo, Japan) and the colony forming units estimated (Bao *et al*., 2010). Survival rate was calculated according to the following equation:

$$Viable\ count = \frac{Number\ of\ colonies * plated\ volume}{dilution\ factor} \qquad [20]$$

3.3.2.3.2. Lactic determination

Organic acids were quantified using HPLC according to the method of Arun *et al*., (2017). The solutions of soursop extracted juice before and after fermentation for each strain and the reference compounds (1mg/mL) were analysed following filtration via a 0.45µm PTFE filter into 2 mL vials then injected into the HPLC system. This evaluation was done on a Prominence UFLC system (LC-20AD, Shimadzu, Tokyo, Japan), with an LC-20AD system controller, Rezex-RHM-

Monosaccharide + H column (300*7.8 mm), a Rheodyne injector (USA) with a loop of 20 µL volume, a refractive index detector (SPD-M20A) and a column oven (SIL-20AC HT).

The mobile phase had solvent A: 0.1% formic acid and solvent B: water with the gradient programs 0–15 min 15% B, 16–20 min 50% B, 21–35 min 70% B, 36–50 min 100% B and lastly the column was restored in 20 min. The volume injected was 10 µL, and the flow rate was 0.8 mL/min. The 36 column was maintained at room temperature and the eluted fractions were monitored at 280 nm. Peaks of the samples analysed were identified via comparison with the retention times of the standard peaks. Data was obtained and analysed.

3.3.2.3.3. Assessment of short chain fatty acids (SCFA)

SCFA production at different time intervals was analysed and quantified by UHPLC following the method of Guerrant *et al*. (1982) with some modifications. The supernatant and the standards where filtered through 0.45 µm PTFE filter; 20 µL was injected into the UHPLC system. The analysis was performed on a Prominence UFLC system (LC – 20AD, Shimadzu, Tokyo, Japan) equipped with LC-20AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) and a diode array detector (SPD-M20A). The mobile phase used was 10% acetonitrile with pH 2 (pH adjusted using ortho phosphoric acid). The flow rate was 0.5 mL/min; the injection volume was 20 µL and the column was kept at 37 °C. The fractions were monitored at 200 nm. Sample peaks were identified by comparing with the retention time of standard peaks and also by spiking the sample with standard SCFA. LC LabSoluton software (Shimadzu Corporation, Tokyo, Japan) was used for data acquisition and analysis.

3.3.2.3.4. Total Polyphenol content (TPC)

Samples were analysed as described in the chapter two.

3.3.2.3.5. pH and Titratable acidity

Samples were analysed according to AOAC method as described by AOAC (2005) in chapter two. All experiments were done in triplicates.

3.3.3. Kinetic Modelling

In the current study, the kinetic models evaluated were rate equations of unstructured models for batch fermentation based on: cell growth rate, substrate utilisation and product formation. The parameters in the models were fitted to experimental data, obtained from the fermentation experiments using MATLAB software (R2018b, MA, USA). The simplest model which is the unstructured distributed model was used based on the following assumptions:

- Cells were represented by a single component which was cell number.
- The population of cellular mass was uniformly distributed throughout the medium. The cell suspension could be regarded as a homogeneous solution. The heterogeneous nature of cells was ignored (Dutta, 2008).
- All other nutrients (except reducing sugars) are presumed to be present in excess of the requirements for growth and, hence, their concentrations do not limit the growth rate.
- All cells in the medium are at the same growth and physiological state.

*3.3.3.1.**Growth kinetic***

Several models are involved in the kinetic modelling of cell growth which were basically derived from Monod's model (Monod, 1949).

- **Monod model**

The Monod equation empirically fits a wide range of data satisfactorily and is the most commonly applied unstructured, unsegregated model of microbial growth (Shuler and Kargi 2002). The Monod equation has remained most widely used in microbiology because of its simplicity and its

similarity to the familiar Michaelis-Menten equation for enzyme kinetics (Owens and Legan, 1987). Monod described the rate of increase of biomass as a function of biomass only (Monod, 1949).

$$\frac{dX}{dt} = fX \qquad [21]$$

Where fX = μX

The specific growth rate, μ, was expressed as a function of the limiting substrate concentration, S, by Monod equation:

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad [22]$$

Hence the first rate equation is as follows

$$\frac{dX}{dt} = \frac{\mu_{max} S}{K_s + S} X \quad [23]$$

While the Monod equation is an oversimplification of the complicated mechanism of cell growth, it often adequately describes fermentation kinetics when the concentrations of those components which inhibit the cell growth are low.

According to the Monod equation, further increase in the nutrient concentration after μ reaches μ_{max} does not affect the specific growth rate. However, it has been observed that the specific growth rate decreases as the substrate concentration is increased beyond a certain level (Dutta, 2008).

- **Logistic model**

Characterization of cell growth in several microbial fermentation processes is well studied using the logistic equation which is a substrate independent model (Rajasekar *et al*., 2015). Most microbial growth processes are simulated by the use of empirical logistic models especially in

cases where the cell population is inhibited by their own growth through nutrient depletion or limitation, accumulation of end product or any other undefined reasons (Kedia *et al*., 2007). Some authors have also modified this logistic model with a product model to obtain the Logistic-Luedeking Piret (LLP) model. The growth pattern of logistic kinetics can be described as

$$\frac{dX}{dt} = \mu_m X(1 - \frac{X}{X_m}) \quad [24]$$

Where X is the biomass concentration (CFU/mL), X_m is the maximum biomass concentration (CFU/mL), μ_m maximum specific growth rate (h^{-1}) and t is the time (h). Keeping the initial conditions of $X=X_0$ at t=0, integrating the above equation gives:

$$X = \frac{X_0 e^{\mu_m t}}{\{1-(X_0/X_m)(1-e^{\mu_m t})\}} \quad [25]$$

Rearranging gives

$$ln\frac{X}{(X_m-X)} = \mu_m t - ln\left(\frac{X_m}{X_0} - 1\right) \quad [26]$$

The value of μ_m and X_0 can be obtained from the slope and y-intercept of the plot between $ln\frac{X}{(X_m-X)}$ and time (t). The value of X_m is determined from the experimental data.

3.3.3.2. ***Production formation kinetics***

The main model for product formation from which all other kinetic models were derived for the same was Luedeking and Piret model (Luedeking and Piret, 1959)

- **Luedeking and Piret model**

The classic study of Luedeking and Piret (Luedeking and Piret, 1959) on lactic acid fermentation by *L. delbrueckii* indicated that the product formation kinetics combined both growth and non-growth-associated contributions.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad [27]$$

3.3.3.3. ***Substrate utilisation/consumption model***

The rate of sugar consumption is mainly based on three factors: the growth rate, lactic acid production and the rate of substrate uptake for cell maintenance. Substrate utilisation kinetics could be expressed as:

$$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\frac{dX}{dt} - \frac{1}{Y_{PS}}\frac{dP}{dt} - m_S X \qquad [28]$$

which considers substrate consumption for biomass, product and cellular maintenance.

The substrate requirement to provide energy for maintenance is usually assumed to be negligible. The rate equations stated in the previous section included many variables and parameters. Variables can be classified as state and operating variables. The state variables are the biomass concentration (X), the limiting substrate concentration (S), and the product concentration (P). The operating variables are the inlet concentrations of the biomass, substrate and product, X_0, S_0 and P_0 respectively.

Table 14: Kinetic models from literature combining biomass growth, product formation and substrate utilization

Microorganisms	Substrate	Product	Biomass growth	Product formation	Substrate utilisation	Reference
L. helveticus	Lactose from whey	Lactic acid	$\frac{dX}{dt} = \mu X$	$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$	$\frac{dS}{dt} = -\frac{1}{Y_{PS}}\frac{dP}{dt} - \left(\frac{\mu_{max}}{Y_{XS}} + m_S\right)X$	Biazar *et al.* (2003)
L. delbrueckii	Glucose	Lactic acid	$\frac{dX}{dt} = \mu X\left(1 - \frac{P}{P_{max}}\right)^h$	$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$	$\frac{dS}{dt} = -\frac{1}{Y_{PS}}\frac{dP}{dt} - m_S X$	Dutta *et al.* (1996)
L. plantarum	Lactose	Lactic acid	$\frac{dX}{dt} = \mu X$	$P = Y_{PS}(S_0 - S) + P_0$	$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\frac{dX}{dt}$	Fu and Mathews (1999)
L. delbrueckii	Glucose	Lactic acid		$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$		Luedeking and Piret (1959)
L. delbrueckii	Sucrose from beet molasses	Lactic acid	$\frac{dX}{dt} = \mu X\left(1 - \frac{P}{P_{max}}\right)$	$\frac{dP}{dt} = \left(\alpha \frac{dX}{dt} + \beta X\right)\left(1 - \frac{P}{P_{max}}\right)$	$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\frac{dX}{dt} - \frac{1}{Y_{PS}}\frac{dP}{dt} - m_S X$	Monteagudo *et al.* (1997)
S. cerevisiae	Sucrose	Ethanol	$\frac{dX}{dt} = \mu X$ $\frac{dX}{dt} = \mu X e^{-hP}$	$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$	$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\frac{dX}{dt} - m_S X$	Starzak *et al.* (1994)

Symbols

Greek letters

μ= specific growth rate (h^{-1})

μ_{max}= maximum specific growth rate (h^{-1})

β= non-growth-associated constant for product formation

α= growth-associated constant for product formation

Roman letters

k_s= saturation constant or half-velocity constant

k_i= substrate inhibition constant

S= substrate concentration (reducing sugar, mg/mL)

P= product concentration **(**organic acid concentration, mg/mL)

P_{max}= finite production concentration (mg/mL)

X= Biomass accumulation (log CFU/mL)

t= time

Y_{xs}= yield of biomass based on utilised substrate

Y_{ps}= yield of product based on utilised substrate

m_s= maintenance energy parameter (mg substrate/(log CFU/mL cell mass . h))

h= parameter used to describe product inhibition

$\frac{dX}{dt}$ =biomass concentration rate

$\frac{dP}{dt}$ =product formation rate

$\frac{dS}{dt}$ =substrate utilisation rate

Table 15: Coefficients of modelling parameters from literature

Microorganism	μmax (h^{-1})	Ks (g/l)	α	β	ms (h^{-1})	Yxs	Yps	Kp (g/l)	Ki (g/l)	Reference
L.helveticus	0.76	-	2.69	0.71	-	-	-			Amrane and Prigent (1997)
L.helveticus	-	-	4.26	0.5	-	-	-			Kulozik and Wilde (1999)
Lactococcus lactis	1.1	1.32	0.932	3.02	-	-	0.93			Boonmee *et al*. (2003)
L.helveticus	0.25	0.9	4.6	0.23	2.65	0.064	0.61			Biazar *et al*. (2003)
L. rhamnosus	0.633	0.3	6.6	0.33	-	1	1			Kwon *et al*. (2001)
L.helveticus	0.49		2.56	0.76	-	-	0.84			Amrane (2001)
L.helveticus	0.82	0.22	4.5	1.62	-	-	0.95			Schepers *et al*. (2002)
L.helveticus	0.21	-	-	-	-	0.08	0.71			Tango and Ghaly (1999)
L.helveticus	-	-	2.2	0.55	-	-	-		250	Luedeking and Piret (1959)
Lactococcus lactis	0.403	0.79	13.2	0.0645	0.0037	-	-	0.016	164	Akerberg *et al*. (1998)
L. delbrueckii	0.831	-	0.235	0.087	-	0.27	0.91			Monteagudo *et al*. (1997)
L. delbrueckii	0.0696	0.0967	0.3853	0.0032	0.00014	-	-			Dutta *et al*. (1996)
L. plantarum	0.364	44.4	-	-	-	-	1.02			Fu and Mathews (1999)
L. casei	0.994	-	1.332	0.035	-	0.409	0.834			Ha *et al*. (2003)
L. casei	1.23	0.203	-	-	-	0.115	0.977			Pinelli *et al*. (1997)

3.4. Results and discussion

3.4.1. Evolution of biological and chemical parameters of *A. muricata* during fermentation

The influence of fermentation on the biological and chemical parameters (cell growth, pH, organic acid, reducing sugar and total polyphenol contents) of *A. muricata* enzymatically extracted juice using *L. casei* (LC), *L. acidophilus* (LA) and a consortium of both microorganisms (LCA) is represented in Figure 22.

3.4.1.1.Viable cell counts

The growth of probiotic bacteria is important, and their viability is crucial for the quality and stability of fermented products. International standards stated that fermented products claiming health benefits must contain a minimum of 10^6 viable probiotic bacteria per gram or milliliter of product at the time of purchase (Kun *et al*., 2008).

In this study, the viable count graphs of *A. muricata* juice fermented with LC, LA and LCA showed similar patterns. During fermentation of *A. muricata* juice, the number of all potential probiotic strains used showed a significant increase (p <0.05) especially in the early stage of fermentation. The cell growth was in the range 2.4 to 9.1; 5.4 to 10.1 and 5.0 to 11.5 (log CFU/mL) for LC, LA and LCA, respectively. Concerning LC, the cell growth increased from 0 h to 24 h where the highest value of 9.1 was recorded then decreased at 72 h. Meanwhile for LA and LCA the maximum cell growth of 10.1 and 11.5 respectively, was obtained at 36 h then decreased til 72 h of fermentation. After 72 h fermentation, the viable counts of LC and LCA presented a higher increase (6.7 and 6.5 log CFU/mL) in fermented *A. muricata* juice than that of LA (4.7 log CFU/mL).

The lag phase was almost insignificant, and this could be attributed to the fact that there was an overnight subculture of the microorganisms in the juice at pH 7 hence the microorganisms had

adapted to the media. There was exponential growth phase for all samples meanwhile a stationary phase was observed after the 24 h for LC and for LA and LCA, then was a decline phase after 36 h.

After adapting to the environment, the microorganisms consumed the nutrients present in the medium and replicated causing cell growth. The cell growth entered decline or death phase upon depletion of nutrients, accumulation of metabolic product such as organic acids, acetaldehyde, oxygen, and hydrogen peroxide. Also, the accumulation of toxic oxygenic metabolites such as superoxide might cause the death of vulnerable cells (Wang *et al*., 2019). The higher bacterial cell concentration of *L. acidophilus* could be attributed to the fact that it is more acid tolerant than *L. casei* meanwhile *L. casei* has been reported to complement *L. acidophilus* justifiable to the highest value obtained for LCA. After 6 h of fermentation for samples LA and LCA and 12 h for LC, the bacterial cell concentration was above the recommended dose of 6.0 log CFU/mL for a probiotic product, acceptable to produce health benefits (Freire *et al*., 2017). Lactic acid bacteria also have as an essential growth factor manganese (Mn^{2+}) which was present in the samples (3.89 ppm) hence contributed to the growth of the various microbes. Dimitrovski *et al*. (2016) also reported the growth of *L. plantarum* CX-15 in Mn^{2+} supplemented Jerusalem artichoke juice. High bacterial viable cell concentration are important to obtain the desired end products such as acid production and reduction in pH, which affects organoleptic properties and shelf-life, and prevents product contamination (Santos *et al*., 2014). The results agree with that published by Fonteles *et al*., (2012) who obtained 8.93 log CFU/mL after 20 h of fermentation of cantaloupe melon juice with *L. casei* NRRL B-442 as well as Pereira *et al*. (2011) with same strain obtained an increment of 8.5 CFU/mL after 24 h in cashew apple juice. The result obtained in this study for *L. casei,* clearly demonstrates that this strain is also pH dependent because at pH 5 where the optimum viable cells

were obtained, it immediately followed with decrease in pH. The findings of Pereira *et al*., (2017) who produced probiotic cupuassu beverage using *L. casei,* registered a faster microbial growth (9 log CFU/mL) requiring lower fermentation periods (18 h) at pH 5.8. Costa *et al*., (2013) observed maximum cell viability using *L. casei* at pH 5.8 for sonicated pineapple juice meanwhile Zheng *et al*. (2014) observed same using litchi juice. The stationary phase of LC after 24h could be attributed to the increased acidity of the medium with *L. casei* not as tolerant as *L. acidophilus* to low pH.

As far as *L. acidophilus* is concerned, the results obtained in this study were in line with the increase cell viability (7.73-8.11 log CFU/mL) obtained by Salmerón *et al*. (2015) using barley, malt and oat. Babu *et al*. (1992) also reported an increase cell viability in skimmed milk supplemented with tomato juice using *L. acidophilus*. Same observation was made by Yoon *et al*. (2005) using tomato juice with neither nutrient supplementation nor pH adjustment.

The use of mixed cultures lactic acid bacteria could present symbiotic and/or synergistic effects which are exploitable. Therefore, *L. casei* and *L. acidophilus* strains which were selected to be co-cultured resulted in a better fermentation with larger probiotic bacterial population and a short fermentation period. A similar trend was observed after 24 h of fermentation of apricot juice with the viable cells (9 – 10 log CFU/mL) higher in the mixed culture than in the monocultures (Bujna *et al*., 2017). The stationary phase observed between 12 h and 24 h for LCA could be described as the diauxic growth phase. This implies that during the first logarithmic stage the microorganisms may have pumped acid into the medium via their metabolism rendering the medium acidic or have consumed a particular type of sugar to completion and needs to readapt before picking up with growth again while consuming another type of sugar.

The results obtained in this study proves *A. muricata* to be a good matrix (no need to supplement with growth requirements such as free amino acids, peptides, vitamins, and fermentable carbohydrates) for the production of probiotic beverage due to the increase cell viability.

3.4.1.2.Reducing sugars

The reducing sugar content is a significant factor in fermentation because it influences the growth of microorganisms, aroma development, and sensory characteristics of *A. muricata* juice amongst others. Fructose, glucose, and sucrose are the major sugars in *A. muricata* juice and can be used by LAB (Kelebek & Selli, 2011; Li *et al*., 2012). The reducing sugar contents were decreased after juice fermentation by LA, LC and LCA. It was noticed to decrease from 0 h to 72 h for all samples beginning with LC from 57.23 to 44.20 (g/L), LA obtained 56.55 to 38.19 meanwhile LCA decreased from 52.73 to 39.43 (g/L). Also, being an acidic fruit (pH less than 4.0), there is protonic activation which reduces the stability of the chain and causes the release of fructose and glucose via hydrolysis of fructo-oligosaccharides (Savedboworn *et al*., 2017). Therefore, the microorganisms consume these sugars while causing microbial growth, increase in organic acid production with consequent decrease in pH hence decrease in reducing sugars from 0 h to 72 h. This results were in accordance with the observation of Nguyen *et al*. (2019) where *L. acidophilus* amongst other lactic acid bacteria consumed fructose (from 2.65 to 1.78 mg/100mL) and glucose (from 2.94 to 2.33 mg/mL) highest amongst other sugars present in pineapple after 24 h of fermentation. Using carrot juice, Claudia *et al*. (2013) observed a very favorable growth in the cell population of *L. acidophilus* LA-5 upon consumption of the reducing sugars present in the juice (25g/L) with the highest sugar been glucose. Consumption rate of reducing sugar was fastest in LA compared to LC and LCA. This is corroborated to the findings of Nagpal *et al*. (2012) and Yoon *et al*. (2005) who reported the same observations by fermenting tomato juice using *L.*

acidophilus and *L. casei* amongst other lactic acid bacteria. The rate of the sugar consumption was higher during the first 18 h of fermentation for LA then slowed down during the next 54 h, meanwhile this could be attributed to pH and increase acidity as fermentation carried on.

3.4.1.3.Lactic acid

Probiotics can catabolise sugars via fermentation leading to the formation of organic acids, and these organic acids are important secondary carbon sources for numerous microbial genera that proliferate during food fermentation. The organic acid content impacts the balance of flavour, taste, colour, chemical stability, pH nutritional properties, acceptability, and storage quality of fermented juice (Ye *et al*., 2014).

The influence of fermentation on the production of organic acids illustrated a continuous increase from 0 h to 72 h for all samples where LC recorded 3.18 to 12.21 (g/L), 4.51 to 9.92 (g/L) for LA and 3.60 to 13.32 (g/L) for LCA. Lactic acid is known to be the main metabolite of lactic acid bacteria and acidification is one of the most desirable effects of their growth (Zheng *et al*., 2014). Claudia *et al*. (2013) reported the accumulation of 9g/L of lactic acid at the end of fermentation of carrot juice using *L. acidophilus* which was considered satisfactory for the shelf-life extension of the end product. The highest lactic acid content recorded for LCA could be as a result of the synergistic effect between the two bacterial strains used, which is linked to the relationship between the inocula and the substrate composition in the development of organoleptic properties of fermented products (Santos *et al*., 2014). It was reported that apricot juice fermented with mixed culture had similar trend attributable to an intense growth and metabolic activity of probiotic bacteria (Bujna *et al*., 2017). The mild sour flavour attribute in fermented beverages has been associated with the lactic acid concentration of the beverage above the threshold value of 0.93 g/L (Freire *et al*., 2017), which was the case in this study.

3.4.1.4.pH

Changes in pH were observed in the juice during fermentation from 7.20 to 4.57 for LC, 6.98 to 4.61 for LA and LCA had a range from 7.31 to 4.96, during the period of 72 h treatment. The sharp drop in pH which was observed in LA and LCA reaching 5.38 at 12 h of fermentation, could be attributed to the fact that *L. acidophilus* is an obligatory homofermentative bacterium that produces a single end product (lactic acid) from the glycolysis of carbohydrates (EM pathway) (Santos *et al*., 2014). Fu and Mathews, (1999) studied the effect of pH on lactose batch fermentation and concluded that low pH values (pH 4) of fermentation media inhibited bacterial cell growth meanwhile higher pH values (pH 5-7) accelerated the bacterial cell growth. Generally, pH drop as low as 4.5, is low enough to inhibit the growth of pathogens and many spoliating microorganisms (Zheng *et al*., 2014). The pH decrease of fermented beverages is related to lactic acid production during fermentation. It is advantageous in that pH between 3.5-4.5 in food formulations helps in increasing the pH of the gastrointestinal tract hence reinforcing the stability and benefits of the probiotic strains consumed (Freire *et al*., 2017). Similar trend of decrease in pH for fruit juices was reported by other researchers with different lactic acid bacteria: *L. casei* in fermented apricot (Bujna *et al*., 2017), cantaloupe melon juice (Fonteles *et al*., 2012), cupuassu beverage (Pereira *et al*., 2017), sonicated pineapple juice (Costa *et al*., 2013) and *L. acidophilus* in noni fruit juice (Nagpal *et al*., 2012).

3.4.1.5.Total polyphenol content (TPC)

One of the most abundant secondary metabolites in plants are polyphenol and very active in defence against ultraviolet radiation damages or pathogenic aggressions. The total polyphenol content (TPC) of fermented soursop juice are shown in Figure 22. The fermentation process was found not to have significantly imparted the total polyphenol content for all samples. There was a

slight increase for LC with values ranging from 1.41 to 2.05 (g/L GAE) meanwhile it was almost constant for LA from 1.53 to 1.77 (g/L GAE) and LCA registered 1.23 to 1.32 (g/L GAE). This stability of total polyphenol content during fermentation could be attributed to the fact that the polyphenol stability is highly favoured by the low pH of the medium. Increase pH increases the rate/risk of the auto-oxidation mechanism (Panda *et al*., 2017).

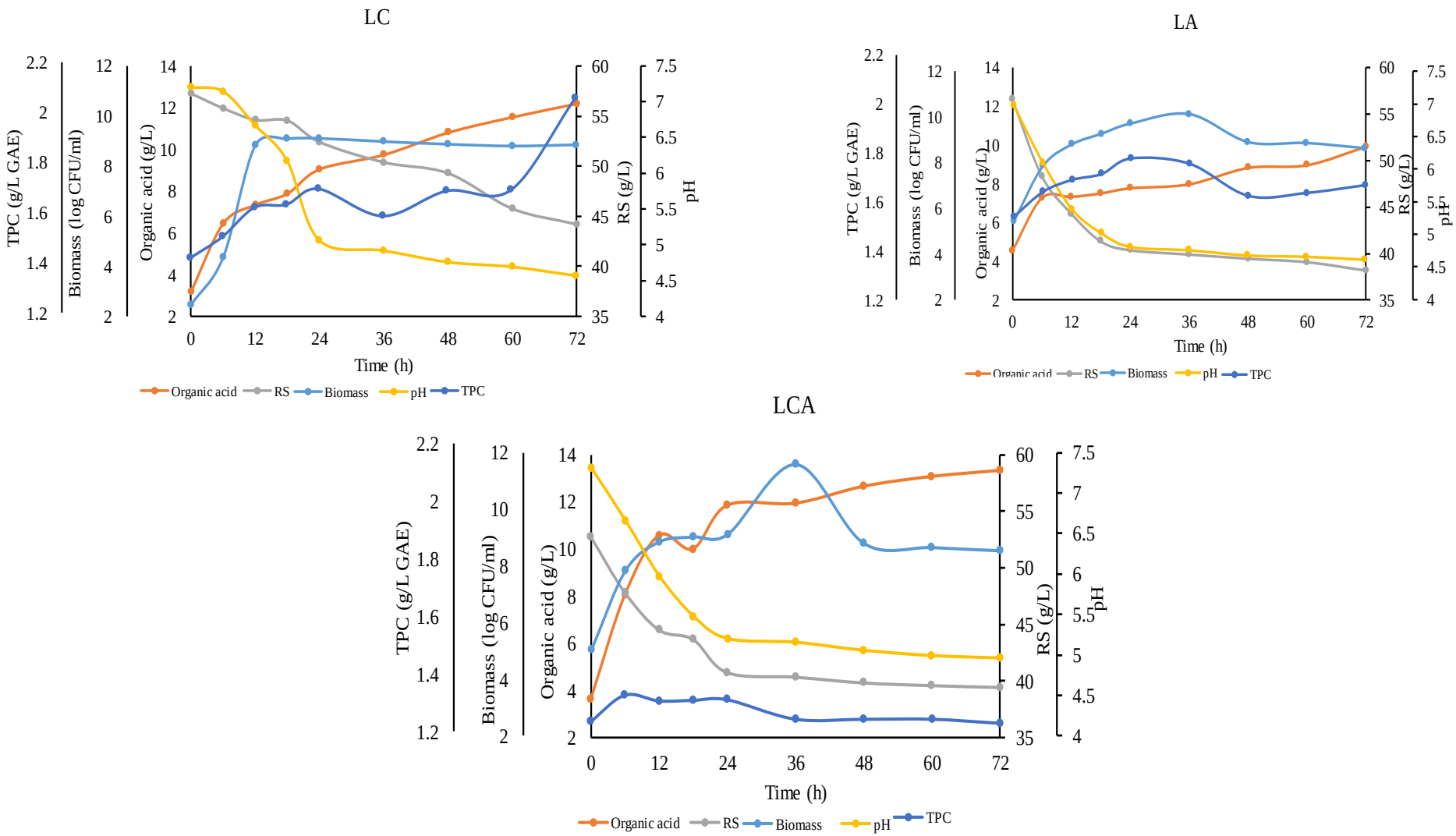


Figure 22: Changes in biochemical parameters during A. muricata fermentation for L. casei, L. acidophilus and a mixture of both microorganisms

3.4.2. Modelling of fermentation kinetic

A clear definition of a model structure (specification of model complexity) is a very essential aspect/element in mathematical modelling of the fermentation process. 'As simple as possible but not simpler' is the rule generally used which implies that the basic mechanisms should always be added and the aim of the model determines the model structure. Therefore, if the aim is to simulate the biomass concentration in a fermentation process (like the case of probiotics), a simple unstructured model may be sufficient. Although these models are completely empirical, they are useful for simple design problems and for the extraction of key kinetic parameter of the growth kinetics. If, on the other hand the aim is to simulate dynamic growth conditions one may turn to simple structured models e.g. the compilation of the compartment models, which are also useful for illustrating the concept of structured modelling.

Several coefficients of parameters or constants are generated from model computation. These parameters could be divided into two types namely the kinetic and the stoichiometric parameters. The kinetic parameters are the Monod parameters (growth model parameters), which are generally the specific growth rate, μ_m and saturation constant or half-velocity constant, k_s, and Luedeking and Piret equation parameters (product formation model parameters) which are α and β. The term α is the growth associated constant meanwhile β is the non-growth associated constant as stated by Luedeking and Piret (1959). Growth associated product formation could be defined as the simultaneous accumulation of end products and growth of microbial cells. Meanwhile the stoichiometric coefficients are the yield coefficients for biomass and product on substrate (substrate utilisation), the main constants are yield of biomass based on utilised substrate (Y_{xs}), yield of product based on utilised substrate (Y_{ps}). The maintenance energy parameter (m_s) is

another parameter mostly generated during modelling. Some assumptions were made as stated in the materials and methods.

3.4.2.1.Model fitting for biomass accumulation, substrate utilisation and product formation

Models developed as well as those from other researchers (Monod, Luedeking and Piret, Tessier, Gompertz etc) were assembled from the works of Biazar *et al*., (2003), Kumar Dutta *et al*., (1996) Monteagudo *et al*., (1997) and Starzak *et al*., (1994). Experimental values were fitted into these models for both biomass accumulation, production formation and substrate utilisation models and the author whose models best fitted from statistical comparisons was retained.

As presented in Table 16, the experimental data best fitted in the models of Biazar *et al*., (2003) which were Monod and Luedeking and Piret models. The determination coefficient, R^2 values as shown in Table 16 was 0.9946, 0.9913 and 0.9946 while the residual sum of square error, SSE was 0.2876, 0.1738 and 0.1589 for LC, LA, and LCA respectively. The growth kinetic parameters included maximum specific growth rate, μ_m which was 0.2876 h^{-1}, 0.1738 h^{-1} and 0.1589 h^{-1} as well as the substrate saturation constant or half-velocity constant, K_s with 9.0680 g/L, 9.9337 g/L, and 9.0709 g/L respectively for LC, LA and LCA. The μ_m was achievable when reducing sugar, s >> Ks and the concentration of all other nutrients were unchanged (Bailey and Ollis, 1986). The substrate saturation constant may be defined as the concentration of reducing sugars (growth rate-limiting nutrient) that supports half the maximum specific growth rate. The K_s indicates the rapidity of the microbial proliferation and its ability to attain μ_m with the reducing sugars being utilised (Dlangamandla *et al*., 2019).

As shown in table 15, the μ_m values obtained by other researchers were in the range 0.06 to 1.23 and 0.09 and 44.4 for K_s, hence the values obtained in this study felt within the above ranges.

For the product formation model, the growth associated parameter (α) was 1.0028, 0.7681 and 1.9127 while the non-growth associated term (β) was 0.0109, 0.0001 and 0.0035 for LC, LA and LCA respectively. From the experimental values, it is demonstrated that the organic acid (lactic acid) production during the fermentation of *A. muricata* was dependent on the growth of *L. casei, L. acidophilus* and the consortium of microorganisms because the values of β were close to zero hence conforms the growth associated product formation model. This results corroborates the findings of Ghosh *et al*., (2012) who obtained β values turning towards 0 during the fermentation process.

For the stoichiometric parameters, the yield of biomass based on utilised substrate (Y_{XS}) was 50.7932, 3.3940 and 61.0202 and yield of product based on utilised substrate (Y_{PS}) was 2.4524, 0.2307 and 0.7415 for LC, LA and LCA respectively. Meanwhile the maintenance energy parameter (m_s) was 0.0128, 0.0001 and 0.0004 with respect to LC, LA and LCA.

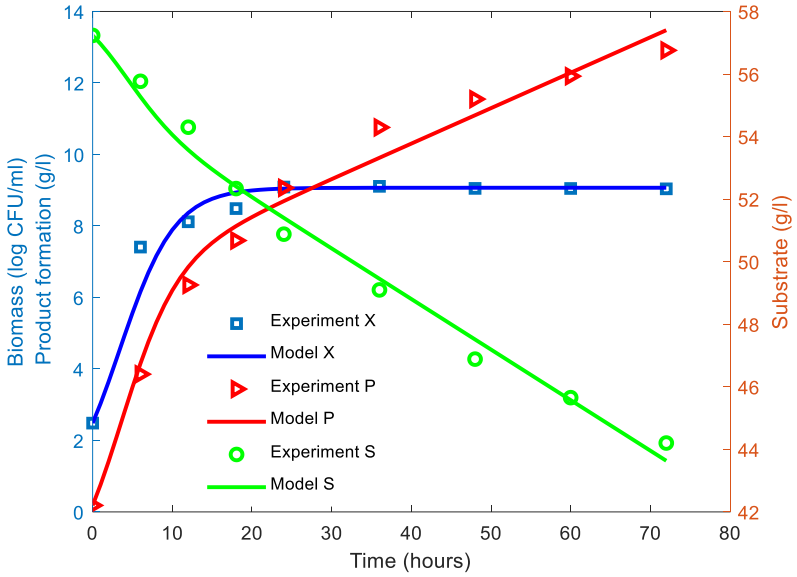
Figure 23 demonstrates the fitting of the experimental and simulated data of the differenct models which implies models were valid due to the good fit. If the concentration of essential medium constituent (reducing sugar) is varied while the concentration of all other medium components are kept constant, the growth rate changes in a hyperbolic fashion as seen in Figure 23 with experimental and simulated plots (hyperbola) for LC, LA and LCA. A functional relationship between the specific growth rate μ and an essential compound (reducing sugars) concentration was proposed by Monod in 1942, of the same form as Langmuir adsorption isotherm in 1918 and the standard rate equation for enzyme-catalysed reactions with a single substrate (Henri in 1902 and Michaelis and Menten in 1913) (Bailey and Ollis, 1986). From the kinetic parameters, this results are similar to the study carried out by Biazar *et al*. (2003) using same models, experimental data

were used and kinetic parameters predicted using Adomian decomposition methods to solve the models responsible for transient responses of the biomass accumulation, substrate utilisation and lactic acid production during a batch production of lactic acid via submerged fermentation of cheese whey with *L. helveticus*.

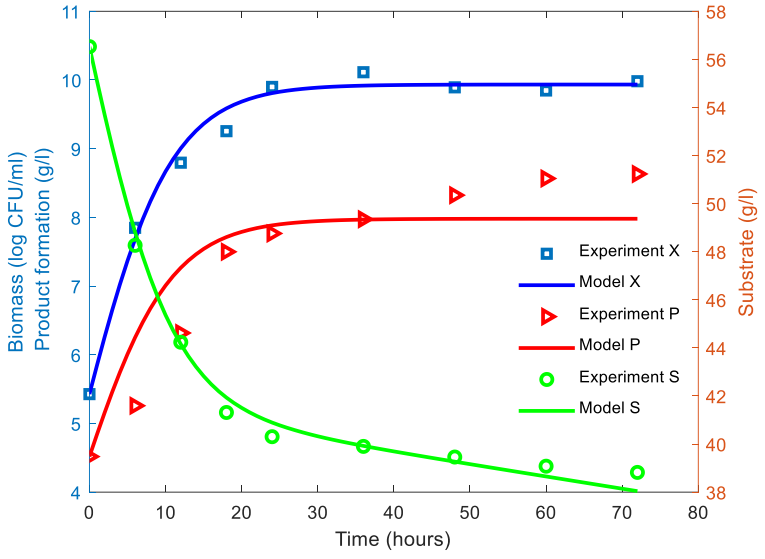
Technically, modelling of a fermentation process is of great importance since the kinetic parameter obtained take into account the process and environmental conditions hence optimal operation conditions from modelling of the fermentation process are necessary for scaling up from laboratory to industrial scale as well as for the conception and design of a bioreactor which will result in a more controlled fermentation process and reduced cost.

Table 16: Kinetic* modelling *parameters of growth, product formation and substrate utilisation

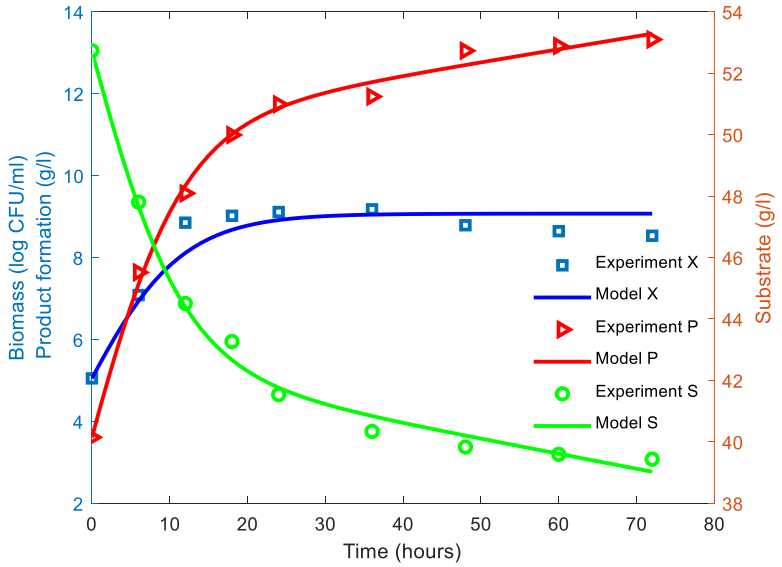
Models	Author	Sample	Kinetic coefficients					Stoichiometric parameters			Statistical coefficients	
			μ_m (h^{-1})	K_s (g/L)	P_{max}	α	β	Y_{PS}	Y_{XS}	m_s	SSE	R^2
$\frac{dX}{dt} = \mu X$	Biazar *et al*. (2003)	LC	0.2876	9.0680	-	1.0028	0.0109	2.4524	50.7932	0.0128	5.6218	0.9946
$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X$		LA	0.1738	9.9337	-	0.7681	0.0001	0.2307	33.9410	0.0001	4.9353	0.9913
$\frac{dS}{dt} = -\frac{1}{Y_{PS}}\frac{dP}{dt} - \left(\frac{\mu_{max}}{Y_{XS}} + m_S\right)X$		LCA	0.1589	9.0709	-	1.9127	0.0035	0.7415	61.0202	0.0004	2.6934	0.9946
$\frac{dX}{dt} = \mu X\left(1 - \frac{P}{P_{max}}\right)^h$	Dutta *et al*. (1996)	LC	2.1972	999.2573	11.3453	0.0005	0.1785	2.5151		0.0146	19.1142	0.9781
$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X$		LA	3.2791	999.7511	8.0957	0.6845	0.0141	0.2125		0.0006	2.8193	0.9940
$\frac{dS}{dt} = -\frac{1}{Y_{PS}}\frac{dP}{dt} - m_S X$		LCA	1.8239	999.9844	12.7605	0.9804	0.1019	0.8015		0.0033	3.9124	0.9914
$\frac{dX}{dt} = \mu X\left(1 - \frac{P}{P_{max}}\right)$	Monteagudo *et al*. (1997)	LC	16.3646	1.0237	3.0758	0.0099	0.9619	0.5710	3.0887	0.0114	211.9433	0.6579
$\frac{dP}{dt} = \left(\alpha\frac{dX}{dt} + \beta X\right)\left(1 - \frac{P}{P_{max}}\right)$		LA	0.0000	4.3735	8.1310	2.2124	0.1548	0.5650	0.2065	0.0000	136.6421	0.6948
$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\frac{dX}{dt} - \frac{1}{Y_{PS}}\frac{dP}{dt} - m_S X$		LCA	0.1678	9.0876	96.1619	2.0684	0.0040	0.4293	7.0897	0.0047	2.6763	0.9946
$\frac{dX}{dt} = \mu X$	Starzak *et al*. (1994)	LC	0.2876	9.0679	-	1.0028	0.0109	-	2.4457	0.0178	5.6218	0.9946
$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X$		LA	0.1654	9.9308	-	0.5582	0.0027	-	0.2785	0.0022	1.3657	0.9973
$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\frac{dX}{dt} - m_S X$		LCA	0.1589	9.0709		1.9127	0.0035	-	0.3877	0.0054	2.6934	0.9946



LC



LA



LCA

Figure 23: Simulated and experimental results of biomass, substrate and lactic acid concentrations

3.4.2.2.Growth kinetic modelling

In the past, several researchers used Monod and the logistic equations which are unstructured models for the macroscopic description of growth processes. Unlike Monod model, logistic equation is substrate independent, implying that, if our interest is strictly the cell viability or biomass which is a very important criterion for probiotic beverages, then logistic model is ideal. This equation is also advantageous in that the biological and geometrical significance are easily calculated, highly manageable, easy adjustment of typical sigmoid profiling as well as possesses the ability to describe auto-catalytic reaction mechanisms (Vázquez and Murado, 2008).

In this study, rather than fitting the entire model parameter set at one time, it was deemed also efficient to perform the fit in parts. The rationale behind this included minimizing the computational burden while allowing the experimenter to examine each portion of the model independently to assess how well the model structure was able to describe the observed phenomena. The computational burden in parameter identification increases with the square of the number of parameters being determined. The opportunity for the experimenter to focus attention on each portion of the model independently provides a basis for improving portions of the model structure if necessary. The R^2 for all samples were good (≥ 0.82) which implies that the data obtained in this study for biomass fits the logistic, exponential and modified exponential equations, which are all derivatives of logistics model. The μ_m and X_{max} for all samples obtained from logistic model was close to those existing in literature for similar studies. The results obtained are represented in Table 17 below.

Table 17: Kinetic parameters of logistic, exponential and modified exponiential equations

Model	Equation	Sample	μ_m	X_{max}	SSE	R^2
Logistics	$\frac{dX}{dt} = \mu_m X(1 - \frac{X}{X_{max}})$	**LC**	0.4043	8.8939	0.6971	0.9934
		LA	0.1654	9.9341	0.2204	0.9966
		LCA	0.2229	8.9252	0.6694	0.9838
Exponential	$\frac{dX}{dt} = \mu_m X exp(1 - \frac{X}{X_{max}})$	**LC**	0.6300	9.9997	0.4838	0.9908
		LA	0.7574	7.9399	0.7744	0.9795
		LCA	0.6296	9.9991	2.6637	0.9039
Modified	$\frac{dX}{dt} = \mu_m X exp(-\frac{X}{X_{max}})$	**LC**	0.6866	9.9995	0.5231	0.9957
exponential		**LA**	0.7574	8.7498	0.7744	0.9795
		LCA	0.6833	9.9965	2.8577	0.9085

This model describes biomass independent of the substrate and product. In this study, the model and its integrated form was used to fit the experimental data for LC, LA and LCA. Experimental data for all three microorganisms were found to fit strictly the biomass model meanwhile experimental data for LC was found to fit the integrated form of this model. The model is as follows:

$$\frac{dX}{dt} = \mu_m X(1 - \frac{X}{X_{max}}) \qquad [29]$$

This equation represents both the exponential (X<<Xmax) and the stationary phase (X = X_{max}) growth. The term (1 - X/ X_{max}) implies that microorganisms inhibit their own growth either by competing for the same carbon and nitrogen sources present in the *A. muricata* juice or by accumulating inhibitory compounds.

There was no pH control during the fermentation process carried out in this study, hence the organic acid produced through various metabolic pathways decreased the pH of the fermented *A. muricata* juice. The parameters indicated that LA medium exhibited a higher maximum specific growth rate followed by LCA then LC with exponential and modified exponential models meanwhile the reverse was observed with the losgistic model, LC had the highest maximum specific growth rate. The maximum cell population (X_{max}) was recorded for LA medium and LA had best fit from the R^2 value. Viccini *et al*., (2001) stated that logistic model in most cases of fermentation fits adequately to the data though not perfect has its own limitations. Even with the adequate description, the biomass steadily decreases during the later stages of fermentation which could be attributed to the death and autolysis of the biomass or endogenous maintenance metabolism. These results indicate that the logistic model could be used to describe *L. acidophilus* and *L. casei* growth in various environmental conditions. It must be pointed out that little information is available in the literature regarding the kinetics of LAB growth in a fermented fruit product. Most published works study the kinetics of the fermentation process for the optimisation of the production of lactic acid in industrial applications.

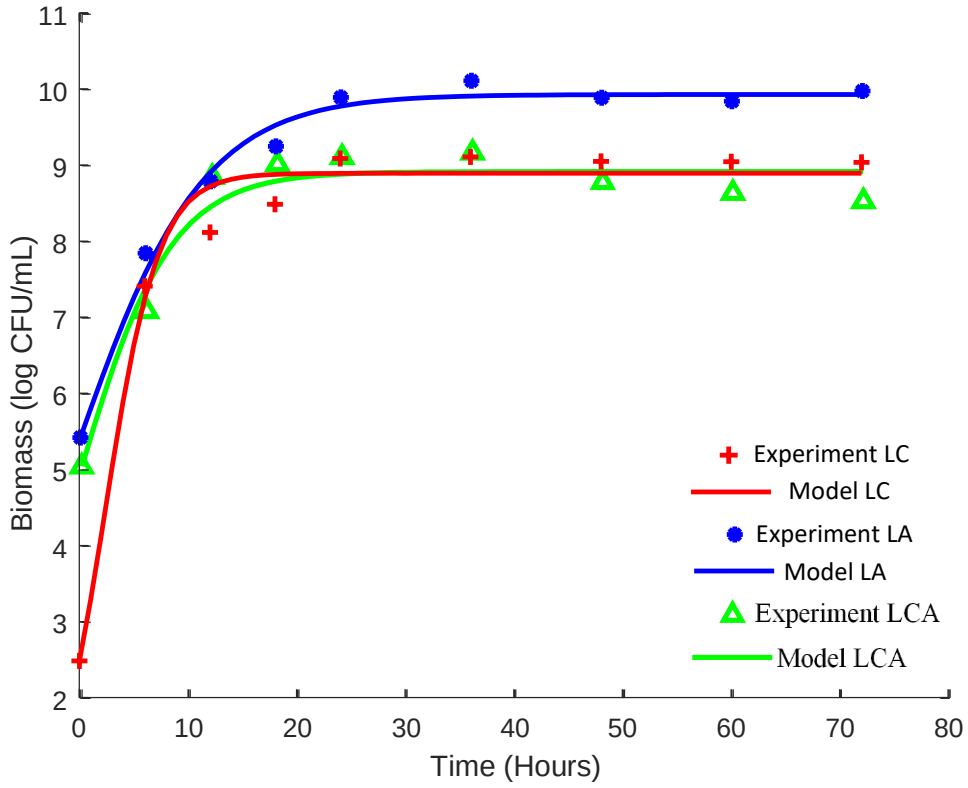


Figure 24: Experimental and simulated data for the growth of lactic acid bacteria a) LC, b) LA and c) LCA predicted by the logistic model

3.5. Conclusion

The chapter which aimed to determine mathematical models that best fit the experimental data that would be able to simulate the kinetics of cell growth, lactic acid production and reducing sugar consumption in *A. muricata*-based fermentations with LAB was attained. From the kinetic modelling, best fitting was obtained from the Monod model, Luedeking and Piret with the maximum specific growth rate been 0.287 for *L. casei* (LC), 0.173 for *L. acidophilus* (LA) and 0.158 for the consortium (LCA) while the saturation constants were in the range 9.067 and 9.999 for the growth parameters of all three samples. The growth associated constants were in the range 0.7681 to 1.9127 meanwhile the non-growth associated constants was between 0.0001 to 0.0109 implying the lactic acid production was dependent on the growth of LC, LA and LCA. The determination coefficients and residual sum of squares were respectively ≥ 0.991 and ≤ 5.621. The results of cell growth for LC, LA and LCA had best fitting to the logistic model for growth kinetics with $R^2 \geq 0.9039$. Therefore, the the fermentation process of *A. muricata* juice using *L. acidophilus*, *L. casei* and the consortium can easily be scaled up from the laboratory to industrial level.

CHAPTER 4: PROBIOTIC CHARACTERISATION AND EFFECT OF REFRIGERATED STORAGE ON FERMENTED *A. MURICATA* JUICE PRODUCED FROM *L. CASEI, L. ACIDOPHILUS* AND A CONSORTIUM OF THESE MICROORGANISMS.

4.1. Abstract

In this chapter, *A. muricata* juice was fermented at optimum time of 24 h for *Lactobacillus casei* (LC) and 36 h for *Lactobacillus acidophilus* (LA) and the consortium of both microorganims (LCA) with an initial pH of 7.0 at 37 ℃ in a 5% supply of CO_2 incubator. Several analyses were carried out on the fermented samples before storage for 28 days: antimicrobial activity, stimulated gastrointestinal tract passage, aroma profile and rheological properties. Meanwhile, during storage at 4 ℃ for 28 days the viable cell count, reducing sugar, lactic acid, antioxidant and total polyphenol contents were evaluated. Day zero recorded a viable cell count of 9.095, 9.186 and 9.017 log CFU/mL and pH of 5.09, 4.80 and 4.92 for LC, LA, and LCA respectively. LA and LCA had the highest antioxidant activity using DPPH assay (160.37 and 249.75 µM TE/mL respectively) whereas LC was highest (345.60 µM TE/mL) with the TEAC assay. Survival under stimulated conditions of the human GIT proved that LCA resisted highest the salivary, gastric and intestinal stimulated solutions compared to LC and LA. Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* revealed that all strains had zones of inhibition greater than 7 mm for both pathogens meanwhile LA was highest (10 mm) against *E. coli*, LCA was highest (15 mm) against *S. aureus* and LC was least against both pathogens. More acids and alcohols were responsible for the aroma profile of the fermented samples compared to more esters for unfermented meanwhile the samples presented similar trend in the rheological properties of samples at 5, 15, 25, 35, and 45 ℃. LC was reported to have the least loss of cell viability from 9.09 to 9.04 log CFU/mL during storage. Reducing sugars content for all samples dropped by the 28^{th} day of storage from 632 to 467 g/L which was evidenced with the slight drop in pH (5.0 to 4.7) and increase in organic acid content from day 0 to day 28 (13 to 14 g/L). Total polyphenol content, on the other hand, remains stable during storage for all three samples. Therefore, this work supports the use of *A. muricata* juice as a medium for storage and passage of probiotic microorganisms.

Key words: *A. muricata*, cell viability, refrigeration, shelf-life/storage

4.2. Introduction

The shelf-life of a food can be defined as the time period within which the food is safe to consume and/or has an acceptable quality to consumers. For probiotics, shelf-life is the time for a product's microbial population to reach 6 log CFU/mL which is determined experimentally (Andres *et al*., 2001). The shelf-life of juices is affected by both the intrinsic and extrinsic factors. Among intrinsic factors pH and water activity are the most influential factors affecting spoilage rates. Bacteria prefer to grow at pH 6.5-7.5 but tolerate a pH range of 4 to 9. Several factors may affect LAB survival which include strains of probiotic bacteria, pH, presence of hydrogen peroxide and dissolved oxygen, concentration of metabolites such as lactic acid and acetic acids, buffering capacity of the media as well as the storage temperature (Champagne and Gardner, 2008; Dave and Shah, 1997; Kailasapathy *et al*., 2008). Although strain selection for addition into foods are based on several criteria (Luckow *et al*., 2005), stability during storage and health benefits are of primary importance. Besides their desired health properties, probiotics should meet the basic requirements for the development of marketable probiotic products including their survival and activity in the product. Although there are instances where non-viable cells have shown health benefits (de Almada *et al*., 2016), it is still considered desirable to have live cultures in the products (Stanton *et al*., 2005). Refrigeration of juices is required to help maintain probiotic viability so that the adequate dose is delivered throughout shelf-life and also to avoid metabolic activity of the probiotic and spoiling of the juice (Fenster *et al*., 2019). In addition, probiotics should not adversely affect the taste or aroma of the product nor acidification during the shelf-life of the product as well as survive both in the food product and passage to reach the small intestine (Champagne and Gardner, 2008). Therefore, the objective of this chapter is to determine the shelf-life of *A. muricata* probiotic juice during storage at 4 ℃ and to predict how long the juice can stay after 28 days with viable count of at least 6 log CFU/mL.

4.3. Materials and methods

Below is the block diagram for the storage of the fermented *A. muricata* juice

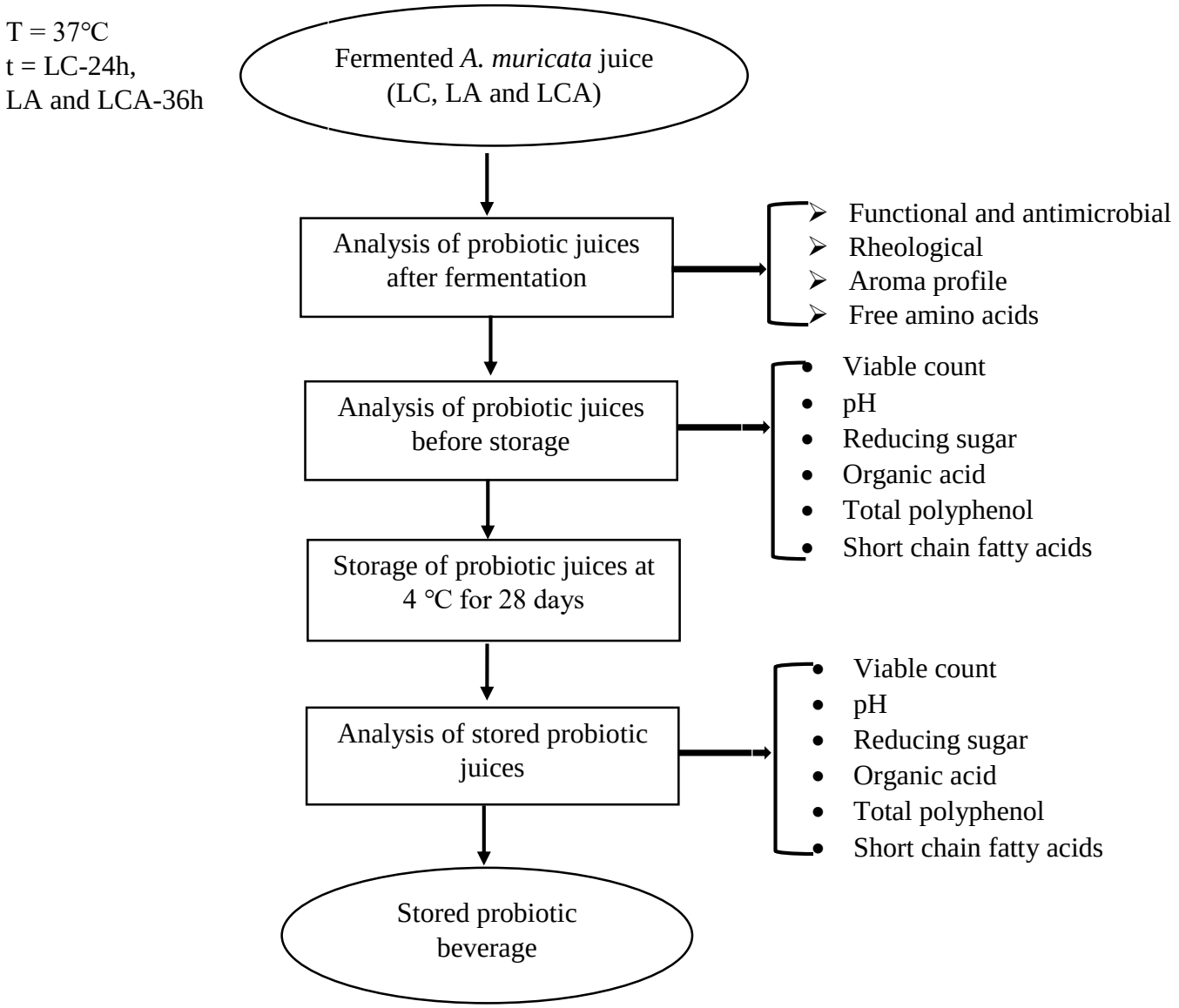


Figure 25: Work plan for the shelf-life study of fermented A. muricata juice

4.3.1. Functional and antimicrobial analyses

4.3.1.1. Acid tolerance test

The acid tolerance test was determined by the method described by Lee *et al*., (2016) with modifications. Juices obtained from the two LAB strains (*L. casei, L. acidophilus* and mixture of both) via fermentation using the optimum condition were analysed for acid tolerance. Ten microliter of juice was added to 1 mL of MRS broth of pH 3 and 7 (control) by adjusting with 1 N HCl or NaOH respectively. The experiment was conducted in triplicate and the survival rate was expressed as percent difference between the variation of optical density (DO) at pH 7.0 (ΔDO pH 7) and the variation of optical density (ΔDO) at pH 3 (Sieladie *et al*., 2011):

$$Survivalrate\ (\%) = \frac{\Delta DO_{pH7} - \Delta DO_{pH\ 3}}{\Delta DO_{pH7}} * 100 \qquad [30]$$

The isolates survived if they demonstrated a survival percentage equal or greater than 50%.

Samples (10 µL) were also collected and inoculated at 0 and after 3 h in MRS agar then incubated for 48 h at 37^{o}C.

4.3.1.2. Bile salt tolerance

Bile salt tolerance was determined according to the method of Vinderola and Reinheimer, (2003). Fermented juices obtained from the two lactic acid bacteria strains during optimum condition were used for bile tolerance assessment. These samples (2% v/v) were used as inoculum for MRS broth containing 0.3% (w/v) bile salts oxgall and a control without bile salt then incubated at 37°C for 24 h. Optical density at 600 nm was measured and compared to the control culture. This experiment was performed in triplicates and the results expressed as the percentage of growth ($DO_{600\ nm}$).

$$\%\ Survivalrate = \frac{OD_{600}}{OD_{600}Control} \times 100 \qquad [31]$$

4.3.1.3. Transit tolerance

4.3.1.3.1. Saliva stimulating solution (SSS)

Saliva stimulating solution was prepared by freshly suspending 2 g/L of sodium chloride (NaCl), 2.2 g/L of potassium chloride (KCl), 0.22 g/L of calcium chloride ($CaCl_2$), 1.2 g/L of sodium hydrogen carbonate ($NaHCO_3$) and 100 mg/L of lysozymes (pH 6.9). The fermented and unfermented juice samples were mixed with SSS in the ratio 1:1 and keep for 5 min then total viable count analysed.

4.3.1.3.2. Gastric stimulating solution (GSS) or Pancreatic fluid tolerance

Simulated gastrointestinal juice was prepared according to the method described by Bao *et al*., (2010) with slight modifications. The simulated gastric juice was prepared freshly by suspending 0.35 g of pepsin in 100 mL of 0.2% NaCl and the pH adjusted to 2.5 with concentrated hydrochloric acid, and sterilized using 0.22 µm filter. Juice samples (1mL) were added into 9 mL of GSS and mixed for 10 s then incubated anaerobically (5% of CO_2) at 37 ℃ for 3 h. Total viable cells were counted at 0 h and 3 h.

4.3.1.2.3. Intestinal stimulating solution (ISS) or intestinal juice tolerance

Intestinal stimulating juice was prepared by suspending 0.1 g of trypsin and 1.8 g of bile salts in a sterile solution of 1.1 g of sodium bicarbonate and 0.2 g of sodium chloride in 100 mL distilled water. The pH value was adjusted to 8.0 with 0.5 M sodium hydroxide. The solution was sterilized by filtering through 0.45 µm filter. One mL of GSS cultures was inoculated into 9 mL of ISS (pH 8.0) and incubated anaerobically at 37 ℃. Total viable counts were done at 0 h and 4 h (Bao *et al*., 2010).

4.3.1.4. Antimicrobial activity against pathogens

The two bacterial strains in the various juices were tested for antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* strains by disc diffusion method assay by Fossi *et al*.,

(2015). An initial inoculum (1 mL) of approximately 1×10^6 CFU/mL of the targeted pathogens (evaluated by plate count) was incorporated into Mueller Hinton soft agar (1%, w/v) plates and well mixed then allowed to solidified. The antibiotic kanamycin (30 mg/ mL) was used as positive control, whilst MRS broth adjusted to pH 6.5 and *A. muricata* juice were used as the negative controls. Sterile filter discs (diameter; six millimetres) were dipped into the fermented juices of LAB for 24 h in a shaker (187 rpm) and placed on solidified Mueller-Hinton agar spread with 18 h cultures of indicator microorganisms. Some of the discs were dipped in MRS broth and *A. muricata* juice which served as controls. The plates were kept at 4°C for 3 h to permit diffusion on the assay material, and incubated at 37°C for 24 h then plates examined for the presence of inhibition zones (Argyri *et al*., 2013). The zones of inhibition (clear zones around the discs) were evaluated by the use of a ruler to measure the diameter of the disk with the surrounding clear area in millimetres (mm). The experiments for functional and antimicrobial activities were done in duplicates.

4.3.2. Rheological analysis

The rheological measurements of both fermented and unfermented samples were done according to Deshmukh *et al*., (2015) with slight modifications. A modular compact rheometer (MCR 102, Anton Paar, Anton Paar Strage Ze 8054 Gruz, Austria) with a measuring cone (CP 25-2, part no.: 79039, diameter: 25 mm, angle: 2.009º, truncation: 105 µm, serial number: 35401, gap: 0.105 mm and normal force: -0.02-.03 N) was used for this analysis. Temperature (5-45 ℃) was controlled using a thermostatic water bath. The unclarified fermented and unfermented *A. muricata* juices were placed at the centre on the surface of the rotor then the external measuring cone was programmed for the measurement mode, it was brought in contact with the sample and the viscometer turn on to start taking the readings. The rheological measurements were carried out in

duplicate and at varying temperatures (5, 10, 15, 25, 35, 45 ℃) with shear rate from 0-500 s^{-1} and generation of different data for shear stress, viscosity and torque hence analysis of flow behavior of fermented and unfermented *A. muricata* juice.

Modelling of fluid flow using rheological models was done. The experimental data of *A. muricata* fermented juice was fitted to a few rheological models, namely; Power law, Bingham and Herschel–Bulkley as shown in Table 4.2. The solver function in MATLAB software (R2018b, MA, USA) was used for the curve fitting. Generalised reduced gradient non-linear regression optimisation code was adopted in determining the rheological parameters. To obtain the best fitted lines, the sum of square errors (SSE) was minimised. The coefficient of determination, R^2 was calculated. The trials were done in triplicates.

4.3.3. Analysis of Aroma profile

The aroma profile of the fermented and unfermented samples was analysed according to Siewe *et al*., (2020) with slight modifications.

- **Extraction of aroma compounds by solid-phase microextraction (SPME)**

Extractions of volatile compounds of fermented and unfermented *A. muricata* juice flavouring models were performed by solid-phase microextraction (SPME). Briefly, 1 µL of 1,2 dichlorobenzene (internal standard, 0.1 mg/mL in methanol) was added to 5 mL each aliquot of samples contained in glass vials (20 mL) sealed with plastic screw caps and thoroughly mixed, and Teflon coated septa. After pre-equilibration at 50 °C for 20 min in a water bath, the aroma compounds were adsorbed at 50 °C for 30 min with the 50/35 µm Carboxen/polydimethylsiloxane/divinylbenzene (CAR/PDMS/DVB) fibre (Supelco Inc., Bellefonte, USA) in the headspace of the vial. The adsorbed fibre was directly introduced into an Agilent GC injector for the subsequent GC–MS.

- **Analysis of aroma by gas chromatography-mass spectrometry**

The fibre with absorbed volatile compounds was directly inserted into the 7890B Agilent GC injector port (Agilent Technologies, Santa Clara, California, USA) for 3 min in splitless mode at 250 °C to desorb the volatile compounds. Separation of the volatile compounds was performed by an RT-WAX capillary column (60 × 0.25 mm, 0.25 µm W Scientific, Folsom, CA). Helium was used as the carrier gas, a flow rate at 1.8 mL/min. The initial temperature of the GC column was set at 40 °C, held for 3 min, then raised to 235 °C with an increment of 5 °C/min, and then maintained this temperature for 10 min. The mass spectrometric detector was operated at a temperature of the ion source of 230 °C and an electron voltage of 70 eV. The transfer line temperature was 250 °C. Record of the chromatograms was performed by monitoring the total ion current in a range of 40–450 amu.

- **Identification and quantitation of the aroma compounds**

Volatile compounds were identified by comparing their mass spectra with the US National Institute of Standards and Technology (NIST) mass spectra database. The determination of approximate quantities of the volatile compounds in each sample was carried out by relating their peak areas to that of internal standard (1,2 dichlorobenzene). The quantitative formula was as follows:

$$W_i = f' * \frac{A_i * m_s}{A_s} / m \qquad [32]$$

Where A_i is the peak area of the compound, A_s is the peak area of internal standard, m_s is the mass of internal standard, m is the mass of the sample, f' is a relative correction factor, assumed to be 1, W_i is the concentration (mg/g) of compounds.

4.3.4. Free amino acid profile using LC-MS/MS

The determination of free amino acid (FAA) content of fermented and unfermented *A. muricata* juice was carried out as per the method of Bidlingmeyer *et al*., (1987). The *A. muricata* samples

(100 mg and 100 mL) were suspended in 20 mL of 5% trichloroacetic acid and stirred for 1 h at room temperature (28 ± 1 ℃), then centrifuged (7780; Kubota, Bunkyo-ku, Tokyo, Japan) at 10,000 g for 15 min at 4 ℃ (solid sample). The supernatants were collected and filtered through a 0.22 µm PTFE membrane filters. Free amino acids (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 standardised using 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). Working standards were prepared from the stock solution by dilution with milli-Q water with a concentration ranging between 0.01-1 µg/mL. The quantification of all the amino acids was carried out using Shimadzu Shim-pack GISS C18 column (150 X 2.1 mm i.d, 1.9 µm) using a mobile phase of 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 ionisation (ESI) was operated in multiple reaction-monitoring (MRM) mode, both positive and negative. The injection volume was 10 µl, and the ion spray voltage was 4 kV. The collision-induced dissociation (CID) gas was 230 kPa. Each calibration solution was analysed in triplicate, and the average value of the results was used as the representative for each point. Amino acids were identified and quantified by comparing with

the authentic standard amino acid mixture. The results obtained were expressed as part per billion (ppb). The experiments for aroma were done in duplicates.

4.3.5. Other analyses

The analyses done during storage (viable count, pH, reducing sugar, organic acid, total polyphenol and short chain fatty acids) have been described in chapters 2 and 3. These experiments were done in triplicates.

4.4. Results and discussion

4.4.1. Characterisation of probiotic beverage from *A. muricata*

4.4.1.1. Technological properties

Table 18 below shows the technological characteristics of the juice samples fermented with *L. casei* (LC), *L. acidophilus* (LA) and a consortium of both microbial strains (LCA).

4.4.1.1.1. Survival under conditions simulating the human gastrointestinal tract

- **Salivary juice**

It is important for the viability of probiotics to be maintained not only in the food product but also during storage and passage along the gastrointestinal tract when consumed, therefore the conditions of the digestive tract (from saliva to GIT) were stimulated to verify if the LAB used in this study could survive digestion be it quick or slow digestion. All the samples were found to have more than 95.98% survival rate in saliva stimulating solution. This implies 2.8% of microorganisms were lost during salivary passage for sample LC, 3.4% for LA and 4.1% for LCA hence microorganisms underwent mouth (salivary) passage without any loss in viability per say. This salivary passage was done during a period of 5 min which is considered an average time of maceration (for solid foods) but the product in this study is liquid and will definitely take less time of passage hence the strains might completely survive passage in the mouth.

- **Gastric juice**

In order for probiotics to survive the adverse conditions of the gastrointestinal tract and reach the intestine in sufficient numbers, they need to be present at a concentration of at least 10^7 CFU/mL in the product at the time of consumption; this corresponds to approximately 10^9 CFU per portion in the case of fruit juices (Nualkaekul and Charalampopoulos, 2011). The pH in human stomach ranges from 1, during fasting, to 4.5, after a meal, and food ingestion can take up to 3 h. (Maragkoudakis *et al*., 2006). Gastric stimulating solution adversely affected the strains where LC

was the most susceptible with 31.25%, followed by LA having 46.73% and lastly LCA which had 53.46% survival rate. *L. acidophilus* being an acidic strain was expected to have survived most in gastric conditions. The highest survival obtained for sample LCA confirms the literature which states that *L. casei* complements *L. acidophilus*. In similar studies, Goldin *et al*. (1992) reported survival of *Lactobacillus* GG at pH 3, Charteris *et al*., (1998) reported almost complete loss of viability for *L. casei* 212.3 and F19 strains and *Lactobacillus* GG within 3 h interval at pH 2.5. Jacobsen *et al*., (1999) reported that out of 44 Lactobacilli, none of the strains could replicate at pH 2.5. The results obtained in this study were in accordance with previous studies where the tolerance of LAB especially *L. casei* to acid was highly strain specific (Mishra and Prasad, 2005). Several studies have showed that the capacity of LAB to survive at low pH varied, even within the same species (Sameh *et al*., 2016). Vinderola *et al*., (2000) reported that *L. acidophilus* was most resistant when subjected to gastric juice compared to other LAB.

- **Intestinal or pancreatic juice**

If probiotics are to respect FAO/WHO 2001 definition, then they "should be resistant to gastric juices and be able to grow in the presence of bile". The survival percentage for intestinal juice tolerance and overall digestion was 60.7%, 85.6% and 96.51% for LC, LA, and LCA respectively. This high survival percentage of pancreatic juice (> 60%) is in line with previous studies which showed that the majority of the strains survived well under such conditions, suggesting a potential recuperation of the initial levels during the passage in the small intestine (Maragkoudakis *et al*., 2006).

- **Acid and bile tolerance test**

In this study, the strains were found to be acid tolerant with survival rate of 92.838%, 94.135% and 92.577% for LC, LA and LCA respectively. The result of LA is similar to those of Jin *et al*., (1998) who studied the acid and bile tolerance of different isolated lactobacillus species (*L.*

acidophilus inclusive). According to Maragkoudakis *et al*. (2006), Lactobacillus strains retained their viability when exposed to pH values of 2.5–4.0, but displayed loss of viability at lower pH values meanwhile Bolin *et al*., (1997) reported the high survival rate of two *L. acidophilus* strains amongst the four strains they studied.

Bile tolerance is an important characteristic since it enables the probiotic strains to survive, grow, and exert their beneficial effects in the host. All samples proved to be tolerant to bile salt with the highest been LC with 96.2% survival rate meanwhile LA and LCA had about 93% survival rate. The high survival rate in bile sensitivity observed in this study is consistent with many reports, including those of Mishra and Prasad (2005), Sameh *et al*., (2016), and Fernandez *et al*., (2003). A consensus, therefore, emerges that wide variation exists in the susceptibility of probiotic cultures to bile and this property is species, as well as strain, specific (Mishra and Prasad, 2005)

4.4.1.1.2. Antimicrobial activity

LAB could produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin during lactic fermentations hence the attribution of antibacterial activity to LAB. All three samples were tested for growth inhibition of *Escherichia coli* and *Staphylococcus aureus* using disk diffusion asssay. Samples had inhibition zones greater than 7 mm for both pathogens meanwhile LA was highest (10 mm) against *E. coli*, LCA was highest (15 mm) against *S. aureus* and LC was least against both pathogens. No inhibition zone at all was observed when the pathogens were grown in the presence of the appropriate control medium (MRS at pH 6.5, negative control), but maximum inhibition was observed with positive control (kanamycin) where *S. aureus* had 35 mm and *E. coli* had 40 mm. Sameh *et al*., (2016) reported that *L. acidophilus* and bifidobacteria exert antagonistic effects on the growth of pathogens such as *S. aureus*. Several authors have reported the inhibition of pathogenic bacteria growth by LAB strains and related it to

the production of bacteriocin or bacteriocin-like compounds (Jacobsen *et al*., 1999; Sameh *et al*., 2016). Great attention has been given to bacteriocins from the generally recognized as safe (GRAS) LAB as a novel approach to control pathogens in food-stuffs (Savadogo *et al*., 2004). The production of bacteriocin-like compounds by different species of *Lactobacilli* that exhibit broad activities against Gram-positive and Gram-negative bacteria has also been reported in previous studies (Coconnier *et al*., 1997; Sameh *et al*., 2016). *L. acidophilus* is said to produce the bacteriocin acidophilin while *L. casei* produces nisin. Thus, the combination of these in sample LCA could be responsible for the maximum growth inhibition of the gram positive pathogen *S. aureus.*

4.4.1.1.3. pH

The quantity and types of organic acids produced during fermentation process with consequent reduction in pH is dependent on the species of LAB or strain, medium composition and growth conditions (Sameh *et al*., 2016). Sample LC had pH of 5.09, LCA of 4.92 and LA had the least which was 4.80 from an initial pH of 7.0 for all samples. This drop in pH after fermentation is attributed to the consumption of sugars and production of organic acids.

Table 18: Technological characterisation of A. muricata juice fermented with L. casei, L. acidophilus and a mixture of both (50:50) using different conditions

	LC	LA	LCA
Plate count (log CFU/mL)	9.095 ± 0.002	9.186 ± 0.010	9.017 ± 0.005
Saliva stimulating solution (%)	97.243 ± 0.386	96.643 ± 0.221	95.983 ± 0.827
Gastric stimulating solution (%)	31.250 ± 0.671	46.736 ± 0.949	53.467 ± 0.987
Intestinal stimulating solution (%)	60.705 ± 2.364	85.681 ± 3.729	96.518 ± 3.686
Bile tolerance (%)	96.207 ± 0.110	93.969 ± 0.001	93.442 ± 0.011
Acid tolerance (%)	92.838 ± 0.190	94.135 ± 0.010	92.577 ± 0.103
Antimicrobial activity (MIZ) in mm			
E. coli	7.0 ± 0.011	10.0 ± 0.031	8.0 ± 0.032
S. aureus	9.0 ± 0.060	10.0 ± 0.102	15.0 ± 0
Antibiotic activity (mm)			
Kanamycin (30 µl)	10.0 ± 0.000	13.0 ± 0.141	16.5 ± 0.070
Kanamycin (10 µl)	7.5 ± 0.020	9.0 ± 0.000	10.5 ± 0.070
pH	5.09 ± 0.014	4.8 ± 0.000	4.92 ± 0.015

4.4.1.2. Rheological profile

The variation in shear stress and shear rate of the fermented and unfermented samples were analysed at 5 ℃, 15 ℃, 25 ℃, 35 ℃ and 45 ℃ as represented in Figure 26. All the samples exhibited almost the same behavior cutting across the various temperatures which was a non-linear relationship, quite typical of non-Newtonian fluids. The viscosity of the juices was decreasing with increasing temperature for all samples. The highest viscosity was recorded by the unfermented sample (SS) at the temperature of 5 ℃ then sample LC, followed by LCA with the least been LA.

This result was similar to what was obtained by Giner *et al*., (1996) who studied the rheology of clarified cherry juice while varying the temperature (5-70 ℃) and soluble solids (22-74 ºBrix) and also Essien and Usoh, (2016) who studied the effect of temperature on the rheological properties of pineapple juice. This could be attributed to the fact that temperature reduces the resistance to flow since it has an inverse relationship with viscosity of liquids hence increase flow ability of liquid foods (Keshani *et al*., 2012; Shamsudin *et al*., 2007).

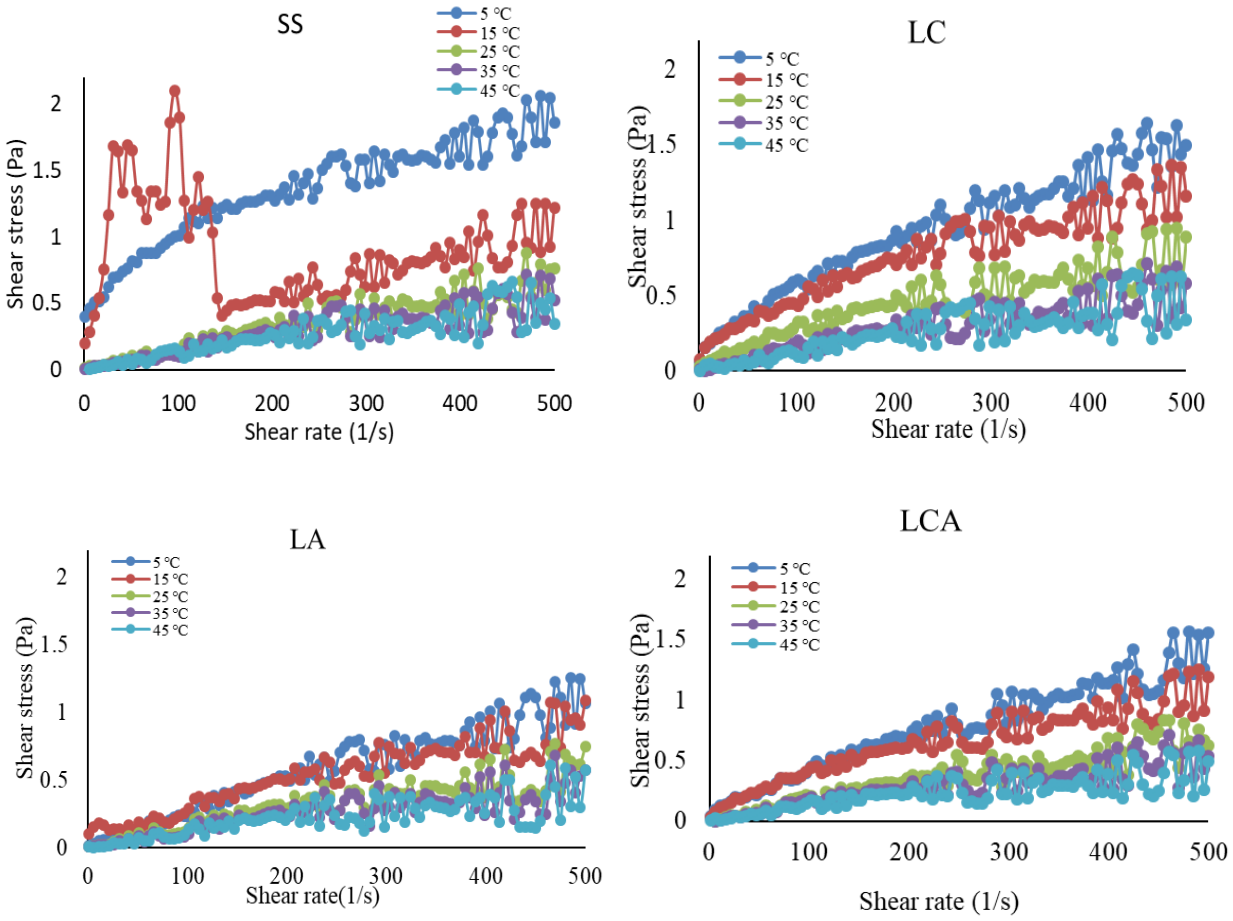


Figure 26: Typical rheogram of unclarified fermented and unfermented soupsop juice at temperature of 5, 15, 25, 35, and 45 ℃

On the other hand, the fact that the viscosity of the sample SS was found to be higher than LC, LA and LCA could be attributed to the total soluble solid content and sugars. For the fermented

samples (LC, LA and LCA), sugars were used up during fermentation hence a drop in total soluble solids. This result is in accordance with other research works where the viscosity was found to increase with increasing total soluble solid content (ºBrix) of the juices (Deshmukh *et al*., 2015; Giner *et al*., 1996; Manjunatha *et al*., 2012).

The above data (Figure 26) were fitted into rheological models (Herschel-Bulkley, Power Law and Bingham) and parameters such as consistency index (K_1), flow behaviour index (K_2), yield stress (K_3) and dynamic viscosity (K_4) were determined. The models were validated using the coefficient of determination (R^2) and the Root Mean Squared Error (RSME). At 5 ℃, all the models showed high values of goodness of fit with $R^2 > 0.9$ for all samples, except for sample SS which differed with the Bingham model. The same was the case for all samples at 15 ℃ and 25 ℃ with $R^2 > 0.8$ for all three models though sample SS did not fit for all models. This could be as a result of the zone presented between 0 s^{-1} and 100 s^{-1} shear rate which does not follow any trend. At 35 ℃, sample LA had $0.7 < R^2 \leq 0.75$ meanwhile all the other samples fitted the three models with $R^2 > 0.75$ and at 45 ℃, SS had $R^2 > 0.7$ and all other samples did not fit with $0.5 < R^2 \leq 0.75$.

Looking at the model parameters, the data obtained in this project had better fiting to the Power Law and the Bingham models since the Herschel–Bulkley, a three parameters model, yielded negative yield stress values for almost all samples which are meaningless in a physical standpoint. Gratão *et al*., (2007) obtained negative yield stress in the laminar flow of soursop juice through concentric annuli.

As far as the Bingham model parameters are concerned, it was observed that the dynamic viscosity for all samples had no significant difference $p<0.05$ with change in temperature but there was a general drop in yield stress for all samples especially after 15 ℃. From the two-parameter Power Law model, sample LC had a significant ($p<0.05$) decrease in the consistency coefficient with

increase in temperature which was not the trend for the other samples. The flow behaviour indices (K_2) for all samples were less than 1 hence the samples could be said to exhibit non-Newtonian shear thinning behaviour of fluid. This model is often sufficient for industrial purposes and provides a reasonable representation of many practical shear-thinning fluids (Gratão *et al*., 2007). This result is similar to what was obtained by Kobus *et al*., (2019) who noticed that *A. muricata* juice had a greater consistency coefficient and was characterised by a higher degree of pseudoplasticity compared to apple juice. It was seen that the R^2 values decrease with increase temperature for each set whereas the probiotic load was same for all samples (LC, LA and LCA). Generally, the higher the temperature the lower the resistance of the fluid hence movement of particles depends on temperature. This inverse relationship has been likened to the incidence of a freer molecule-to-molecule interaction at elevated temperatures (Quek *et al*., 2013).

Table 19: Model fitting parameters for Herschel-Bulkley, Power Law and Bingham rheological models

		Herschel-Bulkley					Power Law				Bingham			
		K_1 (Pa s^n)	K_2	K_3	R^2	RMSE	K_1 (Pa s^n)	K_2	R^2	RMSE	K_4	K_3	R^2	RMSE
SS	5 ℃	0.105	0.444	0.212	0.949	0.088	0.194	0.361	0.947	0.089	0.003	0.729	0.896	0.125
	15 ℃	66.461	000	-65.402	0.008	0.373	1.025	-0.027	0.007	0.373	0.000	0.982	0.023	0.370
	25 ℃	0.006	0.745	-0.027	0.863	0.075	0.004	0.808	0.862	0.075	0.001	0.044	0.854	0.077
	35 ℃	0.009	0.661	-0.042	0.77	0.083	0.004	0.765	0.768	0.083	0.001	0.044	0.755	0.085
	45 ℃	0.005	0.740	-0.019	0.75	0.081	0.003	0.802	0.749	0.081	0.001	0.035	0.743	0.081
LC	5 ℃	0.024	0.654	0.064	0.962	0.077	0.035	0.603	0.961	0.077	0.002	0.287	0.945	0.092
	15 ℃	0.035	0.561	0.026	0.922	0.086	0.041	0.539	0.922	0.086	0.001	0.280	0.892	0.101
	25 ℃	0.019	0.592	-0.011	0.839	0.087	0.017	0.609	0.839	0.086	0.001	0.138	0.817	0.092
	35 ℃	0.007	0.688	-0.022	0.781	0.079	0.005	0.748	0.780	0.079	0.001	0.052	0.770	0.081
	45 ℃	0.007	0.675	-0.028	0.688	0.091	0.004	0.755	0.687	0.091	0.001	0.043	0.677	0.092
LA	5 ℃	0.008	0.792	-0.028	0.931	0.086	0.006	0.835	0.930	0.085	0.002	0.063	0.925	0.088
	15 ℃	0.007	0.762	0.056	0.892	0.083	0.014	0.671	0.890	0.084	0.002	0.140	0.885	0.086
	25 ℃	0.009	0.671	-0.035	0.816	0.079	0.005	0.754	0.815	0.078	0.001	0.054	0.802	0.081
	35 ℃	0.013	0.587	-0.050	0.738	0.077	0.005	0.711	0.734	0.077	0.001	0.050	0.715	0.080
	45 ℃	0.014	0.546	-0.047	0.606	0.086	0.005	0.677	0.603	0.086	0.001	0.049	0.584	0.088
LCA	5 ℃	0.010	0.778	0.044	0.947	0.088	0.015	0.726	0.949	0.088	0.002	0.161	0.941	0.092
	15 ℃	0.025	0.595	0.001	0.908	0.086	0.026	0.594	0.908	0.086	0.002	0.203	0.886	0.096
	25 ℃	0.005	0.791	-0.014	0.876	0.075	0.004	0.824	0.876	0.075	0.001	0.044	0.871	0.076
	35 ℃	0.005	0.728	-0.015	0.781	0.079	0.004	0.772	0.781	0.078	0.001	0.046	0.774	0.080
	45 ℃	0.012	0.573	-0.033	0.627	0.084	0.006	0.668	0.625	0.084	0.001	0.055	0.608	0.085

Where: K_1 is consistency index, K_2 is flow behaviour index, K_3 is yield stress and K_4 dynamic viscosity

4.4.1.3. Aroma profile

It is widely known that fermentation influences the aroma profile of food products such as beer, wine, meat etc. In the case of probiotics, some researchers stated that components from the metabolism of probiotic cultures greatly impacted the aroma and taste of the products (Cruz *et al*., 2013). The establishment of the chemical nature of volatile compounds which in combination or individually can act to produce specific aroma response for any given product is based on the composition of the flavour (Cronin, 1990). *A. muricata* is reported to have more than 50 identified compounds present in both the fruit and the essential oil of the fruit with the dominant being esters of aliphatic acids and the majority being methyl 2-hexenoate, ethyl 2-hexenoate methyl 2-butenoate and methyl 2-octenoate which are responsible for the green, fruity and pineapple-like flavour. Mono- and sesquiterpenes such as α-caryophyllene for woody; 1,8-cineole for mint and sweet; linalool for flower and lavender; R-terpineol for oil, anise and mint; linalyl propionate for floral and calarene (Bicas *et al*., 2011; Jirovetz *et al*., 1998; Padmanabhan and Paliyath, 2016; Pélissier *et al*., 1994; Wong and Khoo, 1993).

About 49 volatile components could be attributed to the aroma of the samples studied which comprised of 18 esters, 15 acids, 5 aldehydes, 3 ketones and 2 alcohols for SS; 17 esters, 23 acids, 3 aldehydes, 2 ketones and 4 alcohols for LC; 14 esters, 18 acids, 5 aldehydes, 5 ketones, 6 alcohols and 1 terpene for LA meanwhile 11 esters, 18 acids, 3 aldehydes, 3 ketones and 6 alcohols were identified for LCA. Generally, most of the values of the volatile compounds were higher in the fermented samples than in the unfermented samples. This implies the treatment method had an influence on the aroma profile of food samples. Ester groups were higher in SS than in LC, LA and LCA, while acids and alcohols content were least in SS but higher in the fermented samples with the most being in LC. On the other hand, the aldehyde and ketone contents were more in LA then in LCA, LC and SS.

Most of the volatile components obtained for SS were similar to those previously reported for *A. muricata* from Malaysia, Sri Lankan, Brazil, Ghana, Ivory Coast, Colombia and Nigeria (Bicas *et al*., 2011; Gyesi *et al*., 2019; MacLeod and Pieris, 1981; Pélissier *et al*., 1994; Prieto Jenifer *et al*., 2019; Santana *et al*., 2017; Wong and Khoo, 1993). Slight differences in the component could be attributed to the geographical and agronomical differences in the samples, fruit maturity and ripeness of samples as well as the differences in the analytical methods used and the equipment involved. It is therefore important to note that, if a particular raw fruit (fresh *A. muricata*) aroma is needed in the end product, appropriate techniques from extraction, pasteurisation right up to fermentation as well as during the isolation process should be applied. Such as inactivating the enzymes that may lead to changes in the volatile components, low temperature extraction for volatile components that are heat liable, under inert gases or in the absence of light for volatile components that are light sensitive (Maga, 1990).

During fermentation various substrates such as glucose are metabolised via different pathways which leads to the production of flavour compounds (aldehydes, acids, alcohols and ketones) (Waché and Dijon, 2013). Another way to produce flavour compounds is through microbial metabolism or biocatalysis. Whole cells, on the other hand, also catabolise components such as carbohydrate, fats, and proteins, converting the breakdown products to amazing flavour compounds (Macedo *et al*., 2010). It is therefore possible to establish a relationship between microorganisms present and the flavour compounds produced since different strains undergo different mechanisms (microbial routes) hence different products (Macedo *et al*., 2010). This could be the possible reason for the variation in the volatile compounds for the selected strains in this study. The strains involved in this study might contain the enzyme alcohol dehydrogenase which converts aldehyde to alcohol (Dan *et al*., 2019). Carboxylic acids are said to be the major volatile

flavour components in most dairy products since they are produced during yogurt fermentation via lipolytic processes or bacterial fermentation (Dan *et al*., 2019). That was the case in this study were the bacterial fermentation led to the generation of more carboxylic acids compared to the unfermented sample. Hexanoic acid and 3-Methylbutanoic acids for instance confers a "cheesy," "rancid," and "sweat-like" flavour while Octanoic acid provides a "soapy" flavour to dairy products (Dan *et al*., 2019).

Amino acids are also responsible for the aroma profile of food samples. The amino acids L-leucine and L-isoleucine for instance which were present in the samples studied could serve for the derivation of isoamyl alcohol and 2-methyl-l-butanol respectively (Gee and Ramirez, 1994). It is worthy of note that the juices obtained after fermentation still possessed their fresh, fruity and sweet aroma which is characteristic of *A. muricata* juice and it high acceptability by consumers.

Table 20: Aroma profile of fermented and unfermented A. muricata juice

			SS		LC		LA		LCA	
	RT	**Compounds**	**Area**	**% area**	**Area**	**% area**	**Area**	**% area**	**Area**	**% area**
1	1.389	Unknown	2823491.25	0.493	151696940	16.595	99804272	15.490	49955573	5.405
2	2.279	Acetic acid, hexyl ester [IV]	4388081	0.767	23137921.5	2.531	16889307.5	2.621	8466946.9	0.916
3	2.760	dl-Alanine ethyl ester [IV]			12587953.45	1.377	12369284.3	1.919	7671764.45	0.830
		2-Hexanone, 5-methyl- [V]	5654122	0.98833						
4	2.820	Propanoic acid, 2-methyl-, methyl ester [IV]	3588907.9	0.627			6310342.1	0.979	8946606.5	0.968
		Unknown hydrocarbon			7083883	0.774				
5	3.095	Butanoic acid, methyl ester [IV]			5687931.25	0.622	3170538.8	0.492	6244548.75	0.675
		Hexanal[II]	10890188.9	1.903						
6	3.175	2-Butanone, 3-methyl-1-phenyl- [V]			11418009.7	1.2490	991894.2	0.153	3459034.4	0.374
		Hexanoic acid, 5-methyl-, methyl ester [IV]	7332032.9	1.281						
7	3.425	Pentanoic acid, 3-methyl-, methyl ester [IV]	3892295.5	0.680	12251623.5	1.340			10954439	1.185
		Cyclobutanol [III]					1063220.65	0.1658		
8	4.360	Butanoic acid, ethyl ester [IV]	3329974.85	0.582	2569508.15	0.281	2781938.8	0.431		
		Pentanoic acid, 4-methyl-, methyl ester [IV]							14979516.5	1.620
9	5.871	2-Butenoic acid, methyl ester, (E)- [IV]	32481371	5.677	41817857.5	4.5747	6810345.6	1.057	3259434	0.352
10	6.706	4-Hexenoic acid, methyl ester [IV]	5648606.75	0.987	8462291.6	0.925			34218634	3.702
		2-Heptanol, 6-amino-2-methyl- [III]					8405097	1.304		
11	7.441	2-Butenoic acid, ethyl ester, (Z)- [IV]	2426336.6	0.424	1782840.4	0.195	2120693.35	0.329	30336654.5	3.282
12	8.132	Hexanoic acid, methyl ester [IV]	24623636.3	4.304	6335324.1	0.693	6792661.25	1.054		

		4-Penten-2-ol [III]							2090078.8	0.226
13	12.008	2-Hexenoic acid, methyl ester, (E)- [IV]			29828517.4	3.263	22513724.9	3.494		
		2-Propanol, 1-amino-, (R)- [III]							2945555.3	0.318
		2-Decenal, (E)- [II]	4151437.25	0.725						
14	12.143	2-Undecenal[II]	3505538.75	0.612					2493926.6	0.269
		Acetoin [V]			2956714.9	0.323	10595562.45	1.644		
15	14.994	1-Hexanol [III]							3093271.15	0.334
		4-Pentenoic acid, 3-methyl-, methyl ester [IV]	52384371.6	9.156			1579883.75	0.245		
		1-Pentanol, 4-methyl-2-propyl- [III]			2537439.5	0.277				
16	16.130	3-Hexen-1-ol, (Z)- [III]			2202432.8	0.240				
		1-Pentanol, 3-methyl- [III]					1164717.75	0.180	3050586	0.330
		Cyclobutanecarboxylic acid, 2-methyl-, methyl ester [IV]	9655927	1.687						
17	19.516	cis-9-Hexadecenoic acid [I]	7787303.4	1.361	9697196.8	1.060				
		4-Penten-1-ol, 2-methyl- [III]					984463.8	0.152		
		4-Nonanol, 2,6,8-trimethyl- [III]							3152585.5	0.341
18	22.813	Cyclopentadecanone, 2-hydroxy [V]	4887192.5	0.854	36190503.05	3.959	2016419.2	0.312	8518663.4	0.921
19	24.043	1-Octen-4-ol [III]	2342707.4	0.409			3046173.05	0.472		
		Octadec-9-enoic acid [I]			14433483.4	1.578			22822344.6	2.469
20	27.049	3-Butenoic acid, 2-oxo-4-phenyl-, methyl ester [IV]	2324041.1	0.406						
		Octanoic acid [I]			2281334.95	0.249				
		2-Decenal, (E)- [II]					1477594.5	0.229	2066182.1	0.223

21	31.566	Cis-Vaccenic acid [I]	3086714.25	0.539	3843203.2	0.420			8407013.4	0.909
		Linalyl acetate [VI]					1772753.35	0.275		
22	35.248	2-Nonenal, (Z)- [II]	4354576.65	0.761			1173656.85	0.182		
		Octanoic acid [I]			5239108.4	0.573			2363568.2	0.255
23	35.853	Cyclopropanecarboxyllic acid, tridec-2-ynyl ester [IV]	2171455.35	0.379	5165626	0.565				
		2-Dodecenal[II]					1761437.65	0.273		
		Dodecanoic acid, 3-hydroxy- [I]							2875187.85	0.311
24	36.473	Nonanoic acid [I]	2564357.9	0.448	2617387.35	0.286				
		2,4-Nonadienal[II]					1467692.4	0.227		
		15-Hydroxypentadecanoic acid [I]							5562911.9	0.601
25	36.793	Pentanoic acid, 4-methyl- [I]	3121055.65	0.545			2586973.8	0.401		
		2-Propenoic acid, 3-phenyl-, methyl ester [IV]			2818293.55	0.308			2598713.85	0.281
26	37.324	3-Methyl-hexanoic acid [I]	2973246.15	0.519			1794451.4	0.278		
		Dodecanoic acid [I]			5567145.7	0.609			4352826.95	0.470
27	37.779	cis-13-Octadecenoic acid [I]	4285792.85	0.749	1877591.2	0.205			6640380.5	0.718
		Tridecanoic acid [I]					3876019.25	0.601		
28	37.959	1-Penten-3-one [V]	3562884.4	0.622			1141634.7	0.177	6609204.5	0.715
		3-Ethylheptanoic acid [I]			4599990.15	0.503				
29	38.354	cis-9-Hexadecenoic acid, trimethylsilyl ester [IV]	8060731.4	1.409					11017285.25	1.192
		Phenol, 2,6-bis(1,1-dimethylethyl)- [III]			2869507.05	0.313				

		Nonanoic acid [I]					1100394.75	0.170		
30	38.844	n-Hexadecanoic acid [I]	2609910.55	0.456	8003922.2	0.875	2109242.85	0.327	3372437.4	0.364
31	39.344	Benzofuran, 2,3-dihydro- [II]	3224726.6	0.563					21974300	2.377
		trans-13-Octadecenoic acid [I]			3194154.45	0.349	1276849.15	0.198		
32	39.464	Oxacyclotetradecan-2-one, 13-methyl- [V]	8309145.1	1.452					20085420.6	2.173
		Phenol, 2,4-bis(1,1-dimethylethyl)- [III]			4942546.6	0.540	1215634.35	0.188		
33	39.584	Octadecanoic acid, 3-oxo-, methyl ester [IV]	3541560	0.619	9205022.4	1.007			97085639	10.504
		2(3H)-Furanone, 5-dodecyldihydro- [V]					2371612	0.368		
34	40.100	9-Octadecynoic acid [I]	11126961.5	1.944	6447915.75	0.705			127966217	13.846
		Benzaldehyde, 3-methyl-[II]					4127893.65	0.640		
35	40.375	9,12-Octadecadienoic acid, methyl ester [IV]	1848853.65	0.323	44614820	4.880	3195441.95	0.495	5956554.25	0.644
36	40.590	Undecanoic acid [I]	2996851.85	0.523					6025675.25	0.651
		9,12-Octadecadienoic acid (Z,Z)- [I]			81347356.5	8.899	9005815.5	1.397		
37	40.685	Pentadecanoic acid [I]	2114545.65	0.369	5104926.35	0.558	16830758.25	2.612	4011885.1	0.434
38	40.870	Tridecanoic acid [I]	31296671.1	5.470	4222175.65	0.461	5369292	0.833		
		13-Tetradecynoic acid, methyl ester [IV]							7606288.6	0.823
39	41.065	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl			42509560.75	4.650				
		ester [IV]	103383442	18.071			28612141.4	4.440	33376364.6	3.611
40	41.195	Octadecanoic acid [I]	102271137.5	17.876	97346107	10.649	43210827.75	6.706	91202089.25	9.868
41	41.405	Unknown hydrocarbon	5417020	0.946	5790721	0.633	5980277	0.928	11490641.5	1.243
42	41.500	Tetradecanoic acid [I]	5515775.5	0.964	4008623.55	0.438	4209676.25	0.653	7969038	0.862
43	41.715	Eicosanoic acid [I]	4985050.75	0.871	13199727.25	1.444	2118364.45	0.328	17737168	1.919

44	41.790	i-Propyl 16-methyl-heptadecanoate [IV]	5696294.4	0.995	13493035.1	1.476	9708864.1	1.506	4314599.75	0.466
45	41.980	L-Ascorbic acid, 6-octadecanoate [IV]	15095812.5	2.638	7336475.5	0.802	22524521.9	3.495	4594544.9	0.497
46	42.191	Z-8-Methyl-9-tetradecenoic acid [I]	15783332.75	2.758						
		Isopropyl palmitate[I]			17294237.75	1.891	4360305.1	0.676	18310817.5	1.981
47	42.606	cis-10-Nonadecenoic acid[I]	3638539.95	0.636	6537037.75	0.715	4728544.45	0.733	13762814	1.489
48	42.866	3-Pentanol, 3-ethyl- [III]	2573963.7	0.449						
		cis-11-Eicosenoic acid[I]			55724753.5	6.096	21362662.7	3.315	32882179.4	3.557
49	43.026	trans-13-Octadecenoic acid[I]	4966599.6	0.868					100414072	10.865
		6-Octadecenoic acid, (Z)- [I]			60222584	6.588	113806998.3	17.663		
50	43.171	6-Octadecenoic acid[I]	7394109.5	1.292						
		Oleic acid[I]			3997464.95	0.437	110615808	17.168	14896564.75	1.611

Table 21: Summary of the aroma compounds found the SS, LC, LA and LCA

Codes	Volatile compounds	SS	LC	LA	LCA	Odour quality
I	Acids	15	23	18	18	Caramel
II	Aldehyde	5	3	5	3	Leaf, green, fat, orange peel, tallow, almond, malt, pungent, citrus, soap, burnt sugar
III	Alcohols	2	4	6	6	Grass, green, flower, whiskey, malt, burnt, resin
IV	Esters	18	17	14	11	Pineapple, fruity, fresh, sweet, orange, grass, banana, apple peel
V	Ketones	3	2	5	3	Soap
VI	Terpenes			1		Flower, lavender

Odour quality was cited from Bicas *et al*., (2011), Santana *et al*., (2017) and Waché and Dijon, (2013).

4.4.1.4. Free amino acids

Amino acid content of the samples (fermented and unfermented *A. muricata* juices) in this study were analysed by LCMS. The general trend presented in Table 22 indicates that all amino acids decreased after fermentation but for tryptophan, phenyl alanine and alanine. This is a clear demonstration that the microorganisms studied used up those amino acids as an integral component of their growth nutrients. It is also worthy of note that amongst the samples, LCA recorded the highest decrease from the initial values (unfermented sample) compared to LC and LA. The microorganisms used in this study (*L. casei*, *L. acidophilus* and

the consortium) utilised serine, leucine, histidine, methionine, aspargine, threonine, cystine, valine, glutamic acid, proline, aspartic acid, glutamine, tyrosine, isoleucine, arginine, hydroxyl

proline, cysteine, and lysine as essential amino acids for their growth. This outcome was similar to the study conducted by KiBeom *et al*., (2014), where *L. salivarius* and *L. plantarum* were found to have consumed these amino acids during fermentation even though *L. plantarum* consumed alanine and tryptophan in addition to those above. Alanine and tryptophan were observed to have increased after fermentation. This indicates that *L. casei*, *L. acidophilus* and the consortium might have produced these two amino acid after consumption of the others hence are nonessential for their growth. *L. salivarius* also released alanine in the findings of KiBeom *et al*., (2014). Phenylalanine content for LC had no significant difference meanwhile it increased for LA and decreased for LCA. This implies that phenylalanine was a nonessential amino acid for *L. acidophilus* but was essential for the microbial consortium. D'Este *et al*., (2018) termed L-tyrosine, L-phenylalanine and L-tryptophan as aromatic amino acids. The samples in this study are most likely to display antioxidant properties since they contained peptides of 5-16 amino acid residues meanwhile methionine, lysine, cysteine, tyrosine, tryptophan and histidine are examples of amino acids displaying antioxidant activity (Hunaefi *et al*., 2013).

Table 22: Amino acid content (in ppb) of unfermented and fermented A. muricata juices

	Enzymatic *A. muricata* juice	**LC**	**LA**	**LCA**
Tryptophan	288.406 ± 1.58	541.899 ± 9.09	422.329 ± 37.80	371.099 ± 3.94
Serine	187.097 ± 3.78	50.566 ± 1.67	50.613 ± 1.42	49.314 ± 1.12
Leucine	315.827 ± 2.88	86.923 ± 0.01	160.148 ± 1.30	93.297 ± 1.73
Histidine	46.556 ± 0.37	15.844 ± 0.47	16.408 ± 0.46	12.898 ± 0.60
Methionine	2.437 ± 0.23	0.085 ± 0	0.341 ± 0.14	0.717 ± 0.14
Aspargine	1434.35 ± 67.82	678.574 ± 7.86	583.48 ± 11.44	233.774 ± 6.35
Cystine	21.667 ± 0.56	14.055 ± 0.28	12.206 ± 1.45	10.707 ± 1.93
Threonine	52.269 ± 1.49	24.737 ± 2.57	27.436 ± 1.92	14.851 ± 1.89
Valine	112.35 ± 2.17	45.513 ± 7.08	62.801 ± 8.65	38.126 ± 0.77
Phenylalanine	321.296 ± 2.57	320.872 ± 0.08	333.678 ± 7.95	222.99 ± 0.36
Glutamic acid	192.862 ± 13.12	90.424 ± 2.05	91.812 ± 1.09	74.561 ± 2.28
Proline	10861.8 ± 261.50	5763.49 ± 165.83	5629.07 ± 167	5323.9 ± 18.43
Aspartic acid	243.349 ± 20.77	84.916 ± 5.50	95.875 ± 6.37	77.421 ± 0.91
Glutamine	101.058 ± 2.85	5.689 ± 0.43	13.277 ± 1.38	11.694 ± 0.30
Lysine	98.518 ± 0.83	11.858 ± 0.45	18.243 ± 0.25	16.537 ± 0.19
Tyrosine	510.144 ± 6.63	202.262 ± 0.39	200.22 ± 1.86	131.935 ± 3.14
Isoleucine	319.297 ± 2.76	88.498 ± 0.91	169.779 ± 10.59	91.622 ± 4.70
Arginine	1611.78 ± 121.90	140.88 ± 0.21	77.635 ± 7.91	90.821 ± 3.62
Hydroxyl proline	285.248 ± 0.94	77.878 ± 0.13	138.327 ± 2.72	82.895 ± 1.64
Cysteine	12.119 ± 0.29	7.051 ± 0.43	8.657 ± 0.88	8.298 ± 0.48
Alanine	9.937 ± 0.67	15.712 ± 2.315	11.078 ± 0.05	19.936 ± 6.28
Glycine	ND	ND	ND	ND

4.4.1.5. Polyphenolic acid content

Some phenolic acids were observed to increase while others decreased as well as some remain unchanged after fermentation for all samples. Catechin, gallic acid, chlorogenic acid, caffeic acid,

epigallo catechin and *p*-coumaric acid decreased after fermentation. Epicatechin, syringic acid, luteolin, kampferol, shikkimic acid, elagic acid and cinnamic acid increased after fermentation. Whereas, quinine, apigenin, hesperitin and chrysine contents remained unchanged. Svensson *et al*., (2010) demonstrated a decrease in caffeic acid, coumaric acid and ferulic acid after fermenting sorghum dough with two binary strains combination of lactic acid bacteria, *L. casei* inclusive, and this was explained by the fact that phenolic acid decarboxylases and phenolic acid reductases which could be present in the microbial strains degrade phenolic acids. Also Chen *et al*., (2018) achieved a decrease in bioactive components after fermentation of papaya fruit juice using *L. acidophilus* and *L. plantarum*. On the other hand, Hunaefi *et al*., (2013) reported similar results and attributed the increase in phenolic acids to the structural breakdown of plant cell walls induced by fermentation. The decrease pH creates an atmosphere for bound phenolic constituents to be released via enzymatic processes hence liberation and/or synthesis of various bioactive compounds.

Table 23: Polyphenolic acid content (in ppb) of unfermented and fermented A. muricata juices

	Enzymatic *A. muricata* juice	**LC**	**LA**	**LCA**
Catechin	38.971 ± 1.52	29.190 ± 0.89	33.171 ± 0.33	27.139 ± 1.59
Quinine	1.194 ± 0.20	1.108 ± 0.35	0.8665 ± 0.12	0.796 ± 0.20
Narigenin	23.506 ± 1.87	21.403 ± 0.68	25.275 ± 1.41	16.3 ± 0.05
Tocopherol	3.149 ± 0.17	3.813 ± 0.62	3.013 ± 0.29	3.101 ± 0.03
Gallic acid	8.435 ± 0.61	6.203 ± 1.65	5.163 ± 0.05	5.302 ± 0.70
Chlorogenic acid	0.924 ± 0.02	0.338 ± 0.03	0.459 ± 0.12	ND
Epicatechin	28.838 ± 0.14	39.263 ± 3.3	51.534 ± 0.69	37.855 ± 3.75

Syringic acid	29.118 ± 1.76	44.741 ± 4.96	40.670 ± 2.35	31.251 ± 2.04
Vanilic acid	9.254 ± 0.27	9.953 ± 0.73	6.926 ± 0.26	7.872 ± 0.21
Caffeic acid	23.738 ± 1.78	4.478 ± 0.42	6.92 ± 0.57	3.877 ± 0.11
Epigallo catechin	0.865 ± 0.13	4.045 ± 0.19	4.012 ± 0.15	4.489 ± 0.20
Ferulic acid	159.503 ± 2.65	164.265 ± 4.45	152.786 ± 6.31	99.248 ± 2.31
Mycertin	1.273 ± 0.28	1.308 ± 0.22	1.180 ± 0.16	1.437 ± 0.07
P-Coumaric acid	137.3 ± 3.75	34.833 ± 2.45	18.278 ± 0.796	11.109 ± 1.40
Luteolin	0.031 ± 0.00	0.099 ± 0	0.323 ± 0.01	0.103 ± 0.01
Apigenin	0.533 ± 0	0.548 ± 0.02	0.539 ± 0.01	0.536 ± 0.01
Kampferol	0.687 ± 0.04	1.220 ± 0.05	1.086 ± 0.06	0.684 ±
Hesperitin	0.927 ± 0.11	0.846 ± 0.02	0.876 ± 0.04	0.828 ± 0
Shikkimic acid	159.327 ± 4.44	204.046 ± 31.12	202.355 ± 5.51	206.332 ± 5.60
Elagic	13.764 ± 2.99	152.332 ± 2.34	160.834 ± 3.07	88.114 ± 2.43
Genstein	0.563 ± 0	0.590 ± 0	0.548 ± 0	0.548 ± 0
Cinnamic acid	27.959 ± 3.02	82.754 ± 0.82	51.190 ± 2.04	33.888 ± 2.22
Chrysine	0.865 ± 0	0.886 ± 0.02	0.880 ± 0.00	0.870 ± 0
Catechol	ND	ND	ND	ND
Morin	ND	ND	ND	ND
Quercetin	ND	ND	ND	ND
Rutin	ND	ND	ND	ND
Diadzein	ND	ND	ND	ND

ND: not detected

4.4.2. Storage study of *A. muricata* fermented juice

The composition of food, types of packaging material and storage environment (storage temperature, moisture content of powders, relative humidity, oxygen content, and exposure to light, etc) have significant influence on the survival of probiotics. Parameters such as cell viability, pH, reducing sugar, organic acids, SCFA and TPC were studied during storage.

4.4.2.1. Viable count

The ability of all two strains and the consortium to survive the effect of storage temperature (4 ℃) for 28 days was evaluated. A decrease was observed after day 0 of storage, which could be attributed to the stress induced from the temperature differences between the fermentation environment (37 ℃) and the storage environment (4 ℃) where the microorganisms should have probably experienced a shock and some rendered non-viable or the development of dormant physiological states due to conditions of environmental stress (Acevedo-Martínez *et al*., 2018; Reddy *et al*., 2015). Nonetheless, this decrease was highest in sample LA which decreased by 0.125 Log CFU/mL significantly different at $p<0.05$, then LCA which was also significantly different at $p<0.05$ by 0.062 Log CFU/mL meanwhile for LC there was a significant difference $(p<0.05)$ only between 0^{th} day and the 21^{st} and 28^{th} day. This could be attributed to the fact that *L. acidophilus* is more anaerobic than *L. casei*. After the 7^{th} day, the microorganisms were said to have adapted to the storage condition since the viable cell count was slightly constant for samples LC and LCA up to the 28^{th} day meanwhile LA decreased further from the 21^{st} to the 28^{th} day by 0.092 Log CFU/mL. Similar losses in viability of two species of *L. acidophilus* were observed by Champagne and Gardner, (2008) were the effect of storage (at 4 ℃) on different probiotic strains in different fruit drinks was studied. The findings of Ranadheera *et al*., (2012) reported a more significant decrease in *L. acidophilus* compared to other strains meanwhile Fernandes *et al*., (2013) found loss of *L. acidophilus* viability in milk dessert during shelf-life.

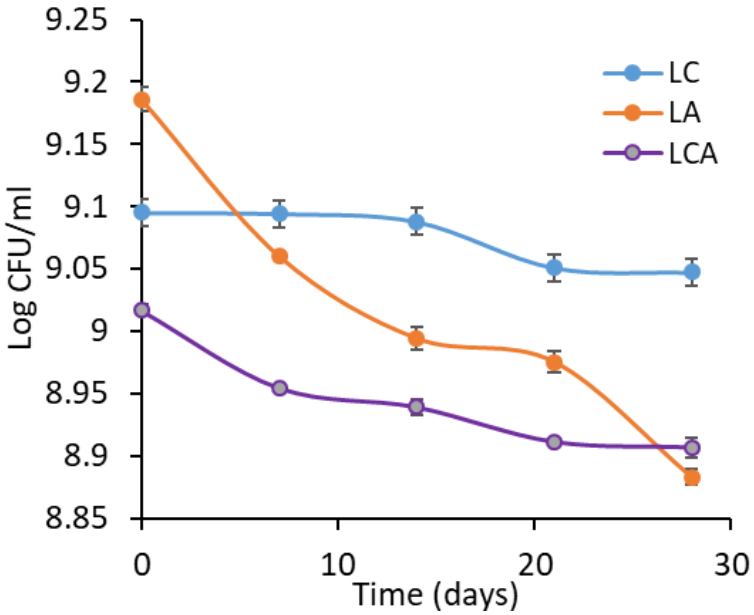


Figure 27: Cell viability of A. muricata juice fermented using L. casei, L. acidophilus and a mixture (50:50) stored at 4 °C for 28 days

Previous studies reported that the factors contributing to the loss of cell viability the most are decreasing pH during product storage (post-acidification) and the accumulation of organic acids as a result of growth and fermentation (Kailasapathy *et al*., 2008). Several investigations showed that bacteria in the logarithmic phase are much more susceptible to environmental stresses as compared to those in stationary phase (Donkor *et al*., 2006) which could probably have been the case for *L. acidophilus* in this study. After a 60 days storage study carried out by Kemsawasd *et al*., (2016) at 4°C, *L. casei* and *L. acidophilus* maintained their survival rates higher than 6 Log CFU/g in refrigerated chocolate. It is worthy of note that the viable cell count at the end of shelf-life was more than 8.8 Log CFU/mL for all samples which is higher than the recommended range (10^6-10^7 CFU/mL) of probiotic (FAO/WHO, 2002). It can be concluded that these beverages after 28 days of storage still possess their viability.

4.4.2.2. pH

During shelf-life study, the pH of the samples were evaluated and represented in Figure 27 showing the impact of the storage condition on the evolution of pH. No great change was witnessed between 0th and 7th day for LC, LA and LCA meanwhile there was a slight increase for LA and LCA on day 14 which continued to day 21 for LA and dropped on the 28th day with the reverse happening for LCA. On the other hand, the pH of LC dropped from day 7 to day 14 by 0.165 (3%), constant til 21st day then further decreased by 0.285 (5%) on the 28th day. This decline in pH was presumed to be as a result of continued fermentation or postacidification (Kailasapathy *et al*., 2008) and pH decrease throughout storage didn't seem to affect the viability of the cells in LC (Donkor *et al*., 2006).

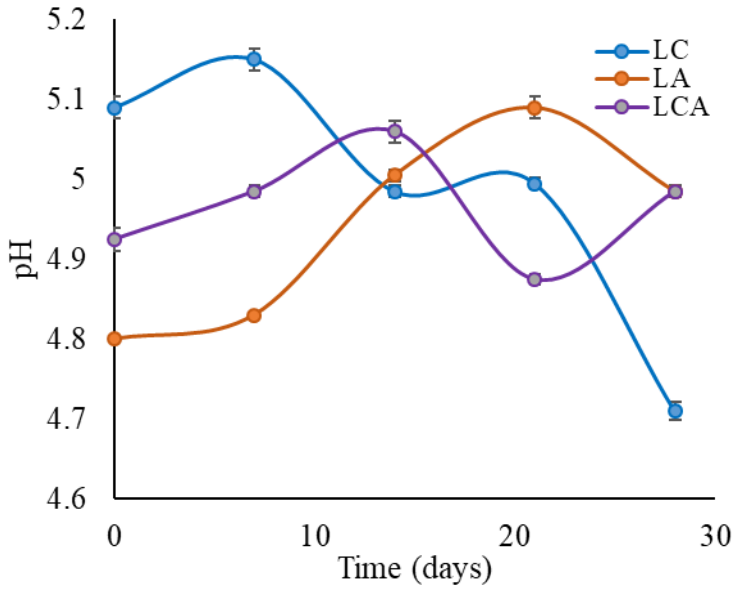


Figure 28: Effect of refrigeration at 4 °C on pH profile of fermented A. muricata juice after 28 days of storage

The physiological state of the probiotic organisms is of special importance when selecting a strain. The termination pH may affect viability due to either one or all of the factors including initial pH,

fermentation time resulting in varying temperature exposure and levels of organic acids produced at the end of fermentation and during storage (Donkor *et al*., 2006). The fluctuating or unusual trend in pH of LA and LCA samples indicated weak metabolic activity during storage at 4 ℃ and this was similar to the observations of Ranadheera *et al*., (2012) and Schlabitz *et al*., (2015). On the other hand, Panesar and Shinde, (2012) observed a decrease in the pH during 28 days storage of *L. acidophilus* in aloe vera fortified probiotic yoghurt and related it to the production of lactic acid, small amounts of CO_2 and formic acid from lactose.

4.4.2.3. Reducing sugars

During storage, the reducing sugar content of all three samples decreased from 0^{th} day to the 28^{th} day; 62.877 to 50.862 g/L for LC, 59.686 to 46.784 g/L for LA and 63.213 to 51.678 g/L for LCA respectively implying refrigeration temperature does not completely stop fermentation (Schlabitz *et al*., 2015). Statistically, there was a significant (p<0.05) decrease between day 0 and all the other days (7-28) for LC and LA meanwhile for LCA, the significant (p<0.05) decrease was only between day 0 with 14 and 21. This could be attributed to the fact that the sugars: glucose, fructose and sucrose (highest sugar in *A. muricata* is fructose then glucose) probably served as a substrate for microorganisms thus was slowly consumed during storage as reported by Pereira *et al*., (2013). LC exhibited similarities to the findings of Costa *et al*., (2013) in which sonicated pineapple juice was used as a substrate for *L. casei* cultivation for probiotic beverage. This consumption of sugars was an indication of post acidification during the storage period. Rodrigues *et al*., (2012) assessed the total sugars in probiotic orange and peach juices over 50 days of storage at 5 °C. These authors also reported that glucose and fructose levels decreased during storage, which was attributed to *Lactobacillus paracasei* L26 growth and sugar fermentation as in the case of LA and LCA.

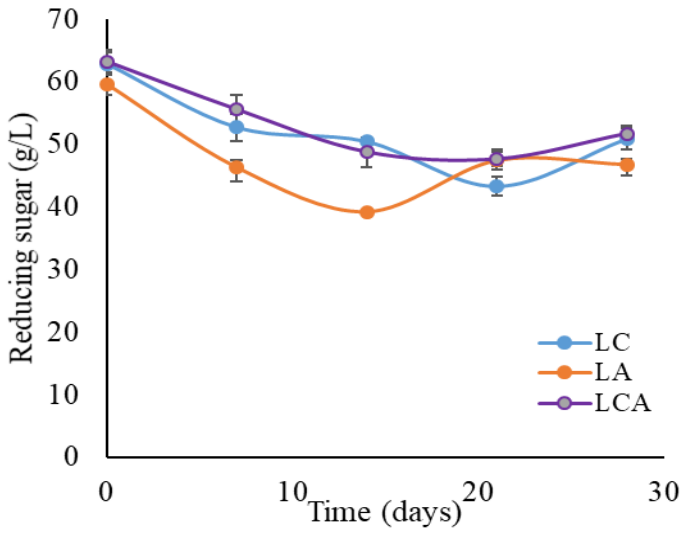


Figure 29: Reducing sugar content of fermented A. muricata juice during storage period of 28 days at 4 °C

4.4.2.4. Organic acid

Figure 30 shows the variation in organic acid content (lactic acid) of the samples during shelf-life study as determined by HPLC. The exact opposite phenomenon of pH was presented here where there was a significant (p=0.05) increase in the organic acid content between day 0 and days 21 and 28 for LC during storage. But a rise and fall trend was witnessed for LA and LCA even though there was a significant difference between day 0 and days 21: 28 (LA) and day 0 and days 14: 28 (LCA).

Interestingly, the highest amount of organic acid was produced by sample LC which exhibited the best cell survival during storage compared to LA and LCA. The result of LC is in accordance with Ertem and Çakmakçı, (2018), Pereira *et al*., (2013) and Tonguc *et al*., (2013), who obtained an increase in the organic acid content in their different studies. The increase in organic acid content during storage could be attributed to the utilisation of available sugars (indicating that metabolism of the available carbon sources was taking place), which is a natural process by lactic acid bacteria

leading to the production of lactic acid and other organic acids as observed by Donkor *et al*., (2006). Lactic acid could have been produced from the metabolism of sugars (as mentioned above), or the metabolism of malic acid through the action of the malo-lactic enzyme, which has been identified in *L. plantarum* (Nualkaekul and Charalampopoulos, 2011). Nevertheless, a decrease was observed for LA sample while a decrease and simultaneous increase was the case of sample LCA. This was possibly due to proteins biodegradation resulting in ammonia generation thus causing the decrease in organic acid content and this corroborates the findings of Schlabitz *et al*., (2015).

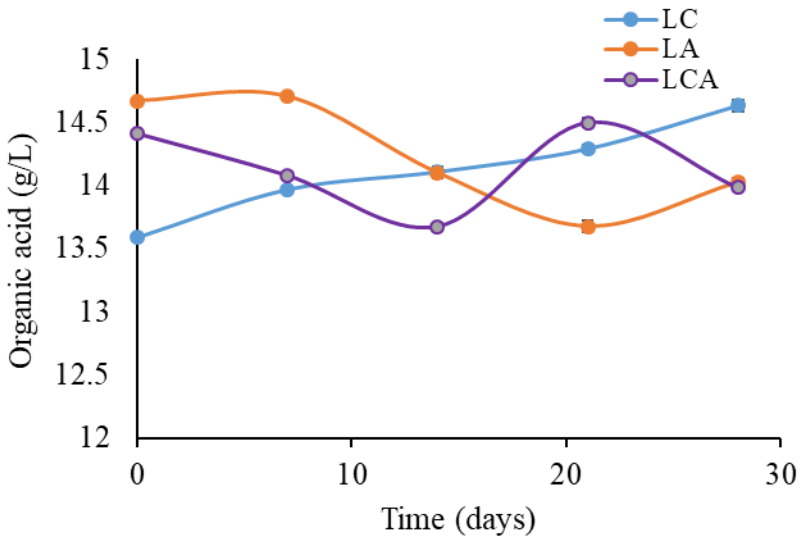


Figure 30: Effect of the storage condition on the of organic acid content of fermented A. muricata during a period of 28 days

4.4.2.5. Short chain fatty acids (SCFA)

Figure 31 gives a representation of the SCFA trend obtained during storage, analysed by HPLC using acetic acid, propionic acid, and butyric acid standards. The samples in this study were found to contain acetic acid and butyric acid but the plot for SCFA used values for acetic acid even though the microorganisms seem not to have produced great amounts. Sample LC was observed

to be significantly different (p<0.05) only on day 21, while for sample LA there was no significant different (p=0.05) for day 0 with 7 - 14 but it existed for the other days, and lastly for sample LCA day 0 and 7 were significantly different (p<0.05) from the days 14 - 28.

Sample LCA had a decrease on day 7 and slightly constant trend up to the end of storage. Sample LA was constant right up to day 14 then decreased on the 21st day while it was the reverse for LC with an increase in the 21st day. The findings of this research are close to the observation by Kennes *et al*., (1991) and Palles *et al*., (1998) who also noticed a more or less consistent concentrations of acetic acid and concluded that the microorganism do not produce much acetic acid during storage and also that they survived better in the acidity.

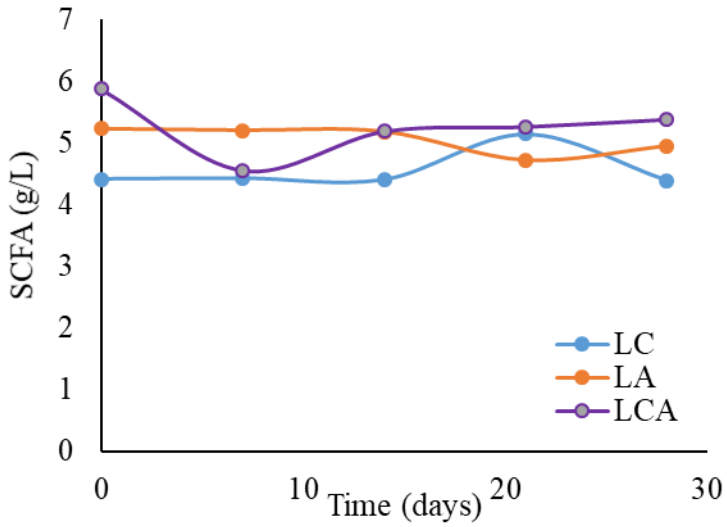


Figure 31: Effect of storage temperature on the short chain fatty acid content of fermented A. muricata juice after 28 days of storage

4.4.2.6. Total polyphenol content (TPC)

Figure 32 shows the TPC pattern during refrigerated storage observing slight increases. LA and LCA exhibited same trend with a significant difference (p=0.05) between day 28 with days 7 and 14 for LA, days 0, 7, 14 significantly different (p=0.05) from 14, 21, 28 for LCA. Meanwhile for

LC a significant difference (p<0.05) was between 0 and 7. However, these discrepancies observed in TPC is dependent on the food matrix, probiotic strain, conditions and time of storage. Kalita *et al*., (2018) observed a decrease in the total polyphenol content and attributed it to the fact that the phenolic compounds found in fresh fruit juice are generally glycosylated with sugar that on fermentation of the juice and sugar consumption by microorganism undergo deglycosylation and release of the free hydroxyl groups and relevant aglycones which can contribute to the improved functional properties of the litchi juice.

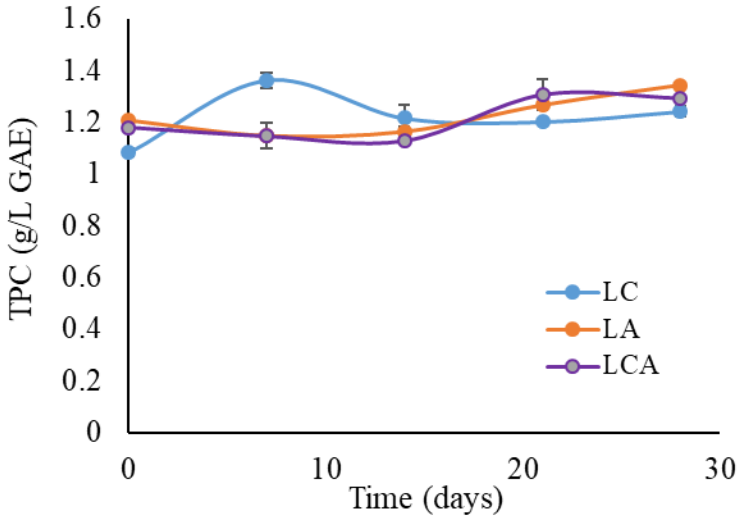


Figure 32: Changes in total polyphenol content during 28 days of storage at 4 ℃

4.4.2.7. Antioxidant activity

The antioxidant activity of the samples during storage at 4 ℃ were analysed by DPPH and TEAC assays. There are principally two groups of antioxidant compounds: primary antioxidants which directly scavenge free radicals and secondary antioxidants which prevent the formation of free radical via Fenton reaction (Muniandy *et al*., 2016). These antioxidant compounds can protect the human host as well as increase the shelf-life of the foodstuffs by retarding the process of lipid peroxidation through hydrogen atom or electron transfer (Sah *et al*., 2015). The reaction

mechanisms involving mainly a hydrogen atom transfer is the basis for 2,2-diphenyl-1-picrylhydrazyl (DPPH)-based assays and both hydrogen atom transfer and single electron transfer for 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC)-based assays (Sah *et al*., 2015). An oxidation process involves a sequence of reactions and antioxidant activity via multiple reaction mechanisms which can be demonstrated by protein hydrolysates hence several methods (assays) are required to be carried out for a better understanding of the antioxidant activities of *A. muricata* (Sah *et al*., 2015).

Figure 32 represents the antioxidant activity of fermented *A. muricata* juice during storage. The DPPH (Fig 32a) results revealed that there was an increase in the antioxidant content of the samples for LC during storage, as well as there was an increase and a decrease followed for LCA with the highest being on the 14^{th} day meanwhile for LA it was almost constant throughout storage. As for the sample LC, there was no significant difference at $p \leq 0.05$ between the 0^{th} and the 14^{th} day while there was a significant difference for day 21 and 28 with the others. For LCA, the 14^{th} day was significantly different from all the samples at $p \leq 0.05$ but for LA, 0^{th} to 14^{th} day was significantly different from each other meanwhile 21^{st} and 28^{th} day were not significantly different from each other but where significantly different from 0^{th} to 14^{th} day. On the other hand, the values for TEAC assay (Fig 4.9b) were almost constant during storage with the highest values being for LC. The 0^{th}, 14^{th} and 28^{th} day were not significantly different at $p \leq 0.05$ whereas the 7^{th} and the 21^{st} day were significantly different.

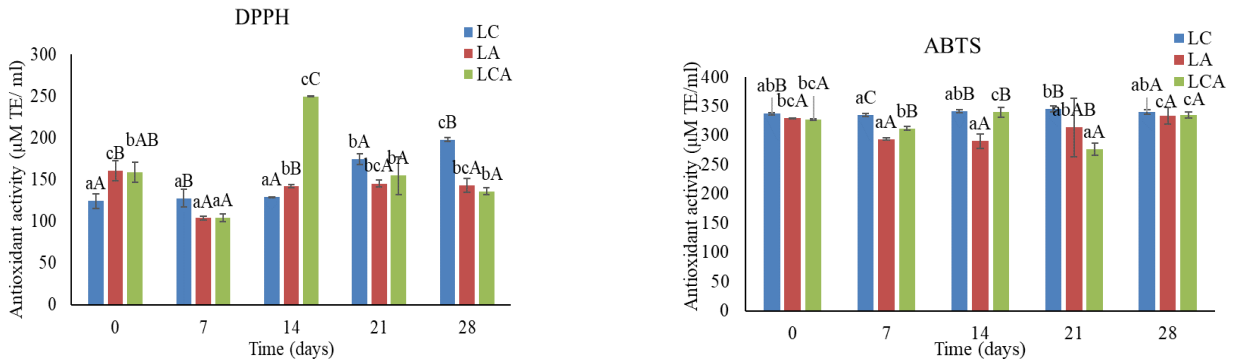


Figure 33: Illustration of the antioxidant activity of A. muricata probiotic juice during storage

In a study by Mishra *et al*., (2015), it was reported that a high antioxidant activity and lipid peroxidation inhibition was exhibited by intact cells and intracellular free extracts of *L. casei* KCTC. Comparing several probiotic strains, *L casei* HY 2782 was reported to contain the highest level of glutathione sulfhydryl (GSH), which is a molecule that donates an electron to the destructive hydroxyl radicals converting it to H_2O (Yoon and Byun, 2004). Hence LAB act beneficially by providing boosting effect of cell glutathione (antioxidant in cell remain neutral after neutralizing free radicals). The juices produced had high content of antioxidant after storage hence can serve as a natural source of antioxidant preventing nucleic acid, proteins and lipid damages caused by reactive oxygen species (Gyesi *et al*., 2019).

4.5. Conclusion

This study was conducted to investigate the shelf-life of *A. muricata* juice fermented with *L. casei*, *L. acidophilus* and the microbial consortium during storage for 28 days, after analysis of the technological, rheological and aroma properties of the probiotic beverages obtained at optimum fermentation time. Overall, during storage a more continuous though slow fermentation occurred in the case of LC compared to LA and LCA. This is observed in the constant trend in viable count, decreasing pH and increasing organic acids contents. The behavior of LA and LCA on the other hand, during storage was fluctuating but a general decrease in cell viability was observed by 0.125 Log CFU/mL for LA and 0.062 Log CFU/mL for LCA which were significantly different at $p<0.05$. *L. acidophilus* was considerably less stable in the fermented *A. muricata* juice than other lactic acid cultures (*L. casei*, and the consortium of *L. acidophilus* and *L. casei*) during cold storage at 4 °C. Therefore, variations in strain stability observed in this study may be due to pH, juice composition or oxygen. In addition, this probiotic *A. muricata* juice produced can be consumed by children, elderly, vegetarians and individuals who are lactose intolerant or on cholesterol-restricted diets. In this way, this probiotic fruit juice developed constitutes a promising functional probiotic product that can be consumed by all age groups.

GENERAL CONCLUSION

The main objective of this project was to extend the shelf-life of *A. muricata* fruit pulp by the production of a probiotic beverage, using *L. casei, L. acidophilus*, and their consortium. The first part of the project aimed to optimise the conditions of extraction of *A. muricata* juice using pectinase-assisted extraction. The extracted juice was further fermented using *L. casei, L. acidophilus* and their consortium and the fermentation process modelled to obtained the kinetic parameters. Finally, the microbial and physicochemical stability of the fermented *A. muricata* probiotic juice was evaluated.

The pectinase-assisted extraction of *A. muricata* juice was optimised by combining the effects of three relevant independent variables to maximise extraction yield, total soluble solids, pH, clarity and minimise titratable acidity. The achieved experimental data were successfully fitted to the theoretical models used to determine the optimal extraction. The optimum for extraction yield (75.20%), pH (3.74), clarity (87.06%T), TSS (7.35 °Brix), and titratable acidity (0.44% MAE) of *A. muricata* juice were achieved with an incubation time of 172 min, an enzyme concentration of 0.04% (w/w), and an incubation temperature of 42.2 °C. The pectinase treatment of *A. muricata* revealed a significant increase in the juice yield as well as the total soluble solids and clarity. The morphological and chemical structures confirmed the action of pectinase in the *A. muricata* pulp which resulted in juice with improved qualities. These attributes obtained renders the fruit a potential substrate for the growth and maintainance of probiotic microorganism.

The study of the fermentation process of *A. muricata* juice obtained via pectinase-assisted extraction clearly revealed that enzymatically pretreated juice provided a better medium for the action of *L. casei*, *L. acidophilus* and their consortium. The LA obtained the highest maximum cell growth of 11.5 (log CFU/mL) after 36 h for LCA; followed by LA with 10.1 (log CFU/mL) after

36 h and 9.1 (log CFU/mL) for LC after 24 h. The Monod, Luedeking and Piret models revealed best fitting to the experimental data for biomass accumulution, product formation and substrate utilisation. Taking into account the definition of probiotics (biomass ≥ 6 log CFU/mL), the logistic equation was employed which predicts the maximum biomass, and showed best fitting for growth kinetics strictly.

The study of the shelf-life of *A. muricata* probiotic beverage showed that this probiotic beverage can stay for 28 days at 4 ℃ on the shelf with maintained functional, antimicrobial and technological properties like other existing probiotics in the market.

For the optimal production of an *A. muricata* probiotic juice, the juice should be extracted from the pulp with 0.04% pectinase concentration for 172 min at 42 ℃ and the juice fermented for 36 h at 37 ℃ with a mixture of *L. casei*, and *L. acidophilus.*

PERSPECTIVES

However, some important aspects of this project could not be addressed and deserve to be studied in order to complete the results obtained in this thesis.

- The enzymatic extraction method used during this project can be employed for juice extraction of other pectinaceous fruits.
- Application of other green and innovative methods for extraction of juice from *A. muricata* pulp.
- Further studies are necessary to evaluate the effect of other factors, such as growth supplements, temperature, pH, and starter cultures load on the fermentation kinetic parameters of *A. muricata* juice with fermented probiotics for scale-up.
- The sensory evaluation of the product must be carried out and if necessary food additives should be added to improve sensory characteristics and shelf-life of *A. muricata* juice fermented juice.
- Multienzyme hydrolysis of pectin should be studied
- Microbiological analyses of the final product should be evaluated.

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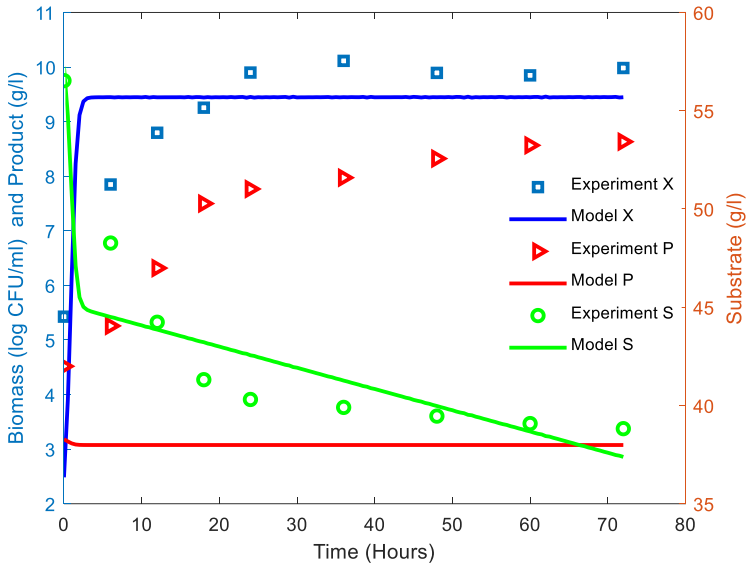
APPENDICES

Appendix 1: Standard curve and determination of total phenolic compound

Tubes number	1	2	3	4	5	6	trial
Gallic acid 0.2g/l (mL)	0	0.01	0.02	0.03	0.04	0.05	
Sample (mL)	/	/	/	/	/	/	0.02
Distilled water (mL)	1.4	1.39	1.38	1.37	1.36	1.35	1.2
Folin-Ciocalteu reagent 1N	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Keep for 3 min							
Na_2CO_3 20% (mL)	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Mix and incubate in water bath at 40°C for 20 min							
OD at 765 nm							
Gallic acid (mg)	0	2	4	6	8	10	



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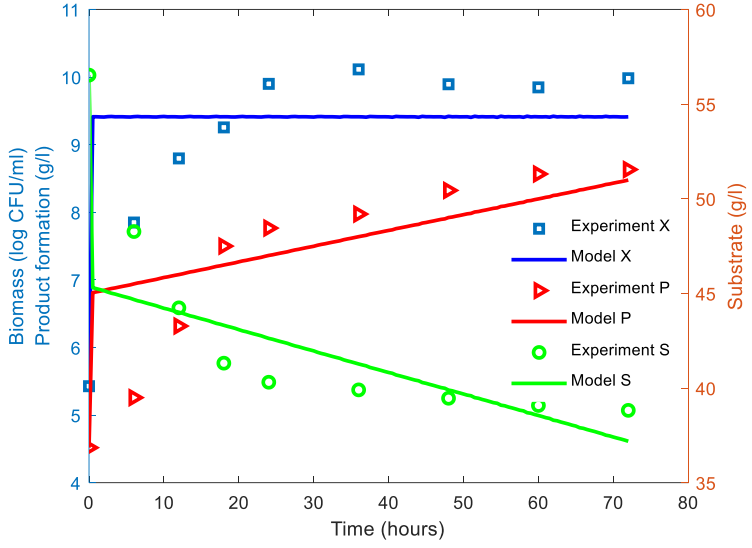

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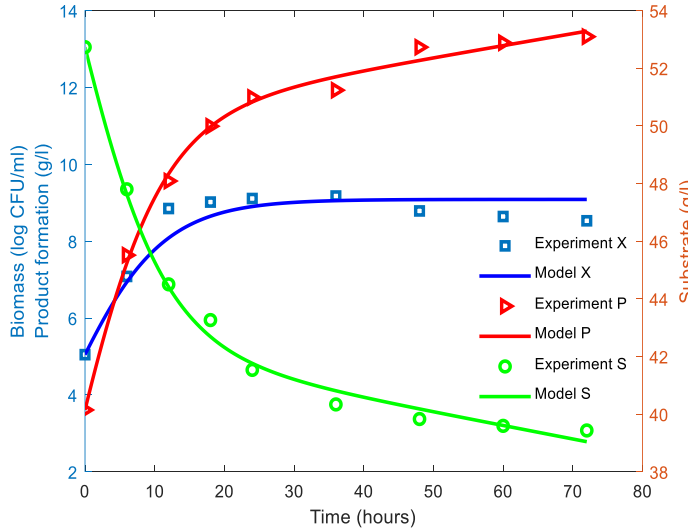
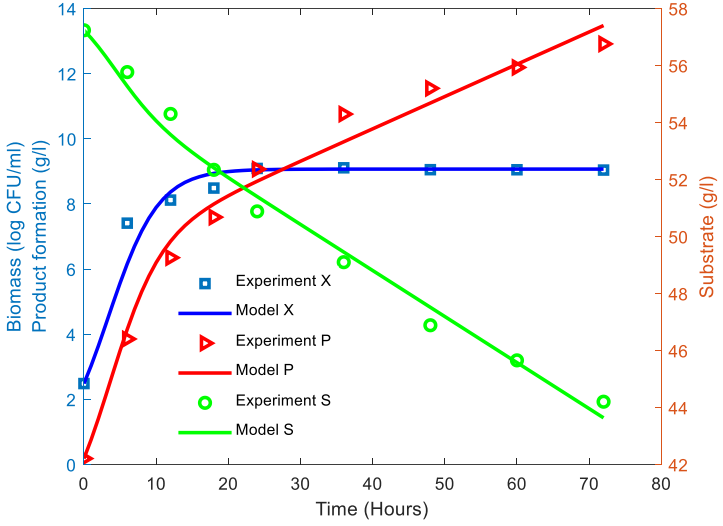


LCA

Appendix 2: Simulated and experimental results of biomass, substrate and lactic acid concentrations (1): Case of Monteagudo et al. *(1997)*

b



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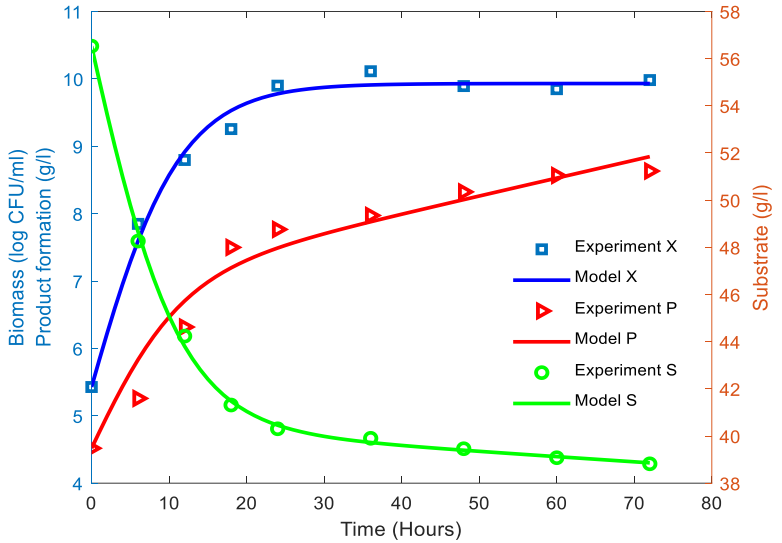

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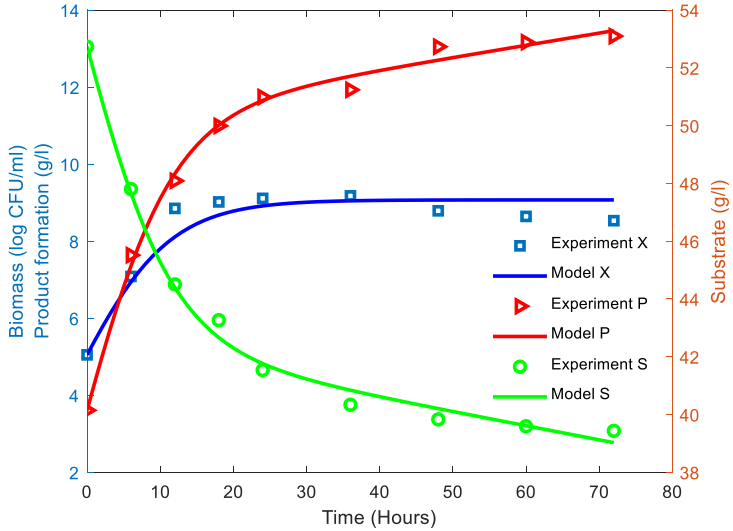
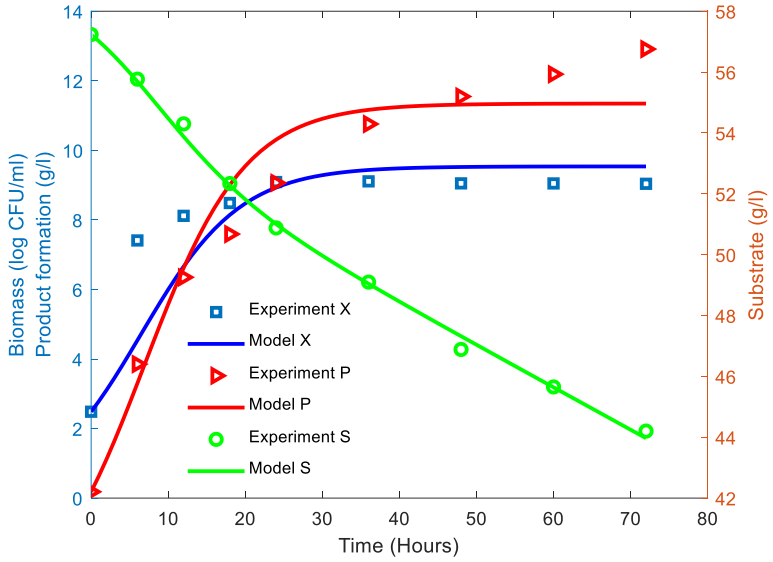


LCA

Appendix 3: Simulated and experimental results of biomass, substrate and lactic acid concentrations (2): Case of Starzak et al., *(1994)*



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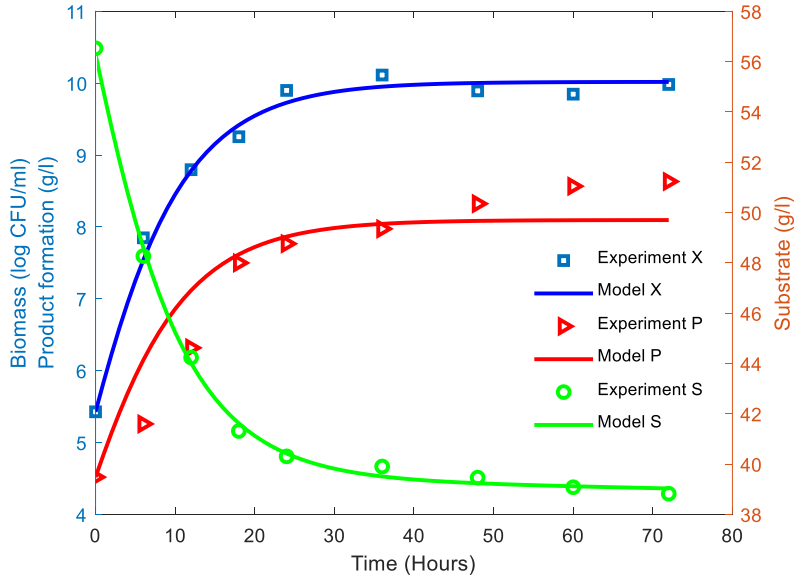

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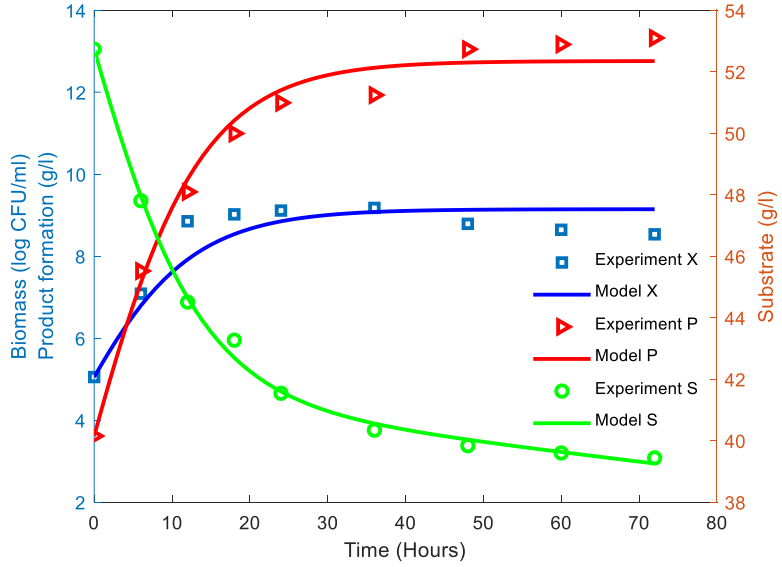
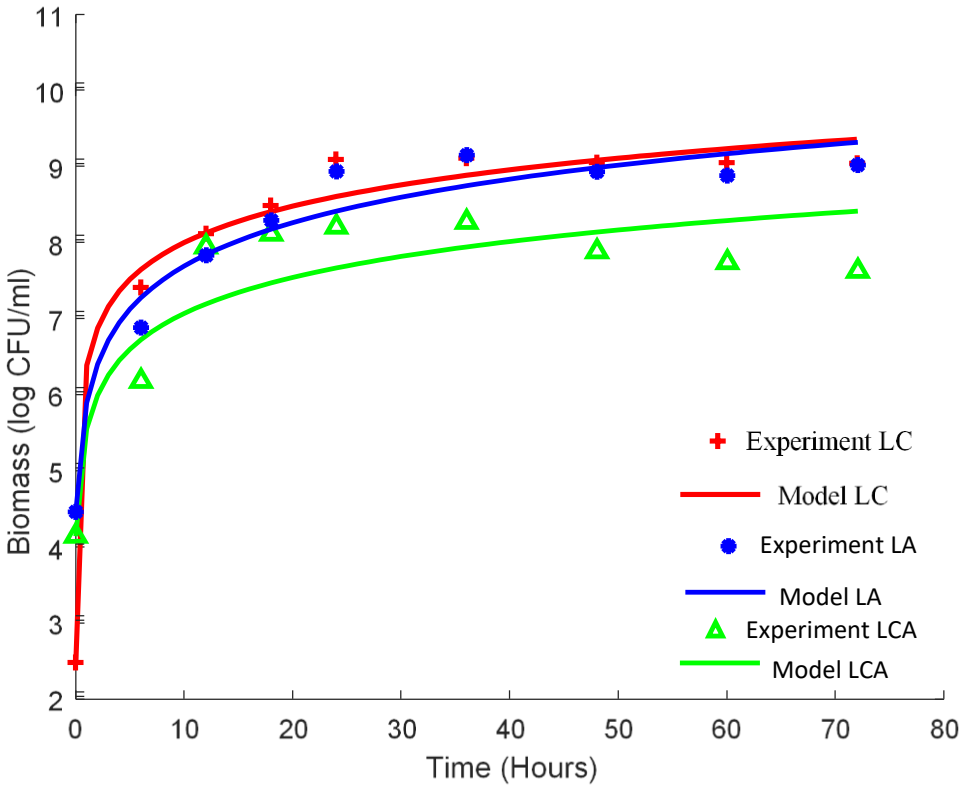


LCA

Appendix 4: Simulated and experimental results of biomass, substrate and lactic acid concentrations (3): Case of Dutta et al., *(1996)*

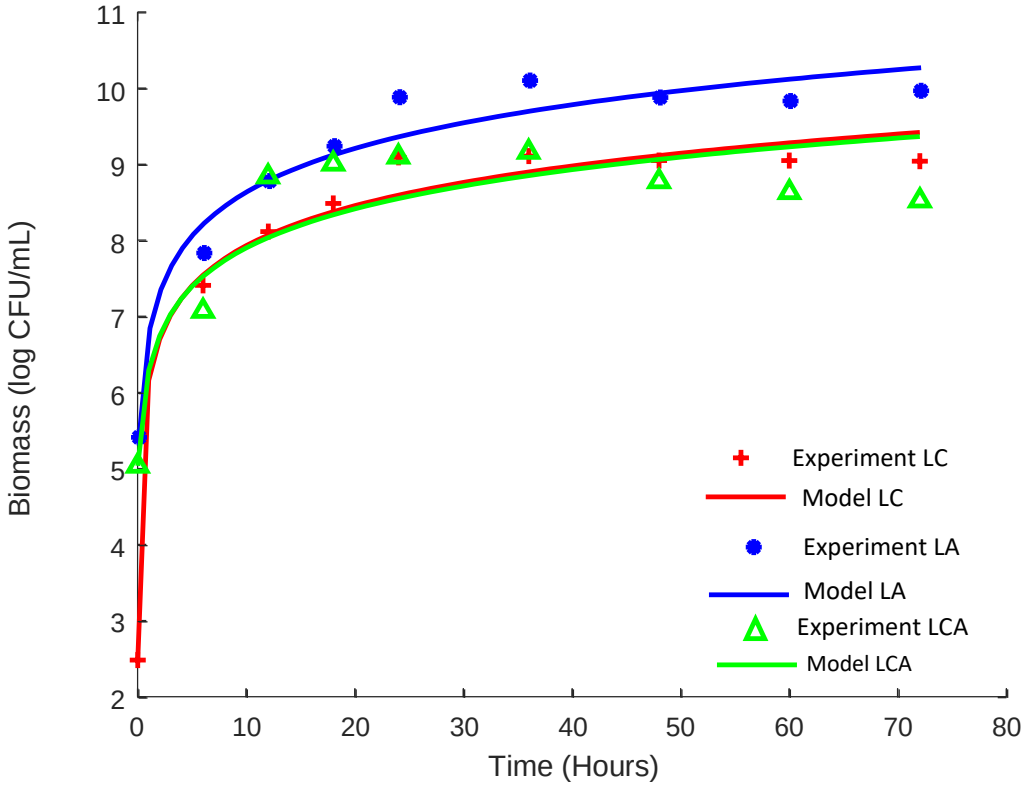
d

b. Exponential equation



Appendix 5: Experimental and stimulated data modelled using integrated forms of the logistic model

Modified Exponential equation



Appendix 6: Experimental and stimulated data modelled using the modified exponential model