SCREENING OF AGRO-WASTES AS GROWTH SUBSTRATE, QUANTIFICATION AND ANTIMICROBIAL ACTIVITY OF BIOACTIVE METABOLITES OF *LACTOBACILLUS* SPECIES

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by

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This thesis is dedicated to…….

My beloved parents, all family members and my wife…...

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LIST OF ABBREVIATION & SYMBOLS

Abbreviation	Full description
ACN	Acetonitrile
Al	Aluminium
AOAC	Association of Official Analytical Chemists
API	Analytical profile index
APS	Ammonium persulfate
ATP	Adenosine triphosphate
$BaCl_2$	Barium chloride
BLAD	Bovine leukocyte adhesion deficiency
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
bp	Base pair
BSA	Bovine serum albumin
BV	Bacterial vaginosis
$C_4H_8O_2$	Acetyl methyl carbinol
Ca	Calcium
$CaSO_4$	Calcium sulphate
CFU	Colony forming units
CH_4N_2O	Urea
CLA	Conjugated linoleic acid
CO_2	Carbon dioxide
COD	Chemical oxygen demand
Cr	Chromium
Cu	Copper
$CuSo_4.5H_2O$	Copper sulphate penta-hydrate
CV	Coefficient of variation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUMPS	Deficiency of uridine monophosphate synthase
E. coli	*Escherichia coli*
e.g	For example
EDTA	Ethylene diamine tetraacetic acid
EEA	European Environmental Agency
EPS	Exopolysaccharides
et al.	et alia (and others)
FDA	Food and drug administration
FDP	Fructose-1-6-diphosphate
Fe	Iron
Fig.	Figure
FTDC	Food Technology Division Culture
g	Gram
GIT	Gastrointestinal tract
GRAS	Generally Recognized As Safe
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid

H_3PO_4	Phosphoric acid
HCD	High energy collision dissociation
HCl	Hydrochloric acid
HMDS	Hexamethyldisilazane
HNO_3	Nitric acid
HPLC	High performance liquid chromatography
hr	Hour
i.e.	That is
IC_{50}	The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.
ICR	Ion cyclotron resonance
IMViC	Indole production, methyl red, Voges-Proskauer and citrate reaction (i inserted for euphony)
K	Potassium
K_2HPO_4	Dipotassium hydrogen phosphate
kDa	Kilo dalton
LAB	Lactic acid bacteria
LAS	Luminescent image analyzer
LC-MS	Liquid chromatography tandem mass spectrometry
MBC	Minimum bactericidal concentration
MEGA	Molecular Evolutionary Genetics Analysis
MFC	Minimal fungicidal concentration
mg	Milligram
Mg	Magnesium
$MgSO_4$	Magnesium sulfate
MIC	Minimum inhibitory concentration
min	Minute
mL	Milliliter
Mm	Millimeter
mM	Millimolar
Mn	Manganese
$MnSO_4$	Manganese sulfate
MRS	Man Rogosa Sharpe
MRVP	Methyl-red and Voges-Proskauer
MS	Mass spectrometry
Na	Sodium
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NFE	Nitrogen free extract
NH_4Cl	Ammonium chloride
NH_4HCO_3	Ammonium biocarbonate
O_2	Oxygen
OD	Opaque density
P	*P*-value
PCR	Polymerase chain reaction
PDB	Protein Data Bank

pH	Potential hydrogen
ppm	Parts per million
QC	Quality control
QRëC	Quality reagent chemical
QTOF	Quadrupole time-of-flight mass spectrometer
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RPM	Round per minute
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS - PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
Se	Selenium
sec	Second
SEM	Scanning electron microscopy
SPV	Sulfo-phospho-vanillin
subsp.	Subspecies
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TTC	Triphenyltetrazolium chloride
USDA	United States Department of Agriculture
v/v	Volume by volume
VP	Voges-Proskauer
w/v	Weight by volume
WHO	World Health Organization
Zn	Zinc
βME	β-mercaptoethanol
µL	Microliter
µm	Micrometer
%	Percent
°C	Degree centigrade

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PENSKRINAN SISA PERTANIAN SEBAGAI SUBSTRAK PERTUMBUHAN, KUANTIFIKASI DAN AKTIVITI ANTIMIKROB METABOLIT BIOAKTIF SPESIS *LACTOBACILLUS*

ABSTRAK

Tujuan kajian ini adalah untuk menskrin dan kenalpasti sisa pertanian yang sesuai dalam membangunkan media pertumbuhan yang selektif untuk *Lactobacillus.* Metabolit bioaktif yang dihasilkan oleh *Lactobacillus* dikira dan aktiviti antimikrob ditentukan. Sebelum permulaan kajian, identiti dua puluh bakteria probiotik yang dibekalkan oleh Pusat Pengajian Teknologi Industri, USM, telah disahkan sebagai *Lactobacillus* melalui ujian morfologi dan biokimia. Daripada dua puluh kultur bakteria, sembilan kultur yang menunjukkan bilangan pertumbuhan melebihi 9.0 $\log_{10}$ CFU/mL telah dipilih untuk ujian toleransi asid dan hempedu. Lima kultur bakteria yang menunjukkan bilangan pertumbuhan melebihi 9.0 $\log_{10}$ CFU/mL dalam media asidik dengan pH 1.5 dan mengurangkan pH media dengan 0.5%w/v garam hempedu kurang daripada 4.5, telah dikenalpasti dengan kaedah SEM, API 50 CHL and 16S rDNA. Identiti *Lactobacillus* telah disahkan sebagai *L. brevis*, *L. plantarum* dan *L. casei.* Nilai nutrisi enam sisa pertanian yang senang diperolehi, murah dan banyak, iaitu kulit tembikai susu, jambu, mangga, kubis, nenas dan betik, ditentukan melalui analisis proksimat dan diskrin sebagai substrak pertumbuhan. Ketiga-tiga *Lactobacillus* tumbuh dalam semua enam sisa pertanian. Bendalir ekstraselular menunjukkan aktiviti antimikrob yang lebih tinggi secara signifikan daripada bendalir intraselular untuk ketiga-tiga spesis *Lactobacillus* yang dikultur dalam kesemua enam substrak sisa pertanian. Bilangan pertumbuhan lactobacilli paling tinggi apabila dikultur dalam kulit nenas. Pengaruh nutrien substrak, glukosa, YSBP, K_2HPO_4, natrium asetat, triammonium sitrat, Tween 80, magnesium sulfat dan manganese sulfat atas pertumbuhan *L. brevis, L. plantarum* dan *L. casei* dinilai.

Pertumbuhan optimum dicapai dengan 1.0%b/v glukosa, 1.0%b/v YSBP, 0.1%b/v K_2HPO_4, 0.1%b/v natrium asetat, 0.2%b/v ammonium sitrat, 0.075%b/v Tween 80, 0.20%b/v magnesium sulfat dan 0.005%b/v manganese sulfat. Medium yang baru dan selektif telah dibangunkan dengan kulit nenas sebagai platform dikayakan dengan nutrien penting. Bilangan pertumbuhan *L. brevis, L. plantarum* dan *L. casei* yang dikultur dalam media kulit nenas baru yang lebih murah dan selektif adalah setara dengan media MRS. Metabolit-metabolit bioaktif bendalir ekstraselular *L. brevis*, *L. plantarum* dan *L. casei*, yang merangkumi asid laktik, lipid, diasetil, eksopolisakarida, hidrogen peroksida dan protein ditentukan. Kandungan asid laktik didapati paling tinggi diikuti dengan protein. Protein bioaktif dalam bendalir ekstraselular *Lactobacillus casei* dipilih untuk identifikasi dengan LC-MS/MS Orbitrap. Ubiquitin dan kasein didapati protein utama dalam bendalir ekstraselular *Lactobacillus casei*. Aktiviti antimikrob bendalir ekstraselular, asid laktik dan protein bioaktif terhadap *S. typhimurium*, *P. aeruginosa, E. coli, K. pneumonia*, *S. aureus* dan *C. albicans* ditentukan dengan membandingkan zon perencatan. Protein bioaktif (bakteriosin) menunjukkan aktiviti antimikrob yang lebih tinggi daripada asid laktik. IC_{50}, MIC and MBC bakteriosin adalah standing dengan erythromycin untuk *S. typhimurium, P. aeruginosa, K. pneumonia* dan *S. aureus*. Protein bioaktif (bakteriosin) lebih berkesan daripada erythromycin terhadap *E. coli*, tetapi kurang berkesan daripada clotrimazole terhadap *C. albicans*. Secara kesimpulan, suatu media kulit nenas yang murah dan selektif yang boleh digunakan intuk laktobasili telah berjaya dibangunkan. Penggunaan sisa pertanian sebagai media pertumbuhan boleh mengurangkan pengumpulan sisa dan pencemaran alam. Di samping itu, protein bioaktif (bakteriosin) boleh dijadikan sumber yang berpotensi untuk penemuan agen antimikrob yang baru.

SCREENING OF AGRO-WASTES AS GROWTH SUBSTRATE, QUANTIFICATION AND ANTIMICROBIAL ACTIVITY OF BIOACTIVE METABOLITES OF *LACTOBACILLUS* SPECIES

ABSTRACT

The objective of this study was to screen and identify a suitable agro-waste in the development of a selective growth medium for *Lactobacillus*. The bioactive metabolites produced by *Lactobacillus* were quantified and the antimicrobial activity determined. Prior to the commencement of study, the identity of twenty probiotic bacteria provided by School of Industrial Technology, USM, was confirmed as *Lactobacillus* using morphological and biochemical studies. From the twenty bacteria cultures, nine that exhibited viable count of above 9.0 $\log_{10}$ CFU/mL were selected for acid and bile tolerance tests. Five bacteria culture that exhibited viable counts of more than 9.0 $\log_{10}$ CFU/mL in acidic medium of pH 1.5 and in a medium with 0.5%w/v bile salt at pH less than 4.5, were identified using SEM, API 50 CHL and 16S rDNA methods. The identity of the lactobacilli was confirmed to be *L. brevis*, *L. plantarum* and *L. casei*. The nutritional values of six easily available, economical, and abundant agro-wastes, namely honeydew skin, guava skin, mango skin, cabbage leave, pineapple skin and papaya skin, were determined through proximate analysis and screened as growth substrate. All the three lactobacilli grew in the six agro-wastes. The extracellular fluid (cell-free fluid) demonstrated a significantly higher antimicrobial activity than intracellular fluid (lysate fluid) for the three *Lactobacillus* species cultured in all the six agro-waste substrates. The viable count of lactobacilli was the highest when cultured in pineapple skin. The influence of substrate nutrients, glucose, YSBP (combination of yeast extract, skim milk, beaf extract and pepton at ratio 1:1:1:1), dipotassium hydrogen phosphate, sodium acetate, triammonium citrate, Tween 80, magnesium sulphate and manganese sulphate on the growth of *L. brevis*,

L. plantarum and *L. casei* was evaluated. The optimum growth was achieved with 1.0%w/v glucose, 1.0%w/v YSBP, 0.1%w/v K_2HPO_4, 0.1%w/v sodium acetate, 0.2%w/v triammonium citrate, 0.075%w/v Tween 80, 0.20%w/v $MgSO_4$ and 0.005%w/v $MnSO_4$. A new and selective medium was developed using pineapple skin as platform enriched with the essential nutrients. The viable count of *L. brevis*, *L. plantarum* and *L. casei* cultured in the newly developed more economical selective pineapple skin medium was comparable with Man-Rogosa–Sharpe (MRS) medium. The bioactive metabolites of extracellular fluid of *L. brevis*, *L. plantarum* and *L. casei*, which included lactic acid, lipid, diacetyl, exopolysaccharide, hydrogen peroxide and protein, were quantified. It was found that the content of lactic acid was the highest followed by protein. The bioactive protein in the extracellular fluid of *Lactobacillus casei* was selected for identification using LC-MS/MS Orbitrap. Ubiquitin and casein were found to be the major proteins in the extracellular fluid of *Lactobacillus casei*. The antimicrobial activity of extracellular fluid, lactic acid and bioactive protein against *S. typhimurium, P. aeruginosa, E. coli, K. pneumonia, S. aureus* and *C. albicans* was determined by comparing the zone of inhibition. The bioactive protein (bacteriocin) showed comparatively higher antimicrobial activity than lactic acid. The IC_{50}, MIC and MBC of bacteriocin were closely comparable to erythromycin for *S. typhimurium, P. aeruginosa, K. pneumonia* and *S. aureus*. The bioactive protein (bacteriocin) was more effective than erythromycin against *E. coli*, but less effective than clotrimazole against *C. albicans*. In conclusion, a selective and economical pineapple skin medium for lactobacilli was successfully developed. The use of agro-waste as growth medium could reduce waste accumulation and environmental pollution. Furthermore, the bioactive protein (bacteriocin) could be potential source for the discovery of novel antimicrobial agents.

CHAPTER 1

INTRODUCTION

1.1 Overview

Agro-waste substrates can be defined as any unwanted residues from industrial agriculture. Agro-waste substrates can be classified into two categories, fruit or plant residues and animal manures. On the other hand, the examples of plant residues are peels, cores, leaves, roots, hulls and plant fibers (Kuan and Liong, 2008). The examples of animal manures are dumps, shells, feathers, scales, intestines, furs, hair and bones. The agro-waste is often treated as useless and discarded (Harris *et al.*, 2001). There are 140 billion metric tons of wastes generated every year globally from agriculture (Rahman, 2013). It was reported that the figure would double in the next decade (Brennan-Tonetta *et al.*, 2014). As such, there is a serious waste disposal problem, which can lead to environmental pollution.

The concept of probiotics was introduced by Metchnikoff (1907). The term "probiotics" was coined by Lilly and Stillwell (1965) to describe substances produced by microorganisms that stimulate the growth of another. The word "substances" in Parker's definition of probiotics resulted in a wide connotation that included antibiotics (Preidis and Versalovic, 2009).

The most widely used bacterial probiotics include species of the following genera: *Lactobacillus, Bifidobacterium, Propioni-bacterium, Enterococcus, Streptococcus, Pediococcus, Lactococcus, Bacillus* and *Leuconostoc* (Fuller, 2012). *Lactobacillus* and *Bifidobacterium* are two major groups of microorganisms which are well known

for their probiotic properties (Elghandour *et al.,* 2014; Fijan, 2014). Probiotics also include yeasts such as *Saccharomyces cerevisiae* (González Pereyra *et al*., 2014).

Lactobacillus is one of the most commonly used probiotics because it is considered as the most beneficial microorganism. The concepts of probiotics and health promoting benefits of consumption of lactobacilli have been known for several years. The ideal characteristics of probiotics are non-pathogenic, non-toxic, maintaining good and valuable effect on the host, adhering to cells, reducing pathogenic adherence, presenting as viable cells in large numbers, stable and capable to remain viable under storage conditions, surviving in the gut environment and safe (Saarela *et al.,* 2000; Walton and Gibson, 2010; Ortiz *et al.,* 2014). In addition, probiotics produce antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocins which are antagonistic to pathogenic microorganisms (Larsen *et al.,* 1993; Shokryazdan *et al.,* 2014).

1.2 Problem statement

Teh *et al.* (2010) reported that Malaysia produced approximately 0.20 million tones of fruits annually. However, only 20% of the whole fruit is edible, while the skin, core, base and rind are discarded as wastes. Nevertheless, these fruit wastes have been reported to contain sugar and can be used as growth substrate for microbial growth. Hills and Roberts (1982) reported that honeydew peel contains primary sugar. Guava peel has ash, lipid, protein, carbohydrate and fibre (Conway, 2002; Owen *et al.,* 2008; Ogoloma *et al.,* 2013). The mango peel contains protein, lipid, mineral and fibres (Gopalan *et al.,* 1999). The pineapple skin contains protein, lipid, carbohydrate, fiber, ash, calcium, magnesium, phosphorus, iron, sodium and

potassium (Sasaki *et al.,* 2012; Ezejiofor *et al.,* 2014). Papaya peel contains protein and carbohydrate (Kumar *et al.,* 1989; Asibey-Berko and Tayie, 2004). The accumulation of these agro-wastes could cause environmental pollution. On the other hand, the use of fruit wastes as growth substrate not only offers an environmental-friendly solution to the organic waste problem but also a way of turning waste into wealth. In addition, the use of a more economical growth substrate can reduce the cost of probiotics production. Fermentation medium cost alone constitutes around 50% of the total cost of lactobacilli production (Hofvendahl and Hahn-Hägerdal, 2000), making the probiotics easily accessible. There has been no comprehensive study to screen and compare the agro-wastes as suitable fermentation medium for *Lactobacillus.* The selected agro-waste can then be compared with commercial medium in terms of viable count.

The extracellular fluid of *Lactobacillus* has been reported to contain bioactive metabolites. The bioactive metabolites have been identified as organic acids (Collado *et al.,* 2013), hydrogen peroxide (Lau and Liong, 2014), diacetyl (Ouwehand and Vesterlund, 2011), fatty acids (O'Shea *et al.,* 2012) and proteins (Sánchez *et al*., 2010). The composition of bioactive compounds of extracellular fluid is highly dependent on the type of fermentation medium. The antimicrobial activity of the extracellular fluid is believed to be attributed to these bioactive compounds. There has been no comprehensive study to evaluate the antimicrobial activity and quantify the content of the individual bioactive compounds of the extracellular fluid produced from *Lactobacillus* cultured in agro-waste medium.

1.3 Experimental work and scope of the study

The aim of the present study was to screen six abundantly and easily available agro-wastes, namely, honeydew skin, guava skin, mango skin, cabbage leave, pineapple skin and papaya skin as suitable fermentation medium for *Lactobacillus.* Experiments were carried out to quantify and evaluate the antimicrobial activity of bioactive metabolites of *Lactobacillus* species. The types of bioactive protein were identified.

The experimental work was divided into various stages which encompassed the following objectives:

1. To confirm the identity of twenty lactobacilli species as potential probiotics culture through morphological and biochemical tests. *Lactobacillus* species with viable count above 9.0 $\log_{10}$ CFU/mL was selected for acid and bile salt tolerance tests.
2. To identify the species of lactobacilli with viable count of more than 9.0 $\log_{10}$ CFU/mL in acidic condition, and pH 4.5 or lower in bile salts, using scanning electron microscopy, biochemical profile (API 50 CHL identification kit) and molecular 16S rDNA genotypic method.
3. To determine the nutritional values of six agro-waste substrates (honeydew skin, guava skin, mango skin, cabbage leave, pineapple skin and papaya skin) and investigate the antimicrobial activity of the intracellular and extracellular fluids of *Lactobacillus* species cultivated in the six agro-waste substrates against *Staphylococcus aureus* and *Escherichia coli.*

4. To evaluate the effect of nutrients on the growth of selected *Lactobacillus* species cultured in an optimum agro-waste. The content of minerals, protein and sugars of the optimum agro-waste was quantified. A new and selective growth medium was developed using the agro-waste as platform enriched with essential nutrients. The newly developed agro-waste medium was compared with a commercial culture medium (MRS) in terms of viable count of *Lactobacillus.*
5. To quantify the bioactive metabolites of the selected *Lactobacillus* species cultured in the newly developed selective agro-waste medium and to identify the bioactive protein using LC-MS/MS Orbitrap.
6. To study the antimicrobial activity of bioactive metabolites against pathogenic bacteria and yeast.

CHAPTER 2

LITERATURE REVIEW

2.1 Agricultural wastes

According to European Environmental Agency (EEA), agricultural waste is one of the five major waste streams in Europe. The accumulation of agro-waste can cause health, safety, environmental, and esthetic concern. According to Subba (1993) and Caprara *et al*. (2011), agro-wastes contain insoluble chemical constituents (e.g., cellulose and lignin) and soluble constituents (e.g., sugar, amino acids, and organic acids). Other constituents are fats, oil waxes, resins, pigment, protein, and minerals (Lim and Matu, 2014).

Waste is an inevitable by-product that arises from various anthropogenic activities. It is also considered as one of the major sources of environmental degradation since it causes air, land and water pollution and contributes to global warming (Peter, 2010). Hence, proper waste management options are vitally important based on the types of wastes and cost effective factors to further reduce environmental degradation and ecosystem destruction.

Leiva-Candia *et al*. (2014) reported the potential use of waste from the agricultural sector for the production of biodiesel through the culture of yeast. One of the outlets for re-using agricultural wastes and/or by-products is in animal feed production. The use of so-called low value crop wastes might be of particular interest. According to their nutritional composition, volume and pollution risk, some wastes have the potential to be utilized in animal feeds (Ulloa *et al.,* 2004).

2.1.1 Honeydew

Honeydew (*Cucumis melo*) is one of the widely cultivated and consumed vegetable crops in the world. The fruits have significant economic value (Gebhardt *et al.*, 1982). Gebhardt *et al.* (1982) reported that honeydew skin contained 89.66% water, 0.46% crude protein, 0.10% crude lipid and 0.60% total ash. The sugar content is known as an important factor in determining the nutritional quality of honeydew (Danyluk *et al.,* 2014).

2.1.2 Guava

Guava (*Psidium guajava* L) is a tropical fruit with a light yellow or green skin and white, pink or dark-red edible flesh. The shape of the fruit is apple or pear-like. Guava is a good remedy for constipation. It has a mild laxative effect and is good for controlling cholesterol level (Ogoloma *et al.,* 2013). Guava is regarded as one of the effective remedies to lower blood pressure (hypertension), since it normalizes the blood pressure. Guava fruit waste is reported to contain high fibre and lignin content. Guava peel waste has ash, lipid, protein, carbohydrate and fibre, which makes it a suitable medium for microbial growth (Conway, 2002; Owen *et al.,* 2008; Ogoloma *et al.,* 2013).

Devasena *et al.* (2004) mentioned that guava fruit waste had the highest fibre and lignin content. Conway (2002) showed that guava peel contained 77 - 86% water, 2.8 - 5.5% crude fibre, 0.43 - 0.7% total ash and 36 - 50 kcal/100 g energy. Adrian *et al.* (2012) reported that guava peel contained 84.9% water, 0.53% crude lipid, 0.88% crude protein and 13.2% carbohydrates. Ogoloma *et al.* (2013) reported that guava

peel had 76.60 - 79.45% water, 0.91 - 1.29% total ash, 1.60 - 2.05% crude lipid, 2.10 - 2.19% crude protein, 0.97 - 2.61 carbohydrate and 12.90 - 17.40% crude fibre.

2.1.3 Mango

Mango (*Mangifera indica* L.) is one of the most important seasonal and tropical fruits in the world. The world production of mango fruits is more than 35 million ton (Berardini *et al.,* 2005). Mango peels and seed kernels are the major by-products. Kernels take up about 17% - 22% (Kittiphoom, 2012), while peel contributes about 15% - 20% (Gopalan *et al.*, 1999). The peel is normally discarded as a waste and causes pollution. Gopalan *et al.* (1999) reported that the peel contained 81% water, 0.6% crude protein, 0.4% crude lipid, 0.4% minerals and 0.7% crude fibre. Most studies on the exploitation of mango peels have been dealing with their use as a source of pectin, which is considered as a high quality dietary fiber (Pedroza-Islas *et al.,* 1994; Tandon and Garg, 1999). The peel was also reported to be a good source of phyto-chemicals, such as polyphenols, carotenoids that exhibit good antioxidant properties (Kim *et al.,* 2010).

2.1.4 Cabbage

The value of Chinese cabbage (*Brassica oleracea* var. *capitata*) as food and in medicine was known to the ancient Greeks and Romans. Rajadevan and Schramm (1989) reported that cabbage contained 92.7% water, 1.46% crude protein, 0.38% crude lipid, 1.11% total ash, 1.65% crude fibre and 2.7% carbohydrate. The Chinese cabbage contains sufficient nutrients of reducing sugar, protein and phosphorous for yeast culture (Choi *et al.,* 2002; Emebu and Anyika, 2011). Therefore, the large amount of Chinese cabbage waste can be used as substrate for microbial growth.

Emebu and Anyika (2011) showed that cabbage waste contained 81.38% water, 3.00% crude fibre, 0.26% crude lipid, 1.33% total ash, 2.36% carbohydrate and 58.46 kcal/100 g energy.

2.1.5 Pineapple

Pineapple (*Ananas comosus*) waste is comprised basically of the residual pulp, peels, stem and skin, making about 40 - 50% of the fresh fruit (Buckle, 1989; Abdullah, 2007). These materials with high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values cause serious pollution if not disposed properly (Ban-Koffi and Han, 1990). However, it contains valuable components of carbohydrates and nutrients which are suitable for recycling into valuable product. Pineapple waste is a material which is rich in sugars consisting mainly of sucrose, fructose, glucose and other nutrients (Rani and Nand, 2004). Cordenunsi *et al.* (2010) showed that pineapple skin contained 78.09 - 86.05% water, 0.42 - 0.47% crude protein and 0.22 - 0.53% total ash. It is also reported that pineapple skin contained protein, lipid, total carbohydrate, fiber, ash, calcium, magnesium, phosphorus, iron, sodium and potassium (Sasaki *et al.*, 2012; Ezejiofor *et al.*, 2014). Ogoloma *et al.* (2013) showed that the pineapple waste contained 82.2 - 86.3% water, 0.36 - 0.76% total ash, 2.0 - 3.50% crude lipid, 0.44 - 0.88% crude protein, 2.0 - 2.25% crude fibre and 6.76 - 7.45% carbohydrate. Upadhyay *et al.* (2013) classified pineapple waste as an alternative feed ingredient for animal.

2.1.6 Papaya

Papaya (*Carica papaya* L.) waste was used to feed animals (Kumar *et al.*, 1989). Papaya waste contains protein and carbohydrate (Kumar *et al.*, 1989; Asibey-Berko

and Tayie, 2004). Asibey-Berko and Tayie (2004) reported that papaya waste contained 91.6% water, 10.9% carbohydrate, 0.1% crude lipid, 0.5% crude protein, 0.2% crude fibre, 0.3% total ash and 40.2 kcal/100 g energy. Papaya peels fed to giant West African snails gave the best results in feed intake, weight gain and shell increment with no adverse effects when compared to other tropical fruit by-product (Omole *et al.,* 2004). It is reported that papaya pulp is successfully converted into medium for the preparation of a yogurt-like product using *L. acidophilus* as the fermenting organism (Pakalwad *et al.,* 2010). The bioconversion of papaya peel to alcohol using *Saccharomyces cerevisiae* was reported by Vikash and Sanjay (2012). Ogoloma *et al.* (2013) presented the proximate composition of papaya waste which contained 88.3 - 91.4% water, 0.46 - 0.62% total ash, 0.44 - 0.88% crude protein, 2.47 - 2.93% crude fibre and 2.90 - 6.66% carbohydrate. Lim and Matu (2014) reported the successful use of papaya agro-waste to produce bio-fertilizers.

2.2 Probiotics

The word 'probiotic' in greek, means 'for life'. Probiotics are defined by Fuller (1989) as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance". This definition emphasizes the requirement of viability for probiotics and introduces the aspect of a beneficial effect on the host. Havenaar and Huis (1992) elaborated the definition of probiotics with respect to host and habitat of the microflora as "a viable mono-or mixed culture of microorganisms which, when applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora". The definition of probiotics was broadened further by Schrezenmeir and de Vrese (2001) who proposed probiotics as "a preparation of a product containing viable, defined

microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host". World Health Organization (WHO, 2002) defined probiotics as "live microorganisms which when administered in adequate numbers confer a health benefit on the host".

The common species of probiotics are presented in Table 2.1 (Holzapfel and Schillinger, 2002; Goldin and Gorbach, 2012; Kumar *et al.*, 2012; Thantsha *et al.*, 2012; Svensson and Hakansson, 2014). The safety of *Lactobacillus* sp. and *Bifidobacterium* sp. as probiotics has been extensively reviewed (Borriello *et al.*, 2003; Snydman, 2008; Svensson and Hakansson, 2014). The characteristics of the common probiotics are shown in Table 2.2.

Table 2.1: Common probiotics species.

***Lactobacillus* species**	***Bifidobacterium* species**	**Other species**
L. acidophilus	*B. bifidum*	*Bacillus subtilis*
L. casei	*B. lactis*	*Saccharomyces cerevisiae*
L. crispatus	*B. longum*	*Saccharomyces boulardii*
L. plantarum	*B. breve*	*Lactococcus lactis*
L. rhamnosis	*B. infantis*	*Streptococcus thermophilus*
L. salivarius	*B. adolescentis*	
L. gasseri	*B. animalis*	
L. buchneri	*B. gallicum*	
L. johnsonii	*B. asteroides*	
L. reuteri	*B. merycycum*	
L. fermentum	*B. magnum*	
L delbrueckii	*B. indicum*	
L. acetotolerans	*B. gallinarum*	
L. acidifarinae	*B. cuniculi*	
L. zymae	*B. boum*	
L. agilis	*B. coryneforme*	
L. alimentarius	*B. choerium*	
	B. ruminale	
	B. minimum	
	B. subtile	
	B. thermaacidophilum.	
	B. psuedocatenulapum	
	B. inopinatum	
	B. catenulatu	
	B. denticolens	
	B. dentium	

Table 2.2: Cell morphology, sizes and arrangements of common probiotics.

Genus	Morphology	Size (µm)	Arrangement	References
Lactobacillus	rod-shaped	2.0 - 10.0 (length) 0.5 - 1.20 (width)	single, pairs, chains	Hammes and Vogel (2012) Ray and Bhunia (2013)
Bifidobacterium	short and irregularly rod-shaped	0.8 - 1·5 (length) 0.7 - 1.0 (width)	single or in pairs	Kiviharju *et al.* (2005)
Bacillus subtilis	rod-shaped	0.87 - 1.41 (length) 0.41 - 0.58 (width)	single or chains	Abusham *et al.* (2009)
Saccharomyces cerevisiae	cocci or ovoid	2.5-10.0 (length) 1.5 - 5.0 (width)	single or budding	Ellis *et al*. (2007) Ojokoh and Uzeh (2005)

2.3 Generally Recognized as Safe (GRAS) microorganism

Probiotics are treated as Generally Recognized As Safe (GRAS) microorganism by the Federal Food and Drug Administration (Mattia and Merker, 2008; Snydman, 2008; Chowdhury *et al.*, 2012). GRAS is a status assigned by the Food and Drug Administration (FDA) to a list of substances not known to be hazardous to health and approved by the FDA for the use in food products. In the Federal Food, Drug and Cosmetic Act, sections 201(s) and 409 Act (Tousley, 1941), GRAS is defined as any substance that is intentionally added to food and is considered as food additives.

The GRAS microorganisms include *Lactobacillus bulgaricus, L. fermentum, L. lactis, L. rhamnosus, L. casei* subsp. *rhamnosus* GG, *L. acidophilus, L. plantraum, L. fermentum, L. reuteri, L. casei, L. acidophilus, L. casei* Shirota, *L. johnsonii, L. reuteri, L. brevis, L. salivarius, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium animalis* subsp. *lactis, B. lactis, B. infantis, B. animalis, B. longum, Bacillus coagulans, B. subtilis* and *Saccharomyces cerevisiae* (Waites *et al.*, 2001; Mattia and Merker, 2008; Giraffa, 2014; Lau and Liong, 2014).

2.4 *Lactobacillus*

Lactobacillus is one of the members of the eight main genera of lactic acid bacteria (LAB), namely, *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc, Bifidobacterium, Carnobacterium, Enterococcus* and *Sporolactobacillus* (Havenaar and Huis, 1992; Barrangou *et al.*, 2011; Hammes and Vogel, 2012).

2.4.1 Characteristics of *Lactobacillus*

Lactobacillus species are members of the family *Lactobacillaceae* (Felis and Dellaglio, 2007; Felis and Pot, 2014). These bacteria consume sugars as their main source of carbon to produce energy. It produces 85% lactic acid, with less than 15% of other products (De Vos *et al*., 2009). In addition, *Lactobacillus* sp. also produces enzymes such as proteases and lipases which help to digest proteins, lipid and vitamins (Saguir *et al*., 2008; Baek and Byong, 2009). Generally, all *Lactobacillus* species are Gram positive, rod-shape, non-motile, non-flagellated and non-spore forming, catalase negative and require complex nutrients for maximal growth (Klein *et al*., 1998; Corsetti *et al*., 2005; De Vos *et al*., 2009; Axelsson, 2011; Khalisanni, 2011). *Lactobacillus* species are able to tolerate bile condition (Hamner *et al.,* 2013; Pato and Surono, 2013), produce hydrogen peroxide and different types of inhibitors such as bacteriocins which have inhibitory actions against various pathogenic microorganisms (Maqsood *et al.,* 2013; Lü *et al*., 2014). *Lactobacillus* species are fastidious and facultative anaerobic and require slightly acidic condition for optimal growth (Willey *et al.,* 2013). *Lactobacillus* species are able to live in acidic environment with pH range of 4.0 - 5.0 or lower and with an optimum growth between 5.0 and 6.0 (Batt, 2014). At low temperature of 15°C and below, the bacteria do not show any growth. However, *Lactobacillus* can generally survive at temperature up to 40°C with an optimum growth at 35°C (Gomes and Malcata, 1999; Batt, 2014).

2.4.2 Identification of *Lactobacillus*

The identification of *Lactobacillus* depends mainly on physiological and biochemical criteria (Stiles and Holzapfel, 1997; Corsetti *et al*., 2005; Jafarei and

Ebrahimi, 2011). The prescreening of different *Lactobacillus* sp. was performed using various methods, such as conventional biochemical and physiological tests (Christopher and Bruno, 2003; Hunt *et al.,* 2006; De Vos *et al*., 2009). It can also be identified by more complex technique such as carbohydrate fermentation patterns using commercially available kits (Molin *et al.*, 1993; Nigatu *et al.,* 2000), or by molecular identification (Christopher and Bruno, 2003; Hunt *et al.,* 2006).

2.4.2.1 Morphological identification

2.4.2.1 (a) Taxonomy

Lactobacillus is the largest genus in lactic acid bacteria group, comprising around 80 recognized species (Saranraj, 2014). The classification of lactobacilli into different species is largely based on their cell morphology, mode of glucose fermentation, growth at different temperature, configuration of the lactic acid produced, ability to grow at high salt concentration, acid or alkaline tolerance, chemotaxonomic markers such as fatty acid composition, constituents of the cell wall, and phylogenetic relationships. A summary of the characteristic of the different *Lactobacillus* species is shown in Table 2.3.

Table 2.3: Characteristic of various *Lactobacillus* species (De Vos *et al.*, 2009).

Species	Characteristics			
	Pantose fermentation	**CO_2 from glucose**	**CO_2 from gluconate**	**FDP aldolase present**
L. acidophilus	-	-	-	+
L. casei	+	-	+	+
L. brevis	+	+	+	-
L. delbruckii	-	-	-	+
L. curvatus	+	-	+	+
L. buchneri	+	+	+	-
L. helveticus	-	-	-	+
L. plantarum	+	-	+	+
L. fermentum	+	+	+	-
L. salivarius	-	-	-	+
L. sakei	+	-	+	+
L. reuteri	+	+	+	-

FDP: fructose-1-6-diphosphate.

2.4.2.1 (b) Cell morphology

Cell morphology is important in the current description of the lactobacilli. The bacteria is rod-shaped form and the arrangement is either in single, pairs or chains (Axelsson, 2011; Batt, 2014).

2.4.2.1 (c) Colony morphology

Lactobacilli produce colonies in the range of 0.7 - 2.0 mm diameter (Iemoli *et al*., 2012). They vary in terms of colour and shape (circular and irregular). The colony morphology may refer to the size, shape, elevation and margin. Colony morphology of lactobacilli species is revealed in Table 2.4.

Table 2.4: Colony morphology of *Lactobacillus* species on specific media.

Medium	Colony Morphology	CD (mm)	CT (hr)	GC	References
MRS agar	cream-white, smooth and convex	1.0 - 2.0	48	FA /35 - 37°C	Iemoli *et al*., (2012)
Rogosa agar	white, smooth and convex	1.0 - 2.0	48	FA /35 - 37°C	Rogosa (1970)
GBL agar	brown, round form, rough surface and irregular edge	0.7 - 2.5	48	FA /37°C	Hammes and Vogel (2012)
S-F agar	circular with irregular border, smooth and convex	1.0 - 2.0	48	FA /35 - 37°C	Vogel *et al.*(1994)
LAMVAB	rod shaped, straight or slightly curved, green colour	1.0 - 1.2	72	FA /37°C	Hartemink *et al.* (1997)
Homohiochii agar	white smooth, round, convex and slimy	1.5 - 2.0	96	FA /30°C	Henick-Kling and Krieger (2001)

CD: Colony diameter
CT: Cultivation time
GC: Growth condition
FA: Facultative anaerobic
MRS agar: De Man Rogosa Sharpe agar
GBL agar: Glucose blood liver agar
S-F agar: San-francisco agar
LAMVAB: *Lactobacillus* anaerobic MRS with vancomycin and bromocresol green

2.4.2.2 Biochemical characterization

2.4.2.2 (a) Indole test

The indole test tells whether an organism is able to degrade the amino acid tryptophan and produce indole. Prior to the indole test, the medium must be filled with a sufficient quantity of tryptophan (Harley, 2013). A species which grows in a medium rich in tryptophan shows that the species has the capacity to degrade tryptophan (MacFaddin, 2000).

2.4.2.2 (b) Voges-Proskaur test

Voges-Proskauer (VP) test is used to differentiate organisms based on their ability to produce acetylmethylcarbinol (acetoin) from glucose fermentation. VP test is a valuable taxonomic characteristic, useful for differentiating microorganism (Pancheniak and Soccol, 2005).

2.4.2.2 (c) Citrate test

The change in test indicator and drop in pH indicated a species is capable of importing citrate and using it as a sole carbon and energy source. Such species is termed as citrate positive (Woodland, 2009).

2.4.2.2 (d) Carbohydrate fermentation test

Phenol red broth medium is used as a test indicator to check the ability of a given species to ferment the specific carbohydrate, with the production of acid, gas or both (MacFaddin, 2000; Mohan, 2010). It has been used to indicate if the microorganism has utilized the sugars.

2.4.2.2 (e) Analytical profile index (API) kit

Analytical profile index (API) 50 CHL fermentation kit contains many types of carbohydrates and related compounds. The principle of API 50 CHL kit is based on the ability of the microorganisms to ferment 49 different types of carbohydrates which is performed for bacterial species identification (Ertekin and Çon, 2011). It is designed to identify the genus *Lactobacillus* (Coeuret *et al.,* 2003; Dimitonova *et al.,* 2008; Herbel *et al.,* 2013). The carbohydrates are fermented in acids leading to decrease in pH, detected by the change in colour of the indicator. The results make up the biochemical profile which is used by api*web*™ identification software with database. In many scientific research studies, this test was used for lactobacilli identification (Nighswonger *et al.,* 1996; Klein *et al.,* 1998; Hedberg *et al.,* 2008).

2.4.2.3 Other conventional tests

2.4.2.3 (a) Gram staining test

Gram staining is a common test to differentiate bacteria by cell wall constituents. This test involves staining of cell with a water soluble dye called crystal violet followed by decolorization and counterstaining. Gram positive bacteria stained deep purple during the decolorization process, as they have a thick outer layer of peptidoglycan. On the other hand, Gram negative bacteria lose the crystal violet stain and are stained pink or red by the safranin in the final staining process, as they have a thin inner layer of peptidoglycan (De Vos *et al.,* 2009; Cappuccino and Sherman, 2013).

2.4.2.3 (b) Spore staining test

The outer coating of spores consists of protein keratin. This keratin coat resists staining. Heating is required to stain the spores. The cells are then decolorized with water and safranin is commonly used as counter stain. As a result, the endospores turn green and the vegetative cells turn red. Schaeffer-Fulton test is the most commonly used staining test (Jones and Versalovic, 2009).

2.4.2.3 (c) Motility test

The ability of bacteria to move is one of the characteristics for classification in microbiology (Christopher and Bruno, 2003). A motile species is easily differentiated from a non-motile species. Motility gelatin medium technique (Kelly and Fulton, 1953) and Hanging-drop wet method (MacFaddin, 2000) can be used to determine the motility.

2.4.2.4 Molecular identification by 16S ribosomal deoxyribonucleic acid (rDNA)

DNA marker is a gene technology tool used for screening chromosome at a known location for identification purpose. It can be described as a variation that can be observed. The common DNA markers include those to determine certain diseases, such as deficiency of uridine monophosphate synthase (DUMPS) and bovine leukocyte adhesion deficiency (BLAD) (Janda and Abbott, 2007). The 16S rDNA is widely used in the molecular identification.

2.4.2.4 (a) Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a revolutionary method developed by Mullis and colleagues in the 1980's. PCR is a process based on using the ability of DNA to

amplify many times to synthesize new strand of DNA complementary to the offered template strand (Dubernet *et al.*, 2002). This process is widely used for detecting and identifying microorganism (Rachman *et al.*, 2003).

Three temperature levels are required in a typical PCR reaction. The first step involves heating to around 94°C. The double stranded DNA is denatured or split into two single strands during this step. The second step involves cooling to approximately 54°C to allow the primers to attach to the single-stranded DNA. The primers determine which section of DNA is copied. They are designed such that they straddle a predetermined specific site on the DNA based on the sequence of the DNA. The third step is performed at around 72°C (Fig. 2.1). The actual copying of the DNA is performed by the DNA polymerase during this step. The DNA polymerase attaches to the primer site and copies DNA using the nucleotides that are in solution as building blocks (Somma and Querci, 2007). The temperature profile of a PCR cycle is controlled by the thermal cycler program which results in a near exponential increase in PCR product accumulation for the first 35 cycles.

Several studies have shown that PCR techniques are far more sensitive, efficient and specific than the morphological technique. PCR method contributes to improvement of the rapid diagnosis and improves the accuracy of the identification of the microorganism **(**Somma and Querci, 2007; Woo *et al.*, 2008).

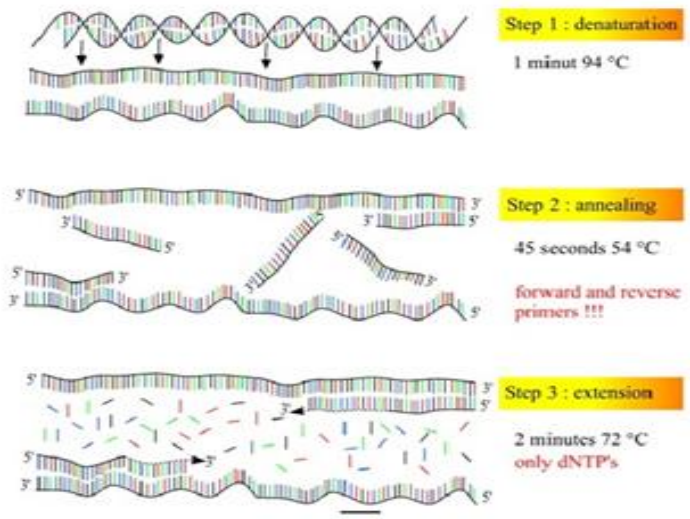

Fig. 2.1: The steps of one cycle in the polymerase chain reaction (PCR).

2.4.2.4 (b) Gel electrophoresis

Gel electrophoresis separates substances based on the rate of movement of the analyte under the influence of an electric field. Gel electrophoresis is a powerful tool to analyse nucleic acids and proteins. Agarose is a polysaccharide purified from seaweed. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA travels from the negative electrode to the positive electrode. Several factors that influence DNA movement in the gel include the strength of the electrical field, the concentration of agarose in the gel and most importantly the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. The different agarose concentrations for separation of different ranges of linear DNA molecules are presented in Table 2.5 (Yılmaz *et al*., 2012).

Table 2.5: The various agarose concentrations for separation of different ranges of linear DNA molecules.

Agarose concentration in gel (% w/v)	Efficient range of separation of linear DNA molecules (kb)
0.3	5.0 - 60
0.6	1.0 - 20
0.7	0.8 - 10
0.9	0.5 - 7.0
1.2	0.4 - 6.0
1.5	0.2 - 3.0
2.0	0.1 - 1.5

2.4.2.4 (c) Sequencing

Organism's DNA is made up from a large number of nucleotides. The arrangement of nucleotides or bases in each organism is very unique. Therefore, the DNA sequence can be used to differentiate one species from the others. For this reason, DNA sequencing is useful to determine the precise order of nucleotides and identify the organism.

The four well-known techniques of DNA sequencing are, i) Sanger method, ii) Maxam and Gilbert method, iii) pyrosequencing (DNA sequencing in real time) method and iv) single molecule sequencing with exonuclease. RNAs are often sequenced either by converting the RNAs into DNA or by sequencing the DNA gene that gives rise to the RNA (Mitchelson *et al.,* 2007; Tamura *et al.,* 2011).