EFFECTS OF DIFFERENT STORAGE CONDITIONS ON
THE PHYSICAL PROPERTIES, MICROBIOLOGICAL
QUALITY AND SHELF-LIFE OF
'SUNDAL BELACAN'

WAN HALIMAH BINTI WAN OMAR

FACULTY OF AGROTECHNOLOGY AND FOOD SCIENCE
UNIVERSITI MALAYSIA TERENGGANU

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Lihat Sebelah

EFFECTS OF DIFFERENT STORAGE CONDITIONS ON THE PHYSICAL PROPERTIES, MICROBIOLOGICAL QUALITY AND SHELF-LIFE OF 'SAMBAL BELACAN'

By

Wan Halimah Binti Wan Omar

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ENDORSEMENT

This project entitled **Effects of Different Storage Conditions on The Physical Properties, Microbiological Quality and Shelf Life Of 'Sambal Belacan'** by **Wan Halimah bt Wan Omar,** Matric No. **UK 17785,** has been reviewed and corrections have been made according to the recommendation by examiners. This report is submitted to the Department of **Food Science** in partial fulfilment of the requirement of the **Bachelor of Food Science (Food Technology),** Faculty of Agrotechnology and Food Science, University Malaysia Terengganu.

.....

(DR. MOHD NIZAM BIN LANI)

Main Supervisor

Date: 14/2/12

DR. MOHD NIZAM LANI
Pensyarah Kanan (DS52)
Jabatan Sains Makanan
Fakulti Agroteknologi dan Sains Makanan
Universiti Malaysia Terengganu
21030 Kuala Terengganu

.....

(MS. ROSHITA BT IBRAHIM)

Co-Supervisor

Date: 18/6/12

ROSHITA IBRAHIM
Pensyarah
Jabatan Agroteknologi
Fakulti Agroteknologi dan Sains Makanan
Universiti Malaysia Terengganu

DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged.

Signature : 

Name : Wan Halimah binti Wan Omar

Matric No. : UK 17785

Date : 13/02/2012

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ABSTRACT

'Sambal belacan' is a type of traditional condiment that popular among Malaysian and usually consumed with 'ulam-ulaman' in a meal of rice and other dishes. This study was conducted to investigate the effect of difference storage conditions on the physical properties, microbiological quality and shelf-life of 'Sambal belacan'. The availability of oxygen in the air and relative humidity were selected as major factors that influence the quality of 'Sambal belacan'. For the effects of anaerobic and aerobic condition in 'Sambal belacan' during storage, 'Sambal belacan' samples were stored at chilled and ambient temperature, respectively. Next, for the effects of low and high relative humidity in 'Sambal belacan' during storage, 'Sambal belacan' samples were stored at chilled and ambient temperature, respectively. All samples were analysed for physical properties (pH, CO_2 concentration, relative humidity (RH) and colour), microbiological quality (Aerobic plate count (APC), yeast and mould count, Lactic acid bacteria (LAB) count, *Psychrotrophic* bacteria count, *Enterobacteriacea* count, *Escherichia coli* count, Coliform count and detection of *Salmonella sp.*), and Shelf-life (APC and yeast and mould count for six weeks storage duration). From all analyses, results showed that there were significant different ($P<0.05$) for all samples due to increasing weeks of storage for both effects of availability of oxygen in air and relative humidity. However, significant different ($P<0.05$) among samples detected in CO_2 concentration, L^*, a^* and $H°$ (physical properties), yeast and mould count (microbiological quality and shelf-life) based on effects of oxygen in 'Sambal belacan'. Besides that, significant different ($P<0.05$) was also detected in percentase of RH, a^*, and $C°$ (physical properties), Lactic acid bacteria count (microbiological quality), APC and yeast and mould count (shelf-life). In conclusion, 'Sambal belacan' treated with low RH and kept at chilled temperature showed the most suitable and the safest storage condition until six weeks storage.

ABSTRAK

Sambal belacan merupakan hidangan tradisional yang popular di kalangan rakyat Malaysia dan biasanya di nikmati bersama ulam-ulaman bersama nasi atau lain-lain sajian. Kajian ini dijalankan untuk menyiasat kesan keadaan penyimpanan yang berbeza pada ciri-ciri fizikal, kualiti mikrobiologi, dan jangka hayat Sambal belacan. Kandungan oksigen dalam udara dan kelembapan telah dipilih sebagai faktor utama yang mempengaruhi kualiti Sambal belacan. Untuk kesan keadaan anaerobic dan aerobik dalam Sambal belacan semasa penyimpanan, sampel Sambal belacan masing-masing telah disimpan pada suhu sejuk dan suhu bilik. Kemudian, untuk kesan kelembapan rendah dan tinggi, sampel Sambal belacan masing-masing telah disimpan pada suhu sejuk dan suhu bilik. Semua sampel telah dianalisis untuk ciri-ciri fizikal (pH, kepekatan CO_2, purata kelembapan (RH) dan warna (L^*, a^*, b^*, $H°$ and $C°$), kualiti mikrobiologi (jumlah plat aerobic (APC), kiraan yis dan kulat, kiraan bakteria laktik asid (LAB), kiraan bakteria *psychrotropik*, kiraan *Enterobactericea*, kiraan *Escherichia coli*, kiraan *koliform* dan pengesanan spesis *Salmonella*) dan jangka hayat (APC dan kiraan yis dan kulat selama enam minggu simpanan). Daripada semua analisis, keputusan menunjukkan adanya perbezaan yang signifikan ($P<0.05$) untuk semua sampel berdasarkan peningkatan minggu penyimpanan untuk kedua-dua kesan kehadiran oksigen dalam udara dan kelembapan purata. Walaubagaimanapun, perbezaan yang signifikan ($P<0.05$) diantara sampel di kesan dalam kepekatan CO_2, L^*, a^* dan $H°$ (cirri-ciri fizikal), kiraan yis dan kulat (kualiti mikrobiologi dan jangka hayat) berdasarkan kesan oksigen dalam Sambal belacan. Selain itu, perbezaan yang signifikan ($P<0.05$) juga dikesan dalam peratusan RH, a^* dan $C°$ (ciri-ciri fizikal), kiraan bakteria asid laktik (kualiti mikrobiologi), kiraan APC dan yis dan kulat (jangka hayat). Kesimpulannya, Sambal belacan dirawat dengan RH yang rendah dan disimpan pada suhu sejuk menunjukkan keadaan simpanan yang paling sesuai dan selamat untuk sehingga enam minggu simpanan.

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LIST OF ABBREVIATIONS

^{o}C	–	Degree Celsius
a_w	-	Water activity
APC	-	Aerobic Plate Count
BPA	–	Baird Packer Agar
BPW	–	Buffered Peptone Agar
CFU/g	–	Colony Forming Unit per gram
CO_2	–	Carbon dioxide
EMB	–	Eosin Methylene Blue
g	–	Gram
L	–	Litre
LDPE	-	Low Density Polyethylene
LIA	–	Lysine Iron Agar
NaCl	-	Sodium chloride
O_2	-	Oxygen
PCA	–	Plate Count Agar
PDA	–	Potato Dextrose Agar
RH	–	Relative Humidity
RTE	–	Ready-to-eat
RVS	–	Rappaport Vasilliadis
TTH	–	Tetrathionate
TNTC	–	Too Numerous To Count
XLD	–	Xylose Lysine Deoxycholate

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CHAPTER 1

INTRODUCTION

1.1 Research Background

'Sambal belacan' is a type of traditional food and very popular condiment among Malaysians that usually eaten with 'ulam-ulaman' in a meal of rice and other dishes and functions as appetizer. 'Sambal belacan' has a unique and spicy taste and normally is eaten together with rice by Malaysian and Indonesian in order to enhance the taste of food and function as appetizer (Fitri Nurdiyana, 2008).

'Sambal belacan' is a type a dishes that can be prepared in a few minutes. According to Noraini (2004), the preparation of 'sambal belacan' does not involve sufficient heat treatment. Therefore, it is identified as raw or semi prepared food. In addition, there are a few ingredients of 'sambal belacan' has potential as source of microbial contamination such as shrimp paste. Based on Steinkraus (2009), insufficient heat treatment of shrimp paste indicated the presence of *Salmonella*. Without using proper heat treatment, there are possible for pathogen to grow. Therefore, application of appropriate extrinsic factors like oxygen concentration and relative humidity are important parameters to control the microbial growth in 'Sambal belacan'.

Nowadays, besides concern about quality of the food that they consume, consumers are also care about how the packages of the product and how it is stored. The use of suitable storage condition and temperature of storage can prolong the shelf life of 'Sambal belacan' by inhibiting the growth of food pathogens.

There are six favourable conditions required for the growth of microorganisms which are food, acidity, time, temperature, oxygen and moisture. Two factors which are moisture or relative humidity and availability of oxygen in the air were chosen as the main factors that affect the storage conditions of 'Sambal belacan' in this study. According to Paull (1999), the impact of relative humidity on quality, such as appearance and texture, was not doubt due to water lost from the food sample. However, the bacterial development in the package is not only influenced by temperature, oxygen availability and water activity also can determine the microbial population and the type of micro-organisms presence in the foods (Labadie, 1999).

Based on research done by Faridah (2009), the shelf life of 'Sambal belacan' was only 7 days at room temperature and 10-days at chiller storage when 'Sambal belacan' was incorporated with vinegar. However, there is no comprehensive study is highlighted on the storage conditions of 'Sambal belacan' as affected by oxygen concentration and relative humidity. Therefore, this study examined both factors (availability of oxygen in the air and relative humidity) that may affect the quality of 'Sambal belacan' during six weeks of shelf-life studies. Using the right treatment and suitable storage condition may reduce the microbial load and prolong the shelf-life of 'Sambal belacan'.

1.2 Problem statement

'Sambal belacan' is easily prepared as home-made food and it is a popular dish among Malaysian, but unfortunately it may potentially cause food poisoning if this food is not prepared and stored properly. It is because improper storage may give a good period for microbial growth and the potential microbial contaminant will be able to reach the total plate count at unsatisfactory level. The major factors that may influence the microbial growth and become microbial contamination in food are the source of raw ingredients, practice in preparation, physical and packaging conditions of 'Sambal belacan' and storage temperature that applied to 'Sambal belacan'.

'Sambal belacan' is a type of condiment that easily deteriorates when did not store properly. The deterioration can occur by reaction of oxygen, higher moisture content and improper temperature storage. However based on current literature, there is no study has been reported about the effects of this storage conditions on the physical properties, microbial quality and shelf life of 'Sambal belacan'.

1.3 Significances of study

This study will generate new knowledge about qualitative and quantitative aspects on proper storage condition of this type of ready-to-eat, (RTE) product. This study will obtain documentation of data that could be used as a basis for commercialization of this product in a large scale. The determination of microbial quality of 'Sambal belacan' during storage conditions may help to determine the shelf life of 'Sambal belacan'. Using the different types of storage, this study is trying to understand how the storage would

affect the physical properties, microbial quality and shelf-life of 'Sambal belacan'. Besides that, this finding may provide guidelines for improving the microbial quality of 'Sambal belacan' in order to increase the shelf life and prepared safe and quality food.

1.4 Objectives of study

The objectives of this study were;

i) To determine the physical properties of 'Sambal belacan' during storage conditions as affected by relative humidity and availability of oxygen in the air.

ii) To determine the microbiological quality and shelf-life of 'Sambal belacan' during storage conditions as affected by relative humidity and oxygen concentration.

CHAPTER 2

LITERATURE REVIEW

2.1 'Sambal belacan'

2.1.1 Previous study on 'Sambal belacan'

'Sambal belacan' has been recently received wide attention since this traditional food has never been scientifically studied before. The previous studies cover on the aspects of formulation and process improvement, texture evaluation, perception and acceptance and affects of acidic ingredients in 'Sambal belacan'.

Table 2.1: Summaries the previous study on 'Sambal belacan'

Title	Author	Important findings
Formulation and process improvement for chili shrimp paste (CSP) using sensory evaluation	Nadia et al, 2010	1. The prefer pH level was 4.0 2. The best acid source was kalamansi juice. 3. The most preferred coarseness of CSP was 120 μm. 4. Dimethyl dicarbonate (DMDC) has no effect on microbial reduction due to the presence of fat globules in CSP which hindered the inactivation.

Title	Author	Important findings
Texture evaluation for a commercial chili-based paste	Sobhi et al, 2010	1. Super mass colloider instrument can substitute the traditional method. 2. Textural measurement methods were found to be reliable and consistent methods that can be applied in quality control for textural properties of chili shrimp paste for commercial-scale production.
Effect of acidic ingredients on microbial load shelf-life of 'Sambal belacan'	Faridah, 2009	1. Addition of vinegar in 'Sambal belacan' has reduced the microbial load, inhibits the growth of *Staphylococcus aureus* and increases the shelf-life of product. 2. Shelf-life of 'Sambal belacan' incorporated with vinegar was stable until 7-days (ambient) and 10-days (chiller)
Perceptions and acceptance of '*belacan*' in Malaysia dishes	Leong et al, 2009	1. Positive and moderate correlation with consumers acceptance and it was significant at P = 0.01. 2. 'Belacan' consumption level was relatively frequent and well acceptance by all races in Malaysia. 3. 'Sambal belacan' was one of the most favourite dishes made from 'belacan'.

2.1.2 Heritage Food

'Sambal belacan' is a traditional Southeast Asian condiment especially in Malaysia, Thailand and Singapore. It is known by different names; *pazon ng api* (Burma), *sambal terasi* (Indonesia), *sambal belacan* (Malaysia), *blachan kapi* or *pherik kapi* (Thailand) and *mam tom* (Vietnam) (Passmore, 1991: Hutton, 1997). It is usually consumed uncooked as a side dish with meal or raw vegetables such cucumber, salad and gingko. There is a demand for convenience foods that taste, smell and feel like home-made food, like 'Sambal belacan'. 'Sambal belacan' traditionally is prepared by housewives at home or at the restaurants using toasted fermented shrimp and fresh red chilies which are ground together with a granite mortar and pestle (Nadia et al, 2010). Combining all these ingredients together will give a superb spicy taste and aroma that probably could not be found anywhere around the world. 'Sambal belacan' is a spicy condiment that can enhance our appetite during having a meal.

'Sambal belacan' can also be classified as ready-to-eats (RTE) food. According to Murcia (2003), cooked RTE products are most often consumed without further cooking, and therefore, the presence of potential food-borne pathogens presents a considerable food safety threat.

2.1.3 Formulation of 'Sambal belacan'

Sambal belacan is produced by mixing chilies, fermented toasted shrimp paste (*belacan*), salt, sugar and kalamansi juice (an organic acid). Shrimp paste need to be

toasted before added into the mixture in order to reduce the number of microbes by exposing it to the heat. The toasted shrimp paste gives a pleasant aroma while kalamansi juice will enhance the taste of 'Sambal belacan'. Table 2.2 is the formulation for preparing 'Sambal belacan'.

Table 2.2: Formulation for preparing 'Sambal belacan'

Ingredients	Amount (%)
Red chili	51.9
Bird- eye chili	5.8
Shrimp paste (*belacan*)	14.4
Acid (kalamansi juice)	13.8
Sugar	12.0
Salt	2.1

Source: Modified formulation from Nadia et al, 2010

2.2 Ingredients in 'Sambal belacan'.

2.2.1 Chilies

Chili or *capsicum,* also known as hot pepper is an important condiment crops grown in the tropical and subtropical region in the world. They are grown commercially in China, Korea, Indonesia, Pakistan, Sri Lanka, Turkey, Japan, Mexico, Ethiopia, Nigeria, Uganda, Yugoslavia, Spain, Italy, Hungary and Bulgaria (Rajput and Parulekar,

1998). The main types of capsicum grown are the bell peppers although there is a variety of colours and shapes available. It is also possible to grow spicy chili peppers. The storage of chili in cold temperatures (10 ± 2°C) increased the shelf life up to 10 days as compared to storage in cool chambers or under ambient temperature (Rajput and Parulekar, 1998).

According to Mandeel (2004), the mycological quality of some spices on the market, especially of pepper, is quite poor, bearing many genera and species of fungi. Most fungi are present on pepper and spices after post-harvest and storage, which developed after harvesting if relative humidity is not controlled during storage. Samples of whole or ground black pepper from various sources yield numerous colonies of several species of *Aspergillus*. In the laboratory, both *Aspergillus flavus* and *Aspergillus ochraceus* are reported to produce mycotoxins. Other species of moulds that is frequently isolated from spices, including those of *Penicillium, Spicaria, Scopulariopsis,* and *Sporendonema* (Mandel, 2004). *Penicillia* and *Scopulariopsis* are abundant in nature, especially upon vegetation in the later stages of decay (Mandel, 2004).

2.2.2 'Belacan'

'Belacan' or shrimp paste is a traditional preparation of salted and fermented minced shrimps or tiny *Acetes* species. It is a thick and having strong pungent odour of shrimp and a colour ranging from grayish pink to grayish purple (Yeoh and Merican, 2011). During the fermentation process, enzyme and protein that contain in the shrimp will react and the microorganisms are present by the presence of savory odour. The

products that are formed will have strong and characteristics odour. Normally 'belacan' is added as flavouring ingredient in most spicy food, such 'kangkung belacan', 'assam laksa', chicken kapitan and others. According to Malaysian Food Regulation 1985, Regulation No. 168 describes that shrimp paste is required to contain with 15% salt, 30% protein and less than 40% moisture. Table 2.3 is the proximate analyses of laboratory samples and commercial samples of 'belacan'. In addition, Malaysia Food Regulation 1985 also stated that the microbial standard of processed fish products should not exceed to 10^6 CFU/g after incubation at 37°C for 48 hours (Regulation 39).

Table 2.3: Proximate analyses of laboratory samples and commercial samples of 'belacan'

	Laboratory samples	**Commercial samples**
pH	7.5	7.2 - 7.8
Moisture, %	27.2	36.3 - 47.2
Ash, %	27.7	21.2 - 27.7
Salt, %	27.9	20.9 - 27.4
Protein, %	37.5	22.2 -236.4
Fat, %	0.6	1.2 - 1.7

Source; (Ali, 2011)

According to Lee et al, (1993), during 7-days fermentation of *trasi* or shrimp paste, the total bacterial load was decreased, where the number lactic acid remained constant. Identification of some important bacteria in *trasi* was *Staphylococcus*. *Bacillus* and *Proteus* were present while *Salmonella, Clostridium, Vibrio* and *Escherichia coli* is absent (Lee et al, 1993). The lactic acid bacteria were increased during early stage of fermentation but decreased at the end of the process as shown in the Table 2.4. High salt-tolerant bacilli content is then dominant microflora present in commercial samples.

Table 2.4 Aerobic Plate Count and Halophiles counts of microorganisms in commercial 'belacan'

Samples	Aerobic Plate Count (1×10^4 CFU/g)	Halophiles Count at 10% salt medium (CFU/g)
Acetes (Semidried)	4.1 - 480	1.1
Fermenting paste (1 month store)	42.0 - 520	50.0
Finish product	1.0 – 13.0	0.2 – 50.0

Source: Steinkraus (1995)

2.2.3 Acidic Ingredients

Addition of acidic ingredients will help to enhance the sour taste to 'Sambal belacan' (Faridah, 2009). There are many type of acidic ingredients are normally added into 'Sambal belacan' such as mango juice, lemon juice, tamarind juice, vinegar and kalamansi juice. But for this study, a market survey was carried out earlier to determine the most suitable acidic ingredient in 'Sambal belacan'. Response from the public had chosen kalamansi juice as the most suitable acidic ingredient in 'Sambal belacan'.

Kalamansi, Calamodian or *Citrofortunella mitis* belongs to Rutaceae family. The composition of whole Kalamansi fruit contains with a small level of carbohydrate (3%), minerals (1%), ascorbic acid (0.1%) and citric acid (3%). From Table 2.5, it shows clearly Kalamansi juice has pH range from 2.5 to 4.5 (Anon, 2011) where the pH does not support the growth of many foodborne pathogens. The use of kalamansi juice in 'Sambal belacan' may inhibit the growth of some foodborne pathogens. According to Mary King (1999), the essential oil from kalamansi had shown promising results against

Staphylococcus aureus and this fruit is kept best in the refrigerator and should be used within a week.

Table 2.5. Approximate pH values permitting the growth of selected pathogens in food

Microorganism	Minimum	Optimum	Maximum
Clostridium perfringens	5.5 - 5.8	7.2	8.0 - 9.0
Vibrio vulnificus	5.0	7.8	10.2
Bacillus cereus	4.9	6.0 -7.0	8.8
Campylobacter spp.	4.9	6.5 - 7.5	9.0
Shigella spp.	4.9	-	9.3
Vibrio parahaemolyticus	4.8	7.8 - 8.6	11.0
Clostridium botulinum toxin	4.6	-	8.5
Clostridium botulinum growth	4.6	-	8.5
Staphylococcus aureus growth	4.0	6.0 - 7.0	10.0
Staphylococcus aureus toxin	4.5	7.0 - 8.0	9.6
Enterohemorrhagic *Escherichia coli*	4.4	6.0 - 7.0	9.0
Listeria monocytogenes	4.39	7.0	9.4
Salmonella spp.	4.2[1]	7.0 - 7.5	9.5
Yersinia enterocolitica	4.2	7.2	9.6

Source: USFDA, (2001)

2.3 Storage conditions of food

2.3.1 Influence of storage condition on food quality

The important parameters of storage conditions are gas composition (oxygen, O_2 and carbon dioxide, CO_2), relative humidity (%RH), pressure or mechanical stresses, light

and temperature (Gallik et al, 2011). Only gas composition, relative humidity (%RH) and temperatures are important aspects of storage conditions that used in this study.

Gas composition in the package can be reduced by using vacuum packaging and modified atmosphere packaging (MAP). Vacuum technique was used in this study. The function of vacuum technique is used for elimination of O_2 from the packaging and prepared adequate refrigeration in order to inhibit the growth of aerobic microorganisms, proteolytic bacteria, yeast and mold (Murcia et al, 2003). Relative humidity is about the moisture content. 'Sambal belacan' is a type of food that contains high moisture content. The presence of moisture content in the product will cause the product become soggy and mushy because the moisture content is too high.

Temperature that used for the storage of the product will affect the permeability of a film. Films that near ideal headspace at ambient condition may not necessarily provide the same headspace similar to those have in the package in chiller storage (Martino, 2003). Temperature that used for storage also may affect the shelf life of that product. Lowering the temperature of foods will reduce the respiration and transpiration, delay senescence, prevent the wilting and shriveling and thus extending the shelf life of product (Burton and Twyning,1989; Beit-Halachmy and Mannheim, 1992).The quality of the storage product will determined by sensory evaluation or by microbial count (Gallik et al, 2011). Table 2.6 shows that existing and emerging of preservation techniques employed to preserve food.

Table 2.6: Existing and emerging preservation techniques employed to preserve foods and to achieve desired shelf-life

Objective	Preservation factor	Method of achievement
Reduction or inhibition of growth	• Low temperature	• Chill and frozen storage
	• Low water activity	• Drying, curing and conserving
	• Restriction of nutrient availability	• Compartmentalization in water-in-oil emulsions
	• Lowered oxygen	• Vacuum and nitrogen packaging
	• Raised carbon dioxide	• Modified atmosphere packaging
	• Acidification	• Addition of acids and fermentation
	• Alcoholic fermentation	• Brewing, vinification, fortification.

Sources: Gould (1996)

2.3.2 Quality changes associated with the storage condition

The qualities that associated with packaging will change due to their storage time. Several quality changes that can be observed is from the colour, texture, sensory changes, moisture loss and overall appearance that associated with packaged food in ambient and chiller storage conditions.

Foods susceptible to moisture damage need to be packaged in a high humidity barrier material. A certain amount of moisture, however, can be trapped in the packaging or develop during distribution. If not removed, this moisture will be absorbed by the product or condensation will be formed, causing microbial spoilage and lower the consumer appealing. On the other hand, excessive water evaporation through the packaging material might result in desiccation of the packed food stuff or it may favour lipid oxidation (Vermeiran et al, 1999)

2.4 Microorganisms in Food

'Sambal belacan' is a type of RTE-food, which has no sufficient treatment. It has been reported in previous study that it has higher potential of causing food poisoning if insufficient acidic ingredient to inhibit pathogenic microorganisms. Besides that, according to the findings by Nadia et al (2010), the percentage of fat in 'Sambal belacan' is around 0.41% and the protein content is around 7.67%. The high content of protein will serve a good media for microorganisms to grow. Besides that different preparations, which include the handling and practice in food preparations are the factors that influence the growth of microorganisms and contribute to the cause of foodborne illness (Faridah, 2009).

According to Fitri Nurdiyana (2008), high numbers of microbial loads in the 'Sambal belacan' were found at different premises in Kuala Terengganu. The microorganisms present in the samples contained high number of yeast and mold count, followed by Aerobic Plate Count (APC), *Lactobacillus* count, *psychrotrophic* count,

Staphylococcus aureus count, coliform count, *Enterobacteriaceae* count and *Escherichia coli*.

There are several outbreak episodes of food poisoning had reported in Terengganu. Based on, Metro Ahad (2006), 83 students from Maktab Rendah Sains Mara (MRSM) had rushed to the hospital Hulu Terengganu (HHT) because they believed to have food poisoning after eating sandwiches that prepared for afternoon tea. As a result, 10 students were confirmed having several toxicity and were rushed to Hospital Kuala Terengganu. The food which had eaten in few hours showed an immediate effect when the victim suffers from food poisoning.

2.4.1 Foodborne Pathogen

Pathogenic microorganisms can cause illness and disease to the victim that act as their host. The pathogenic microorganism does not show the effect on the odour, taste or appearance of food which are characteristic that normally shown in spoilage food. Pathogen is a microorganism that capable causing disease in a host (Jensen et al., 1997).

There are three types of foodborne illness, which is intoxication, infection and toxin –mediated infection. Foodborne illnesses are food infection caused by bacteria that enter into the body through ingestion of contamination of food and the reaction of the body their presence and metabolites. Table 2.7 describes the different types of foodborne illness that usually occurred.

Table 2.7: Classification of foodborne illness

Type of foodborne illness	Description
• Infection	• Caused by ingestion of food contaminated by either virus, bacteria or parasites ingested food invade and multiply in the intestinal mucosa and/or other tissues.
• Intoxication	• Caused by ingestion of food already contaminated by a toxin that produced by certain bacteria, poisonous chemical and naturally found in animals, fungi and plant.
• Toxin-mediated infection	• Caused by ingestion of food contaminated by either virus, bacteria or parasites in ingested food invade and multiply in the intestinal tract and then release a toxin or toxins that damage surrounding tissues or interfere with normal organ or tissue function.

Source: Anon (2011)

The best method of preventing any foodborne disease that causing by bacteria, viral, parasitic worm or protozoan is by controlling the source of infection and preventing the contamination of soil, food and water (Parry and Pawsey, 1984). The precautions steps that can be implemented to avoid from foodborne disease is pasteurization of milk, egg and cream, sedimentation, filtration and chlorination of water, efficient sewage removal and effective treatment, education in food hygiene and protection of raw foods such as vegetable from faecal contamination (Parry and Pawsey, 2002).

Cooking is not just a useful procedure to eliminate foodborne bacteria but also to virus and parasites. Foodborne toxins, however, may be unaffected by heating. Most mycotoxins are stable to normal cooking procedures, as some bacterial toxins such as those produced by *Staphylococcus aureus* and *Bacillus cereus*. Some pathogenic bacteria such as *Clostridium perfringens, Clostridium botulinum* and *Bacillus cereus* produce heat-resistant spores. These will not be killed by conventional cooking procedures and could resume growth after cooking if the food is stored for too long at an inappropriate temperature. Foods processed commercially which have received a moderate heat treatment specifically designed to eliminate non-spore forming pathogens. Besides cooking, freezing, high salt conditions, drying and high acid condition are stress conditions that affect microbial growth and multiplication.

2.4.1.1 *Escherichia coli*

Ecological investigations have substantiated that *E. coli* originates from the intestinal tract of man and warm- blooded animal, although it may survive and even proliferate in other suitable niches (Bruckner, 2008). Since the presence of this microbe in foods indicates that faecal pollution may have occurred, and consumer might be exposed to enteric pathogen when investigating the food (Davvid et al., 1991). According to Michael et al, (1997), *E. coli* strain that can cause diarrheal illness are categories into specific group based on virulence properties, mechanisms of pathogenicity, clinical syndromes and distinct O:H serogroups.

Faecal coliforms which include *E.coli* is easily destroyed by heat and may die during freezing or frozen storage food. In many raw materials of animal origin, small number of *E.coli* can be expected because the close association of these foods with animal environments. The likelihood of contamination of carcasses is come from fecal material, hides or feathers during slaughter and dressing procedure (Michael et al, 1997). In addition, according to Michael (1997) the presence of *E. coli* in heat- process is might caused by improper and failure during handling by employees.

2.4.1.2 *Salmonella spp.*

Salmonella is a type of Gram-negative bacteria. *Salmonella* can lead to salmonellosis disease for human. Normally these bacteria can be found in vegetables, beef, egg, poultry and milk. Contaminated foods that infected by this pathogenic microbe usually look and smell normal. Besides that, *Salmonella* can be infected to human through improper processing or food handling, unsanitary of food handler, feces of some pets and livestock (Anon, 2011). *Salmonella* are non-fastidious as they can multiply under various environmental conditions outside the living hosts. They do not require sodium chloride for growth, but can grow in the presence of 0.4% to 4% (Pui et al, 2011). Most *Salmonella* serotypes grow at temperature range of 5°C to 47°C with optimum temperature of 35°C to 37°C, but some can grow at temperature as low as 2°C to 4°C or as high as 54°C (Gray and Fedorka-Cray, 2002). They are sensitive to heat and often killed at temperature of 70°C or above. *Salmonellae* grow in a pH range of 4 to 9 with the optimum between 6.5 and 7.5. They require high water activity (a_w) between 0.99 and

0.94, but it can survive at a_w <0.2 such as in dried foods. Complete inhibition of growth occurs at temperatures <7°C, pH <3.8 or water activity <0.94 (Hanes, 2003; Bhunia, 2008).

According to van Leusden *et al.* (1982) *Salmonella* would not grow at pH 4.5 and thus the drop in pH value seen in lactose broth may inhibit the recovery of *Salmonella*. However, there is considerable variation in the minimum pH values reported in the literature at which *Salmonella* will grow (Stokes & Bayne 1957; Prost & Rieman 1967) and Chung & Goepfert (1970) indicating that several factors affected the ability of *Salmonella* to grow at low pH value. These researchers showed that *Salmonella* were able to initiate growth at pH 4.05 but that the acid used to lower the pH had a remarkable effect on the ability of the cells to initiate the growth. When hydrochloric acid was used *Salmonella* grew at pH 4.05 and above whereas, when propionic acid was used, the minimum pH of *Salmonella* was pH 5.5 (Fricker, 1987).

3.4.2 Foodborne Spoilage

Spoilage microbes are often common inhabitants of soil, water, or the intestinal tracts of animals and may be dispersed through the air and water and by the activities of small animals, particularly insects (Doyle, 2007). Spoilage is characterized by any change in a food product that renders it unacceptable to the consumer from a sensory point of view. Spoilage is manifested by a variety of sensory cues such as off-colors, off-odors, softening of vegetables and fruits, and sliminess. However, even before it becomes obvious, microbes have begun the process of breaking down food molecules for their own metabolic needs. Sugars and easily digested carbohydrates are used first, plant pectins are

degraded. Then proteins are attacked, producing volatile compounds with characteristic smells such as ammonia, amines, and sulfides (Doyle, 2007). At the point of sensory rejection (spoilage), the so-called spoilage microflora (or spoilage association) is composed of microorganisms that have contributed to the spoilage and microorganisms that have grown but not caused unpleasant changes (Gram et al, 2002).

3.4.2.1 Aerobic Plate Count (APC)

The aerobic plate count (APC) indicates the level of microorganisms in a product (Maturin and Peeler, 1998). Aerobic plate counts useful to indicate quality, shelf life and post heat-processing contamination. Table 2.8 shows the guideline levels for determining the microbiological quality of ready-to-eat (RTE) food assessed by Aerobic plate count (APC).

Table 2.8: Guidelines on safety levels for determining the microbiological quality of ready-to-eat (RTE) food

Test on APC	Microbiological Quality (CFU/g)			
	Satisfactory	Marginal	Unsatisfactory	Potentially hazardous
Level 1	$< 10^4$	$<10^5$	$\geq 10^5$	-
Level 2	$< 10^6$	$<10^7$	$\geq 10^7$	-
Level 3	N/A	N/A	N/A	-

N/A - SPC testing not applicable. This applies to foods such as fresh fruits and vegetables (including salad vegetables), fermented foods and foods incorporating these (such as sandwiches and filled rolls)
Source ICmSF, 2001

2.4.1.2 Yeast and mold

Fungi, which is include yeast and molds are eukaryotic organism that poorly differentiated body called thallus. Both types of fungi reproduce asexually through spore formation or by budding.

Mould which filamentous fungi, growth is initiated when ripe spore is able to germinate and start the mycelium to grow. The food which had affected by mould showed the changes on the food which is colored, musty, softer and sticky or slimy. Mold is aerobic microorganism, so generally the spoilage is begun at the surface and the mycelium later will penetrate deep into the foods. As well as causing spoilage the more perishable foods, mold are often associated with the spoilage of 'dry' food especially those stored under damp conditions and those food containing high concentrations of sugar and salt (Parry and Pawsey, 1984).

According to Yousef and Carlstrom, (2003), fungi can grow in mesophilic to psychrophilic range, osmotic and acid tolerant. Yeast and molds are capable to reproduce visible colonies on plate incubated for 5 days (120 hours) at room temperature (25±1°C). The lowest temperature that fungi can grow is around -7°C to 0°C for such species *Fusarium, Cladosporium* and *Penicilium* (Yousef and Carlstrom, 2003). Besides, thermotolerant fungi such *Aspergillus flavus* which can grow between 8°C to 45°C are having ability to grow both in moderate and also in high temperature (Pitt and Hocking, 1999).

2.4.2.2 *Psychrotropic* bacteria

Psychrotrophic bacteria is defined are those with maximum growth temperature below 25°C and normally having an optimum growth rate around 20°C. Many *psychrotrophic* is also able to grow at temperature below 0°C (Mossel et al, 1995). The growths of visible colonies in plate are incubated for 10 days at 7 ± 0.5°C. The *psychrotrophic* does not constitute a specific taxonomic group of microorganisms. These bacteria include Gram-positive and Gram-negative bacteria, rod, cocci or vibrious, sporeforming and can grow either as aerobic or anaerobic microorganisms (Faridah, 2009). Besides that, several yeast and mould may present together with *psychrotrophic* bacteria on the plates that can affect the quality of 'Sambal belacan'.

2.4.3 Other Microorganism

2.4.1.3 Lactic acid bacteria

LAB were identified as the major spoilage population of vacuum-packaged emulsion-type sausages and other processed meats stored at refrigeration temperatures (Korkeala and Bjorkroth, 1997; Samelis et al., 2000).These types of microbes can grow in anaerobic and facultative environment and have varying shape from long and slender straight rods to cocco-bacilli. They can convert glucose to lactic acid but lactose is not fermented (Parry and Pawsey, 1984). They can be found in many foods such raw milk, egg products, red meat and fresh fruit. They may present in high number after low-

temperature storage of cooked or fresh meat in vacuum packed with low oxygen permeability, and also in fresh, British-type sausage after storage (Mosseel et al, 1995).

Lactobacillus spp. has ability to produce lactic acid and their lack or low in pathogenic made them useful in food that form during lactic acid fermentation: the low pH prevents pathogenic or another spoilage bacteria colonizing the food. So, they are useful in production some dairy product (yogurt and cheese), fermented type sausage and fermented vegetable product such sauerkraut (Mosseel et al, 1995).

2.5 Shelf-life study

Shelf life of a food is the time during which it remains stable and retains its desired qualities. Some spoilage is inevitable, and a variety of factors cause deterioration of foods such endogenous enzymes in plants oxidizing phenolic compounds which causing browning effect or degrading pectins causing softening and rendering food undesirable microbes likes bacteria, molds, yeasts that growing on and metabolizing foods (Doyle, 2007). Qualitative and quantitative test can be used to quantify the quality attributes of food.

According to Taoukis et al. (1991), temperature, time or duration of storage would become the useful parameter in monitoring the quality of food during storage and distribution. Previous study was carried out by Faridah (2009) on effects of the addition of acidic ingredients in 'Sambal belacan' shows that the incorporation of 'Sambal belacan' and vinegar can prolong the shelf life of 'Sambal belacan'. From that study, the

shelf-life of 'Sambal belacan' was stable until 7 days at room temperature storage and 10 days at chiller storage. A study from Ahmad et al. (2002) had highlighted on the effect of microbial qualities of thermal treatment of red chili puree at different temperature (60°C, 70°C, 80°C and 90°C) and stored condition (5, 25 and 37°C) showed that prepared red chili paste stored up to 6 months were microbiologically safe.

2.5.1 Generation time

The generation time is the time needed for doubling the initial bacterial population during the exponential growth phase (Muller, 1998). This is often referred to as 'doubling time' because it is the time required for a bacterial population to double in cell number. Generation time varies among bacterial species and ranges from about 10 minutes to more than 24 hours. It is directly linked to the specific growth rate, which is slope of the logarithm of the growth curve in the exponential growth phase.

The doubling time of cell number is directly can read from the plotting graph. The plotting graph with logarithm ($\log_{10}$) of organism's number versus time interval between cell divisions is equally the curve that shows in straight line. The generation time of bacteria cell can directly calculate with the followed equation;

Equation 2.1: Mean growth rate constant (k) by Prescott et al. (2005)

$$\text{Mean growth rate constant (k)} = \frac{\log N_t - \log N_0}{0.301t}$$

Equation 2.2: Mean generation time (g) by Prescott et al. (2005); Tortora et al (2001)

$$\text{Mean generation time (g)} = \frac{1}{k}$$

Equation 2.3: Generation time (gt) by McMeekin et al. (2003). The calculation is directly from linear equation given from R-squared formula.

$$\text{Slope} = \frac{\log_{10} 2}{gt}$$

$$gt = \frac{\log_{10} 2}{gt}$$

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Equipments

Table 3.1.: List of equipment and materials used for 'Sambal belacan'

Materials	Equipments
Red chilies, bird-eye chilies, Shrimp paste ('belacan'), Kalamansi juice, Salt, and Sugar	Food processor, bowl, spoon and oven

Table 3.2: List of equipment and materials used for storage and physical analysis of 'Sambal belacan'

Materials	Equipments
'Sambal belacan', glass bottle, polypropylene (PP) pouch, low density polyethylene (LDPE), silica gel.	Vacuum packaging machine, pH meter, RH meter (humidity alert II), gas analyser (Oxybaby®), colorimeter (Konica Minolta CR-310 Japan), pedal sealing machine, distilled water and chiller.

Table 3.3: List of equipment and materials used for microbiological analysis of 'Sambal belacan'

Materials	Equipments	Reagents
'Sambal belacan' from different condition and temperature storage	Aluminum foil, analytical balance (A & D COMPANY, LTD., Japan), autoclave (HICLAVE™, HVE-50, HIRAYAMA, Japan), beakers (100mL and 250mL), chiller (ASECO® MODEL LCD617D), cotton wool, Durham tubes, hockey stick (L- shaped spreader), incubator large (MEMMERT, Germany), laminar flow cabinet (ERLA-CFM SERIES, VFM-4, Malaysia), marker pen (for labeling purpose), lighter (for turn on the heat), measuring cylinder (500-1000mL), micropipette (100µL- 1000µL Eppendorf Research) and tips, Petri dishes, pipettes (1mL and 100mL), spatula, spirit lamp, sterile ladles, stirring hotplate (FAVORIT, HS 0707V2), stomacher (BAG MIXER® - INTERSCIENCE, France), stomacher bag (sterilesed), and Universal bottle.	Distilled water, 0.1% Buffered Peptone Water (MERCK, Germany), Alcohol 70 Eosin Methylene Blue (EMB) – Lactose Sucrose Agar or Levine (CM 0069 – OXOID, England), Lauryl Tryptose Broth (CM 0451 – OXOID, England), MRS agar (MERCK, Germany), Plate Count Agar (MERCK, Germany), Potato Dextrose Agar (MERCK, Germany), nutrient agar (MERCK, Germany), Eosin Methylene Blue agar (MERCK, Germany), Xylose Lysine Dextrose agar (MERCK, Germany), BPA (MERCK, Germany), HE agar (MERCK, Germany), VRBD agar (MERCK, Germany), RV broth (MERCK, Germany), TTH broth (MERCK, Germany), Lacose broth (MERCK, Germany), 10% tartaric acid (MERCK, Germany), potassium iodide and brilliant green.

3.2 Methods

3.2.1 Sample preparation

Kalamansi juice was used in this study due to the most preferred ingredient which incorporated in 'Sambal belacan' after a market survey was carried out. This market

survey was carried out with 200 at Jerteh, Bus Station Kuala Terengganu, MBKT and University Malaysia Terengganu.

All raw materials were obtained from Hock Kee Seng, a local hypermarket in the state of Kuala Terengganu, Malaysia. All the chilies were washed under running tap water, destalked manually and blanched for 1 minute in hot water at 100°C. The blanching time was based on preliminary trials that resulted in complete inactivation of peroxidase enzyme in chilies as suggested by (Ahmed et al., 2002). Sample was prepared in large batch by using food processor (model National MK- 5070M). Preparation of 'Sambal belacan' was based on modified formulation from the Nadia et al (2010). Then, the prepared 'Sambal belacan' was divided into two different packaging for every sample categories. The reason for equal sample divisions was to maintain the quality of product and to avoid contamination during microbiology analysis and physical analysis. Besides that, this step is important in order to avoid from failure during microbial counting of 'Sambal belacan' experimentation. Tables below showed the reformulated formulation of 'Sambal belacan' from Nadia et al (2010).

Table 3.4: Formulation of 'Sambal belacan'

Ingredient	Percentage (%)
Red chilies	51.9
Bird eyes chilies	5.8
Roasted 'belacan'	14.4
Acid (kalamansi juice)	13.8
Sugar	12.0
Salt	2.1

(Source; Modified formula from Nadia et al, 2010)

The most risk ingredient for 'Sambal belacan' is 'belacan'. In this preparation, square shaped of 'belacan' was used. 'Belacan' was roasted before mixed with all the ingredients in order to reduce microbial load. As the precaution steps, an initial trial standard microbiological of 'Sambal belacan' were done to ensure that the microbial count is not obliged the regulation of Malaysia Food Regulation 1985, Regulation No. 39 and No.163. Since the sample was prepared as raw food, it was taken aseptically during experiments. Sample was prepared in hygienic condition. All the equipments that used during samples preparation was sterilized using hot water.

3.2.2 Experimental Design

3.2.2.1 Overall Experiment

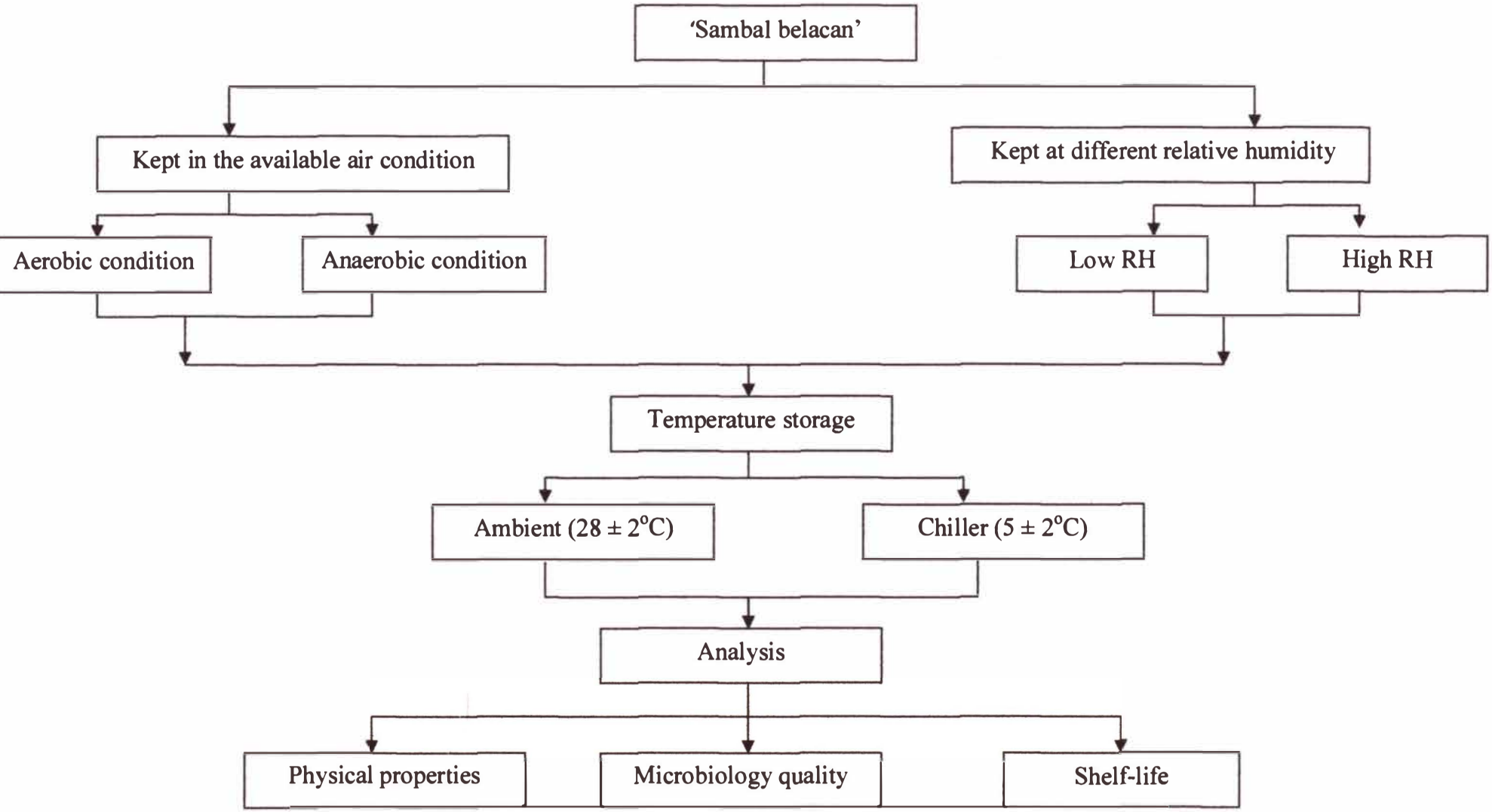


Figure 3.1: Overall experiment of 'Sambal belacan' study

3.2.2.2 Unit of Experimental

Table 3.5: Tables of unit of experiment

Samples	**'Sambal belacan'**
Independent variables	1. Type of storage condition (RH and O_2)
Dependent variables	1. Microbiology analysis
	2. Physical analysis
	3. Shelf- life
Replication	1
Arrangement	1- way ANOVA
Design of experimental	Completely- Randomized Design (CRD)
Experimental unit	40

3.2.2.3 Completely Randomized Design (CRD)

Table 3.6: Tables of Completely Randomized Design (CRD)

Day 1	**Day 2**
A_2X_2	A_3X_1
A_1X_1	A_4X_1
A_2X_1	A_4X_2
A_1X_2	A_3X_2

Description;

A_1	=	with oxygen	X_1	=	Chiller (5±2°C)
A_2	=	without oxygen	X_2	=	Ambient (25±2°C)
A_3	=	Low relative humidity			
A_4	=	High relative humidity			

3.2.3 Storage Condition

'Sambal belacan' was stored at different conditions and temperatures. Two major factors that influence quality of 'Sambal belacan' were chosen to be studied the effect of storage conditions of availability of oxygen in the air and percentage of relative humidity which can be absent and present in the samples and high and low humidity in the food package. All the samples then were stored at difference temperature which is ambient (28 ± 2°C) and chilled (5 ± 2°C) temperatures.

3.2.3.1 Availability of Oxygen in the air

100 g of 'Sambal belacan' was filled into polypropylene pouch for 48 sets of samples. Since the samples need to be away from contamination during performing analysis, the samples were duplicated for each category. 24 pouches were undergone different anaerobic conditions (12 pouches were stored at ambient temperature and 12 pouches were stored chiller temperature) and another 24 pouches were kept in the storage

with the storage with the available of oxygen in the air (12 pouches were stored at ambient temperature and 12 pouches were stored chiller temperature).

For keeping the samples without the present of oxygen in the air, 100 g 'Sambal belacan' was filled with aseptically into polypropylene (PP) pouch. Then, the packages were sealed with 7 unit of standard pressure for all package using vacuum packaging machine (model Hancovac, Johannesburg and Gauteng). Food package was sealed using vacuum packaging machine in order to eliminate the presence of oxygen from the package. Next, all samples were stored at respective temperatures.

For keeping the samples with the present of oxygen in the air, 100 g 'Sambal belacan' were filled with aseptically into polypropylene (PP) pouch. Food packages were sealed using pedal sealing machine (model JIASAI, China). Next, all samples were stored at respective temperatures.

Microbiological, physical and shelf-life analyses were done for all samples and analyses were carried out for every week until 6 weeks storage.

3.2.3.2 Relative Humidity (%RH)

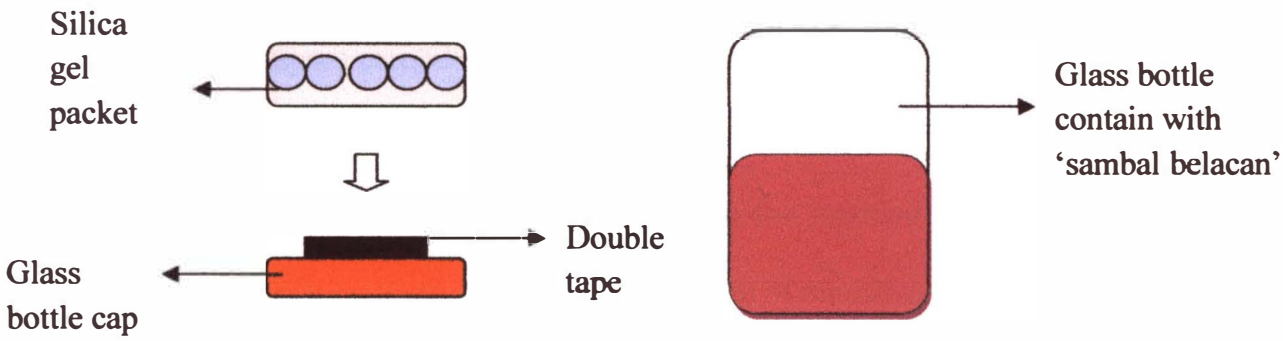
100 g of 'Sambal belacan' was filled into glass bottle for 48 sets of samples. 24 bottles which had low RH with silica gel sealed underneath cover cap of glass bottle (12 bottles was stored at ambient temperature and 12 bottles was stored chiller temperature) whereas another 24 bottles which had high RH without the used silica gel (12 bottles was stored at ambient temperature and 12 bottles was stored chiller temperature).

Firstly, all glass bottles were sterilized using dry hot air. Sterilization is a method use to eliminate all viable microorganisms (Varabadan and Parkinson, 2010). In this study, dry heat sterilization was done using a hot air oven for 30 minutes at 180°C.

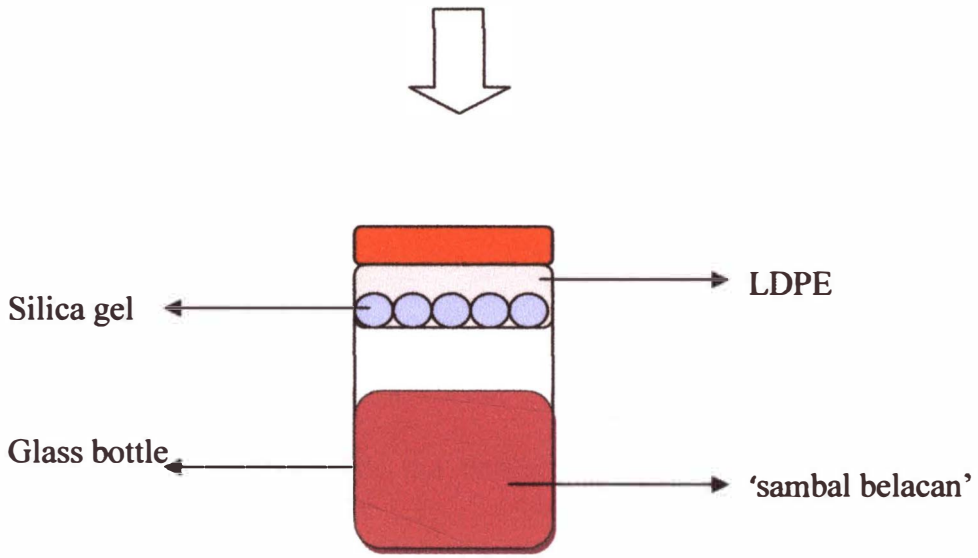
For high relative humidity conditions, 'Sambal belacan' was filled into glass bottles. The headspace of bottle was not less than 10% from height of packaging. The initial percentage of relative humidity that contained inside the glass bottle with samples was measured using RH meter (Extech Instrument, United States). Then, all the samples were stored at respective storage temperatures.

For low relative humidity condition, 'Sambal belacan' were filled into glass bottles. The headspace of bottle was not less than 10% from height of packaging. Then, 5 g silica gel was packed into low density polyethylene (LDPE) and sealed properly. LDPE has high permeability of water vapour properties. According to Vermeiren et al (1999), silica gel can be used to control excess of humidity. This was done by placing humectants between two layers of a plastic film which was highly permeable to water vapour. The initial percentage of relative humidity that contained inside the glass bottle with samples was measured by using RH meter (model Extech Instrument, United States). Then, silica gel packet was attached by double tape underneath the cover cap of glass bottle and closed tightly. Finally, all the samples were stored at respective storage temperature.

Physical properties, microbiological quality and shelf-life analyses were done for each week until 6 weeks of storage.



1) Silica gel packet was attached underneath the cover cap of glass bottle using double tape.



2) Then, the cap was closed tightly.

Figure 3.2: Illustration of low relative humidity storage condition

3.2.4 Physical Analyses

Physical analyses carried out in this study were measurement of pH, percentage of relative humidity (%RH), percentage of carbon dioxide (% CO_2) and colour. These measurements were important in order to measure the reaction that mimic the actual storage conditions of sample that influence the growth of microbes.

3.2.4.1 pH measurement

5 g samples were prepared by diluted into 20 ml distilled water (Kailasapathy, 2006). Before the pH reading was taken, pH meter (model 340i WTW, Germany) was calibrated with standard buffer solution of pH 4 and pH 7. Finally, three readings per samples were taken and the mean of pH values were determined.

3.2.4.2 Relative Humidity (%RH) measurement

The measurement of %RH inside the bottle was taken by using RH meter or Humidity Alert II. Three readings per samples were taken. The reading was taken for samples that used for RH treatment.

3.2.4.3 Carbon dioxide concentration measurement (%CO_2)

The measurements of percentage CO_2 inside the packages was taken by using gas analyser (oxybaby® 6.0, United States). Three readings per samples were taken and

average for each samples were determined. The reading was taken for samples that treated with Oxygen treatment.

3.2.4.4 Colour measurement

Colour measurements were taken with a colorimeter (Minolta CR 300, Osaka, Japan). The samples were placed into Petri dishes before the readings were taken, and there was no gap between the sample and the Petri dish lid. The lens of the colorimeter was placed against the Petri dish lid, and three readings were taken and averaged value for each sample was determined. L^*, a^*, b^* values were also determined (Kayaardı and Gok, 2003).

Colour coordinates were determined by the CIE-LAB system and the results were expressed as lightness (L^*), redness (a^*) and yellowness (b^*). In addition, hue angle, which describe the hue or colour (H^o) was calculated [$H^o = \tan^{-1}(b^*/a^*)$], while the saturation index or chroma (C^*) [$C^* = (a^{*2} + b^{*2})^{0.5}$], describes the brightness or vividness of colour (Rubio et al, 2008).

3.2.5 Agar, broth and enrichment diluents preparation

All media, agar, and broth were prepared following the instructing given by manufacturing. The preparation of agar, broth and enrichment were important to fulfill the experimental requirements. Table 3.7 shows the media, diluents and broth cultures used for microbiological analyses.

Table 3.7: Media, diluents and broth cultures microbiological analysis

Media/Broth	Uses
0.1% Buffered Peptone Water (BPW)	Important to homogenization of the sample and do the serial dilution and used for medium growth or as the basis of fermentation media [a]. Besides, BPW also acts as pre-enrichment medium for isolation of injured microorganisms from various food sources [b]
Plate Count Agar (PCA)	Used for determination of plate count of microorganism in food, water and waste water. This medium does not contain any indicator or inhibitors [b]
Baird- Parker Agar (BPA)	A selective medium for isolation of *Staphylococci* from food sample. It is based for preparation of egg-tellurite-glycine-pyruvate agar [a]
Acidified Potato Dextrose Agar (PDA)	Used for cultivation and enumeration of yeast and mould
Escherichia coli (EC) broth	Describe for examination of water, waste water and various foods for fecal coliform at 37°C and *E. coli* at 45.5°C [b]
Lauryl Sulphate Tryptose (LST) Broth.	Detection of coliform organism in the presumptive phase and enumeration of coliform bacteria [a]
Violet Red Bile Dextrose (VRBD) agar	For selective isolation of *Enterobacteriaceae* or bile tolerant gram-negative bacteria from non-sterile pharmaceutical products, foodstuffs and other sample materials
MRS Agar	For enrichment, cultivation and isolation of *Lactobacillus* species[b]
EMB - Levine Agar	Used for selective isolation and differentiation between *E. coli* and Enterobacter [b]

Media/broth	Uses
Nutrient Agar (NA)	Used for the cultivation of bacteria and for the enumeration of organisms in water, sewage, feces and other materials[a]
Brain-Heart Infusion (BHI) broth	For the growth of pathogenic cocci and other microorganisms[a]
Xylose Lysine Dextrose (XLD) agar	Selective differential medium for the isolation of Gram-negative enteric pathogens from fecal specimens and other clinical material[a]
Hektoen Enteric (HE) agar	Selective and differential media for the isolation of enteric pathogens from fecal material and food products[b]
Bismuth Sulfite Agar (BSA)	Used for the selective isolation of *Salmonella* spp[b]
Lactose broth	Used for the cultivation of *Salmonella* and coliform bacteria from food, dairy, water and pharmaceutical products
Tetrathionate broth (TTH)	Used with iodine for the recovery of *Salmonella* spp[b]
Rappaport-Vassiliadis medium	selective media used for the enrichment of *Salmonella* species[a]

Source : [a] Bridson (2006)
[b] Merck Microbiology Manual (2007)

3.2.5.1 0.1% Buffered Peptone Water (BPW)

BPW used was purchased from Merck, Germany. This pre-enrichment medium consisted of 10.0 g peptone from casein, 5.0 g sodium chloride (NaCl), 9.0 g disodium hydrogen phosphate (Na_2HPO_4) dedocahydrate and 1.5 g potassium dihydrogen phosphate (KH_2HPO_4) for every (g/L) composition. Medium was mixed with distilled water. The medium was be filled into suitable bottle and capacity depended on the test requirements. The BPW then sterilized in autoclave at 121°C for 15 minutes at 15 psi.

Final pH was 7.2 ± 2 at 25°C. The prepared medium was stored at 2 -8°C. The prepared BPW was clear and yellowish.

3.2.5.2 Plate Count Agar (PCA)

PCA used was purchased from Merck, Germany. This medium contained of 5.0 g peptone from casein, 2.5 g yeast extract, 1.0 dextrose and 15.0 g agar for every g/litre. 22.5 g of PCA in mixture of suspended 1000 ml distilled water in the suitable bottle. The medium prepared was autoclaved at 121°C for 15 minutes at 15 psi pressure. The final pH was 7.2 ± 2 at 25°C. The sterilize PCA was cooled until 45-55 °C before it was filled into sterile Petri dishes. The medium was swirled before filling into Petri dishes in order to allow the mix completely. As the result, the plates were clear and yellowish in color.

3.2.5.3 Potato Dextrose Agar (PDA)

This medium contained of 4.0 g of potato infusion (from 200 g potatoes), 20.0 g D(+)glucose and 15.0 g agar. PDA was purchased from Merck, Germany. Each preparation for 1 litre required 39 g PDA media. Media was mixed with distilled water and autoclaved at 121°C for 15 minutes at 15 psi pressure. The final pH of PDA agar was reduced to approximately pH 3.5 by adding 10% tartaric acid. The medium should be swirled before filling into Petri dishes in order to allow the mix completely. The plates were clear and yellowish brown.

3.2.5.4 MRS Agar

This type of agar was purchased from Merck, Germany. This medium consists of 10.0 g peptone from casein, 4.0 g yeast extract, 2.0 g diammonium hydrogen citrate, 5.0 g sodium acetate, 0.2 g sodium sulfate, 14.0 g agar for every liter of dilution. For preparation of 1 litre of distilled water, there were 68.2 g MRS agar were needed. In addition, MRS agar was added with 1.0% (w/v) calcium carbonate. The final pH was 7.4 ± 0.2 at 25°C. This agar then was autoclaved at 121°C for 15 minutes at 15 psi pressure. After that, it was cooled at 50 - 55°C and agar was stirred before pour into Petri dish. The appearance of end product was brown colour and clear.

3.2.5.5 EMB - Levine Agar

This medium consist of 10.0 g of peptones, 2.0 g di-potassium hydrogen phosphate, 10.0 g lactose, 5.0 g sucrose, 0.4 g eosin yellowish, 0.07 g methylene blue, 15.0 g agar for every litre. This medium was purchased from Oxoid, England. 37.5g of EMB medium was mixed with distilled water to produce 1 litre of solution. The final pH was 6.8 ± 0.2 at 25°C. This medium then was autoclaved at 121°C for 15 minutes at 15 psi pressure. After sterilization process, this agar was cooled until 50 - 60°C and it was stirred in order to oxidize the methylene blue and the precipitate was suspended before it was poured into Petri dishes. The prepared plates were reddish-brown to violet - brown and formed in clear agar.

3.2.5.6 Nutrient Agar (NA)

This medium was purchased from Merck, Germany. This media was contained of 5.0 g peptone from meat, 3.0 g meat extract and 12.0 g of agar per litre. Preparation of 1 litre of agar required 20.0 g of this medium. Then the medium was diluted with distilled water and autoclaved at 121°C for 15 minutes at 15 psi pressure. The pH of agar was adjusted to 7.0 ± 0.2 at 25°C and allowed to cool at 50- 55°C. The agar was mixed well before poured into Petri dishes. To make the slant agar, agar was filled into tubes. Then, the tubes were laid slantly on the table as soon as after autoclaved and continued that position until agar was truly solidified. The prepared slant agar was yellowish and clear.

3.2.5.7 Violet Red Bile Dextrose (VRBD) Agar

VRBD Agar is used for selective isolation of *Enterobacteriacea* or bile tolerant Gram-negative bacteria from non-sterile pharmaceutical products, foodstuffs and other sample materials. Medium was purchased from Merck, Germany. This medium contained 7 g pancreatic digest of gelatine, 3 g yeast extract, 1.5 g bile salts, 5 g NaCl, 10 g glucose with H_2O, 30 mg neutral red, 2 mg crystal violet and 15 g agar per litre of prepared media. Preparation of 1 litre VRBD agar required 39.5 g of medium. Then, the medium was diluted with distilled water before boiled it on hot plate for 45 minutes. Magnetic bar or magnetic stir was used in order to avoid media from solidified. The final pH of media was pH 7.4 ± 0.2. After that process, this medium was allowed to cool until 50 - 60°C and then the medium was poured into Petri dishes. The prepared plates were reddish to violet color and form in clear agar.

3.2.5.8 Lactose broth

Lactose Broth is used for the cultivation of *Salmonella* and coliform bacteria from food, dairy, water and pharmaceutical products. This medium was bought from Merck, Germany. For every litre of prepared broth, 13 g of medium were required that contained with 5 g of enzymatic of gelatine, 3 g of beef extract and 5 g of lactose. After added with distilled water, that broth was autoclaved at 121°C for 15 minutes at 15 psi pressure. Final pH was 6.9 ± 0.2 at 25°C. The prepared medium was stored at 2 - 8°C. The appearance of lactose broth was pale to light yellow and clear with light precipitation.

3.2.5.9 Rappaport Vassiliadis (RV) broth

Rappaport-Vassiliadis Broth is a selective media used for the enrichment of *Salmonella* species. This media was purchased from Merck, Germany. 41.8 g medium was needed to prepare 1 litre broth. For every litre of broth, there were contained with 4.5 g soya peptone, 29.0 g magnesium chloride hexahydrate, 8.0 g sodium chloride, 0.4 g dipotassium phosphate, 0.6 g potassium dihydrogen phosphate and 0.036 g malachite green. After added with distilled water, the broth was filled up until 15 ml per bottles into several universe bottles. Then, it was continued to autoclave at 121°C for 15 minutes at 15 psi pressure. For storage, RV broth was stored at 2 - 8°C and away from direct light. Final pH of broth was 5.2 ± 0.2 at 25°C. The appearance of RV broth was clear and blue in color.

3.2.5.10 Tetrathionate broth (TTH)

This media was purchased from Merck, Germany. Tetrathionate Broth (TTH) is used as a selective enrichment for the cultivation of *Salmonella spp.* that may be present in small numbers and compete with intestinal flora. 46 g of media were needed for every litre of prepared broth which contained with 2.5 g of enzymatic digest of casein, 2.5 g of enzymatic digest of animal tissues, 1 g of bile salts, 10 g of calcium carbonate and 30 g of sodium thiosulfate. TTH solution was then boiled using hot plate for 15 minutes. Magnetic bar or magnetic stir was used in order to homogenize the mixture. Next, the TTH broth was allowed cooling until 50 -60°C before added with potassium iodide (KI) and brilliant green into the broth. Before that, KI and brilliant green solution were needed to be prepared separately. For every litre of TTH broth, there were only 20 ml of KI and 0.1g/10 ml of brilliant green. Both of them were autoclaved separately at 121°C for 15 minutes at 15 psi pressure. Final pH of TTH broth was 8.4 ± 0.2 at 25°C. Lastly, the broth was filled into several universe bottles for 15 ml per bottles. Appearance of prepared broth was milky white to slightly green-white and opaque. For storage, this broth was stored at 2 – 30°C and container was closed tightly in order to protect from moisture and light.

3.2.5.11 Bismuth Sulfite Agar (BSA)

Bismuth Sulfite Agar was used for the selective isolation of *Salmonella* spp. This medium was purchased from Merck, Germany. For every litre of BSA, 55 g of medium were required contained with 5 g of enzymatic digest casein, 5 g of enzymatic digest

animal tissue, 5 g of beef extract, 5 g of dextrose, 4 g of disodium phosphate, 0.3 g of ferrous sulfate, 8 g of bismuth sulfite indicator, 0.025 g of brilliant green and 20 g of agar. After solution was prepared, it was boiled using hot plate for 15 minutes. Magnetic bar or magnetic stir was used in order to avoid the media from solidified. Final pH of media was 7.5 ± 0.2 at 25°C. After that process, this media was allowed to cool until 50 - 60°C before poured into Petri dishes. Prepared medium was opaque and may have a few dark particles suspended in medium, and green-yellow to green-beige.

3.2.5.12 Xylose Lysine Deoxycholate (XLD) agar

XLD Agar is a selective differential medium for the isolation of Gram-negative enteric pathogens from fecal specimens and other clinical material. For every litre of XLD agar contained with 5 g of yeast extract, 3.5 g of xylose, 5 g L-lysine hydrochloride, 7.5 g of lactose, 7.5 g of sucrose, 2.5 g of bile salts, 4 g of sodium thiosulfate, 0.8 g of ferric ammonium citrate, 13.5 g of agar and 0.08 g of phenol red. After solution was prepared, it was boiled using hot plate for 15 minutes. Magnetic bar or magnetic stir was used in order to avoid the media from solidified. Final pH of medium was 7.4 ± 0.2 at 25°C. After that process, this medium was allowed to cool until 50 - 60°C before poured into Petri dishes. Prepared medium was reddish colour.

3.2.5.13 Hektoen Enteric (HE) agar

HE agar is a selective and differential media for the isolation of enteric pathogens from fecal material and food products. In every litre of XLD agar contained with 12 g of peptic digest of animal tissue, 3 g of yeast extract, 9 g of bile salt, 12 g of lactose, 12 g of sucrose, 2 g of salicin, 5 g of sodium chloride, 5 g of sodium thiousulfate, 1.5 g of ferric ammonium citrate, 14 g of agar, 0.1 g of acid fuchsin and 65 mg bromthymol blue. After solution was prepared, it was boiled using hot plate for 15 minutes. Magnetic bar or magnetic stir was used in order to avoid the media from solidified. Final pH of media was 7.6 ± 0.2 at 25°C. After this process, this media was allowed to cool until 50 - 60°C before poured into Petri dishes. Prepared medium was firmed, translucent, and green in color.

3.2.6 Enumeration Method of Microorganisms

The enumeration of microorganism is important in order to compare the actual amount of microorganism present in different samples. Microbial enumeration is the measurement of the number of microorganism cells per milliliter, gram, or cubic meter of a sample. The units of enumeration is depends on the nature of the samples (Gary, 2007). There are a number of methods that can be used to determine the number of cells/unit and these methods can be divided up into different categories. There are four categories of microbial enumeration which are indirect method and viable cells, direct method and viable cells, direct method and total numbers, indirect method and total numbers.

Normally, enumeration is using direct and viable count. This method is referring to standard plate count method which is used to determine the number of viable bacterial cells per unit volume. The colonies are referred to as colony forming units (CFU). The procedure of determine the number of CFUs on the plate is according the formula below;

Equation 3.1: Calculation for colony counting (CFU/g or CFU/ml) (Lani, 2007)

$$\text{Colony count} = \frac{\text{Average number of colonies from duplicate plates}}{\text{Dilution factor} \times \text{Volume plate}}$$

The highest dilutions will produce the lowest number of CFUs and the lowest dilutions will produce the highest number of CFUs. The plate with the countable number of colonies should be selected to count. Normally, only plates with 30 - 300 colonies are counted because this range is considered statistically significant (Prescott et al., 2005). The colonies fewer than 30 colonies is easier to count but if the colonies more than 300 colonies and become crowded to form distinct colonies were known as too numerous to count (TNTC). Dilutions with fewer than 30 colonies are easy to count, but often produce inaccurate results since one or two contaminating colonies can cause a significant overestimate of the cell count.

3.2.7 Sample preparation and serial dilution

The samples were prepared from different storage conditions for the period of 6-weeks of storage. The microbial analyses were done aseptically at Food Science Laboratory in UMT. Research was done at room temperature using aseptic technique in each step of analyses including homogenization, weighing, handling and preparation of serial dilution. The aseptic technique was important to avoid cross contamination which may lead to false microbial count of 'Sambal belacan' samples during experiments

For the serial dilution, firstly 25.0 ± 0.1 g of the 'Sambal belacan' was filled into sterile stomacher bag and weighing using analytical balance. The sample then was added with 225 mL of sterile buffered peptone water (BPW) and then homogenized using stomacher. The serial dilution at this position was 1/10 (10^{-1}) from the original sample. The 1 mL clear dilution from serial dilution 1/10 (10^{-1}) then was transferred into 9 mL of sterile buffered peptone water (BPW) in universal bottle using micropipette with sterile tips. The dilution prepared was 10^{-2}. A sample was dilute serially until dilution 10^{-5}. The duplication of serial dilution was very important in experimentation.

3.2.7.1 Homogenization

Homogenization is a process of reducing a substance, to extremely small particles and distributing it uniformly throughout fluid. In this study, homogenization processed was done by using stomacher. 'Sambal belacan' was mixed by peptone water before homogenization using stomacher. This technique released the organisms within in food into the peptone water that contained in stomacher bag (Interscience, France).

Homogenous suspended of food and microorganisms were prepared by blending or stomaching which permitted the preparation of dilutions appropriate for enumeration procedures (Faridah, 2009). These steps were continued by doing several dilutions of suspensions for microbial load analysis.

3.2.8 Microbial Analyses of 'Sambal belacan'

'Sambal belacan' were sampled and analyzed for aerobic plate count (APC), yeast and mould count, Lactic acid bacteria, *psychrotrophic* bacteria and *Enterobacteriaceae* count, Coliform count, *Escherichia coli* counts and detection of *Salmonella spp.* following the procedure of the USFDA (2010). All analyses for microbial load of 'Sambal belacan' samples were done in duplicate. Then, data collected were expressed by mean and standard deviation of microbial counts (CFU/g).

The microbiological analyses of 'Sambal belacan' were analyzed for every week and it was continuing until 6 weeks. This analysis is very important in order to measure the most suitable storage conditions that can be applied to 'Sambal belacan'. Temperature and duration of storage would serve as useful parameters for monitoring quality of food during storage (Singh, 2000) until the samples reached to unaccepted condition and did not fulfill the requirements of Malaysia Food Act 1983 & Food Regulation 1985 (2009).

3.2.8.1 Aerobic Plate Count (APC)

25 g samples of 'Sambal belacan' were added with 225 ml 0.1% peptone water (buffered) before homogenizing using a stomacher. The original sample had a dilution factor of 10^{-1}. Then, 1.0 ml of dilution 10^{-1} was transferred into a 9.0 ml universal bottle containing 0.1% buffered peptone water for dilution 10^{-2} and serial dilution was continued until 10^{-5} dilutions. Duplicate Petri dishes were labeled at each dilution with 10^{-1}, 10^{-2}, until 10^{-5} dilution and 0.1 ml of inoculum was pipetted onto a Plate Count Agar (Merck, Germany) using spread plate method of each dilution. Each dilution was done in duplicate. Plates were inverted to prevent condensation that can cause contamination. Then, they were incubated at 37°C for 24-48 hours. After 24-48 hours, plates were examined and numbers of colonies were calculated according to the rules applicable for colony counting.

3.2.8.2 Yeast and mould count

25 g samples of 'Sambal belacan' were added with 225 ml 0.1% buffered peptone water and prepared by homogenization using a stomacher. The original sample had a dilution factor of 10^{-1}. Then, 1.0 ml of dilution 10^{-1} was transferred into a 9.0 ml universal bottle containing 0.1% buffered peptone water for dilution 10^{-2} and serial dilution was continued until 10^{-5}. Duplicate Petri dishes were labeled at each dilution with 10^{-1}, 10^{-2}, until 10^{-5} dilutions. Next, 0.1 ml of inoculum was inoculated onto Plate Dextrose Agar contain with 10% tartaric acid using spread plate method for each dilution. Each dilution was done in duplicate. Plates were inverted to prevent condensation that can cause

contamination. Then, the plates were incubated at 25°C for 120 hours (5 days). Plates were examined and numbers of colonies were calculated according to the rules applicable for colony counting.

3.2.8.4 Lactic Acid Bacteria count

MRS Agar is the selective agar used to detect the presence of Lactic acid bacteria. The original sample had a dilution factor of 10^{-1}. Then, 1.0 ml of dilution 10^{-1} was transferred into a 9.0 ml universal bottle containing 0.1% buffered peptone water for dilution 10^{-2} and serial dilution was continued until 10^{-5} dilutions. Duplicate Petri dishes was labeled at each dilution with 10^{-1}, 10^{-2}, until 10^{-5} dilutions. Next, 0.1 ml of inoculum was inoculated onto a plate of MRS Agar using spread plate method for each dilution. Inoculate was spread on agar using sterile hockey stick. Each dilution was done in duplicate. Plates were inverted to prevent condensation that can cause contamination. Then, they were incubated at 37°C for 24-48 hours. Plates were examined and numbers of colonies were calculated according to the rules applicable for colony counting.

3.2.8.5 *Escherichia coli* count

25 g samples of 'Sambal belacan' were added with 225 ml of 0.1% buffered peptone water before homogenizing using a stomacher. The original sample had a dilution factor of 10^{-1}. Then, 1.0 ml of dilution 10^{-1} was transferred into a 9.0 ml universe bottle containing 0.1% buffered peptone water for dilution 10^{-2} and serial dilution was

continued until 10^{-5} dilutions. Duplicate Petri dishes were labeled at each dilution with 10^{-1}, 10^{-2}, until 10^{-5} dilutions. 0.1 ml of inoculum was pipetted onto a Eosin methylene blue, EMB agar (Merck, Germany) using spread plate method of each dilution. Each dilution was done in duplicate. Plates were inverted to prevent condensation that can cause contamination. Then, they were incubated at 37°C for 24 - 48 hours. After 48 hours, plates were examined and numbers of colonies were calculated according to the rules applicable for colony counting.

3.2.8.6 *Psychrotrophic* bacteria

25 g samples of 'Sambal belacan' were added with 225 ml of 0.1% buffered peptone water and was prepared by homogenization using a stomacher. The original sample had a dilution factor of 10^{-1}. Then, 1.0 ml of dilution 10^{-1} was transferred into a 9.0 ml universe bottle containing 0.1% buffered peptone water for dilution 10^{-2} and serial dilution was continued until 10^{-5} dilution. Duplicate Petri dishes was labeled at each dilution with 10^{-1}, 10^{-2}, until 10^{-5} dilution. Next, 0.1 ml of inoculate was inoculated using spread plate method for each dilution onto a Plate Count Agar, PCA (Merck, Germany). Each dilution was done in duplicate.

Plates were inverted to prevent condensation that can cause contamination. Then, the plates were incubated at 5 ± 1°C for 240 hours (10 days). Plates were examined and numbers of colonies were calculated according to the rules applicable for colony counting. Colonies grown are depending on the purpose of medium used (Merck, 2007). Normally, colony obtained is round, opaque to yellowish coloured.

3.2.8.7 *Enterobacteriaceae* count

25 g samples of 'Sambal belacan' were added with 225 ml of 0.1% buffered peptone water before homogenizing using a stomacher. The original sample had a dilution factor of 10^{-1}. Then, 1.0 ml of dilution 10^{-1} was transferred into a 9.0 ml universal bottle containing 0.1% buffered peptone water for dilution 10^{-2} and serial dilution was continued until 10^{-5} dilutions. Duplicate Petri dishes were labeled at each dilution with 10^{-1}, 10^{-2}, until 10^{-5} dilutions. 0.1 ml of inoculum was pipetted onto a Violet Red Bile Dextrose, VRBD agar (Merck, Germany) using spread plate method of each dilution. Each dilution was done in duplicate. Plates were inverted to prevent condensation that can cause contamination. Then, the plates were incubated at 37°C for 24 - 48 hours. After 48 hours, plates were examined and numbers of colonies were calculated according to the rules applicable for colony counting.

3.2.8.8 Coliform count

25 g samples of 'Sambal belacan' were added with 225 ml of 0.1% buffered peptone water before homogenizing using a stomacher. The original sample had a dilution factor of 10^{-1}. Then, 1.0 ml of dilution 10^{-1} was transferred into a 9.0 ml universal bottle containing 0.1% buffered peptone water for dilution 10^{-2} and serial dilution was continued until 10^{-5} dilutions. Duplicate Petri dishes were labeled at each dilution with 10^{-1}, 10^{-2}, until 10^{-5} dilutions. 0.1 ml of inoculum was pipetted onto a Mac Conkey agar (Merck, Germany) using spread plate method of each dilution. Each dilution was done in duplicate. Plates were inverted to prevent condensation that can cause contamination.

Then, the plates were incubated at 37°C for 24 - 48 hours. After 48 hours, plates were examined and numbers of colonies were calculated according to the rules applicable for colony counting.

3.2.8.9 Detection of *Salmonella spp*

25 g samples of 'Sambal belacan' were added with 225 ml of lactose broth in stomacher bag. Then, after the samples were homogenated well with the broth using stomacher, they were incubated for 24 hours at 37°C. After incubation, stomacher bag that containing samples and lactose broth were shake necessarily. 1 ml of the incubated pre-enrichment was transferred into 10 ml of tetrathionate (TTH) broth and 0.1 ml of incubated pre-enrichment was transferred into 10 ml of Rappaport-Vassiliadis (RV) broth. Next, both of them were incubated 24 hours at 37°C. After incubation, a loopful of TT broth was streaked onto Xylose Lysine Dextrose (XLD) agar, Hektoen Enteric (HE) agar and Bismuth Sulfite Agar (BSA) plates. The same techniques were repeated using RV broth. All plates were incubated for 24 ± 2 hours at 37°C. Then, five typical colonies from each plate were chosen and incubated on TSI and LIA slants at 24 ± 2 hours at 37°C. Cap tubes were loosen to maintain aerobic conditions while incubating slants to prevent excessive H_2S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI. As the result in LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. The distinct yellow in butt of tubes as acidic (negative) reaction was considered. The culture that produced discolouration in butt of tube solely on this basis was not

eliminated. Most *Salmonella* cultures produced H_2S in LIA. Some non-*Salmonella* cultures produce a brick-red reaction in LIA slants.

For other steps, five typical colonies that presented on the BSA after the 24 hours incubation were taken but the incubations of BSA were continued for another 24 hours if there were not having any colonies growth. After 48 hours incubation, at least five typical colonies were taken from BSA and if colonies were picked from the BS agar plates incubated for 24 hours give non-like *Salmonella* reactions in TSI and LIA slants, the culture were being discarded as not being *Salmonella*.

3.2.8.10 Gram staining

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by colouring these cells red or violet. Gram-positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retain the crystal violet of these cells in the cells. Alternatively, Gram-negative bacteria stain red, which are attributed to a thinner peptidoglycan wall, which does not retain the color of crystal violet during discoloring. (Bruckner, 2008).

A colony was heat fixed to form a smear than it was stained with crystal violet dye for 30 seconds and rinsed then with tap water. Next, iodine was added to form complex between crystal violet and iodine for one minute. Then, that slide was rinsed with tap

water again. After that, the smears were decolourized using ethanol or acetone and allowed than to react for 10 - 30 seconds and rinsed with water again. Finally, the smears were coloured with safranin for I minute before rinsed with water. Then the slide was observed under microscope to determine whether the presence of bacterium was Gram-positive and negative.

3.2.9 Shelf-life Analysis

Experimentation for shelf-life of 'Sambal belacan' was done continuously until six week of storage. Two microbiological tests were used to determine the shelf-life of samples; Aerobic plate count (APC) and yeast and mould count. The measurement of physical properties was carried out together with the storage conditions of 'Sambal belacan'.

3.2.10 Statistical analysis

Minitab-16 was used for the interpretation of physical properties, microbiological qualities and shelf-life of 'Sambal belacan' that used different storage conditions. The calculated significant difference values was compared using Tukey test from One-way ANOVA test result with the level of significant differences was defined at $P<0.05$.

T-test (for independent test) was also used to differentiate significant difference between two samples that treated with different CO_2 concentration.

Microsoft Excel 2007 was used to enter the statistical result from Minitab-16 to obtain the graph for each analysis. Microsoft Excel 2007 helped the statistical analysis of generation time of shelf-life analysis by linear regression of R-squared formula that using scattered chart.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Preparation of 'Sambal belacan'

In preparation of 'Sambal belacan', there were few steps must be taken in order to produce the product that was safe for consumption and has a prolonged shelf life. Firstly, all the equipments and the table used must be cleaned and sanitized. This step was very important because it reduced the number of microorganisms present and possibility of cross-contamination can be avoided. Then, chilies and kalamansi fruit were washed properly under running water in order to remove physical and microbiological hazards such as undesirable dirt and soils. After the stalks of the chilies were removed and drained for 10 minutes, they were blanched for 1 minute in hot water at 100°C. According to Susan and Gavranich (1994), the purpose of water blanching is to destroy the peroxide enzyme and the entire microorganisms on the surface of vegetables.

The preliminary for microbial load in prepared 'Sambal belacan' was done using APC. The roasted 'belacan' was used in this study. Shrimp paste was roasted at 180°C for 15 minutes in order to reduce the microbial load to safe level. Besides, this process had led the shrimp paste become dried. This step is crucial for flavour development of the

'belacan', which was resulted in a flavourful of 'Sambal belacan' (Nadia et al, 2010). According to Faridah (2009), microbial count for roasted 'belacan' in her study was less than 2.0 x 10^2 CFU/g whereas for unroasted 'belacan' was 2.0 x 10^3 CFU/g.

Figure 4.1: 'Sambal belacan' prepared for experiments

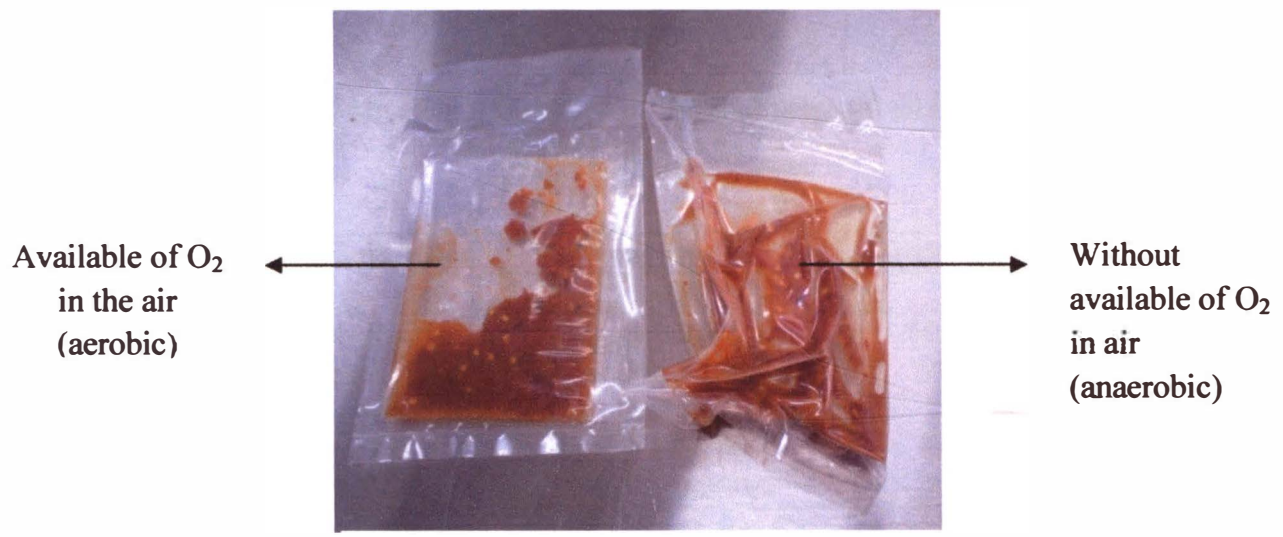
Finally, all ingredients were mixed well and blended together for 45 seconds at speed 1, using food processor (Panasonic, Malaysia). The yield of blending ingredients was weighed to 60 gram for microbial analyses and shelf life studies and 30 gram for physical analyses (Refer Appendix A).

4.2 Storage condition of 'Sambal belacan'

'Sambal belacan' was stored according to two main factors, which under availability of oxygen in the air and relative humidity (RH). For each factor, there were four different conditions of storage were applied. For storage the effect of available O_2, 'Sambal belacan' was kept under aerobic condition and anaerobic condition, and both of them were stored at two different temperatures, chiller (5 ± 2°C) and ambient (25 ± 2°C). Another factor, effect of RH on 'Sambal belacan', there were two conditions of RH; low

RH and high RH. Both of them were also stored at two different temperatures which were chiller (5 ± 2°C) and ambient (25 ± 2°C). Desiccator is a possible solution to control humidity in packaging (Mahajan et al, 2008). So, in this case silica gel was used in order to control the humidity in the packaging of 'Sambal belacan'.

a) RH factor



b) O_2 factor

Figure 4.2: Figure (a) shows that 'Sambal belacan' samples were kept in glass bottle for RH factor determination using glass bottle and Figure (b) shows that 'Sambal belacan' samples were kept in polypropylene (PP) pouch for oxygen determination

4.4 Physical Properties of 'Sambal Belacan'

There were eight samples were tested for physical analyses that obtain from six weeks of samples storage. The ability of microorganisms to grow and survive in 'Sambal belacan' influence by on several factors such acidity (pH), availability of oxygen in air and relative humidity that can lead to quality changes of 'Sambal belacan'.

Table 4.1 (a): Results of physical properties of 'Sambal belacan' as affected by the available of oxygen in the air

Temperature (°C)	Storage condition	Physical analysis for available of Oxygen in the air	
		pH	CO_2
Chiller (5± 2)	Anaerobic	4.5093 ± 0.2078[a]	-
	Aerobic	4.4307 ± 0.2189[a]	1.307 ± 1.307
Ambient (25±2)	Anaerobic	4.4747 ± 0.2246[a]	
	Aerobic	4.4040 ± 0.1933[a]	31.8 ± 22.4

Values were mean ± SD of three replicates storage for 6 weeks, values followed by different letters in same column differs significantly ($P < 0.05$)

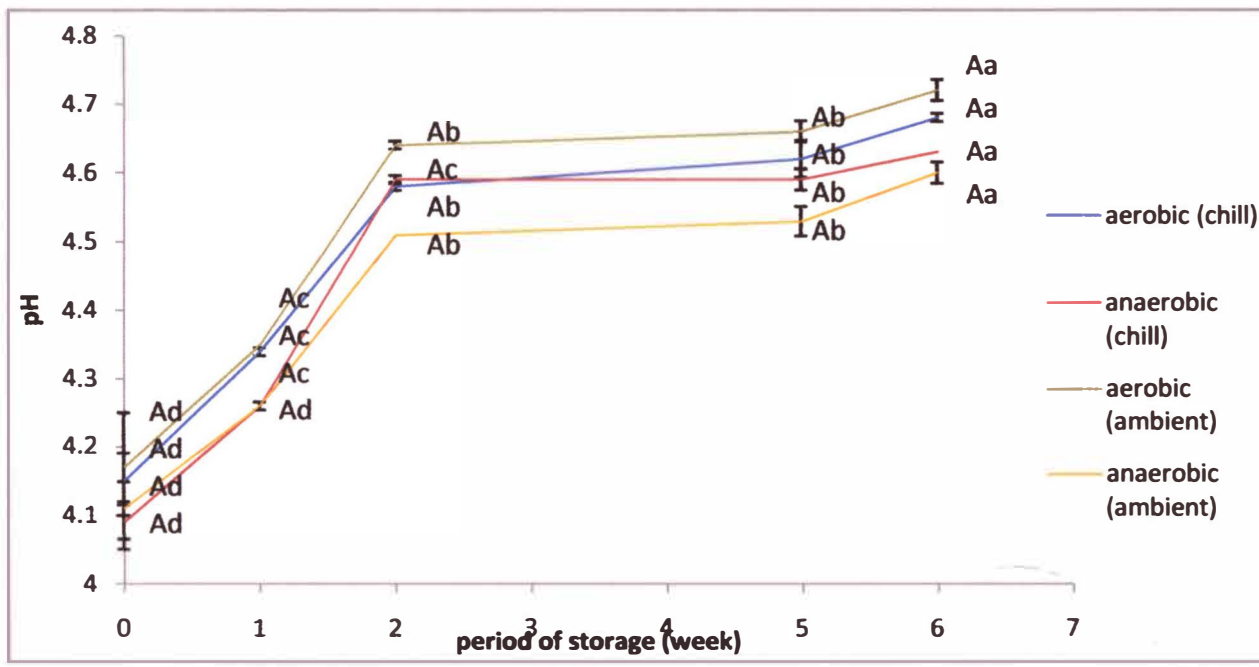
Table 4.1 (b): Results of physical properties of 'Sambal belacan' as affected by relative humidity factor

Temperature (°C)	Storage condition	Physical analysis for relative humidity factor	
		pH	RH (%)
Chiller (5± 2)	Low RH	4.4 ± 0.1693[a]	69.000 ± 10.406[ab]
	High RH	4.367 ± 0.1834[a]	69.800 ± 9.002[ab]
Ambient (25±2)	Low RH	4.462 ± 0.1667[a]	64.533 ± 9.164[b]
	High RH	4.511 ± 0.2698[a]	76.667 ± 5.827[a]

Values were mean ± SD of three replicates storage for 6 weeks with 5 interval, values followed by different letters in same column differs significantly ($P < 0.05$)

4.4.1 pH of 'Sambal belacan' as affected by oxygen and relative humidity

The pH of 'Sambal belacan' samples were monitored in this study. The results of pH measurement for both factors were showed in Table 4.1 (a) and (b). From that table, there were no significant difference (P>0.05) among the samples. No significant difference was detected on pH measurement as affected by both factors since similar organic acid, kalamansi juice were used in preparation of samples. Furthermore Figure 4.3 and Figure 4.4 show that there were significant difference (P<0.05) of pH measurement during the six week storage of 'Sambal belacan' for both factors. Both of figures showed that there were small increases of pH but still below pH 5.0. Study by Adams and Nicolaides (1997) showed that bacteria growth generally occurs optimally at pH values in the range pH 6 to 7.



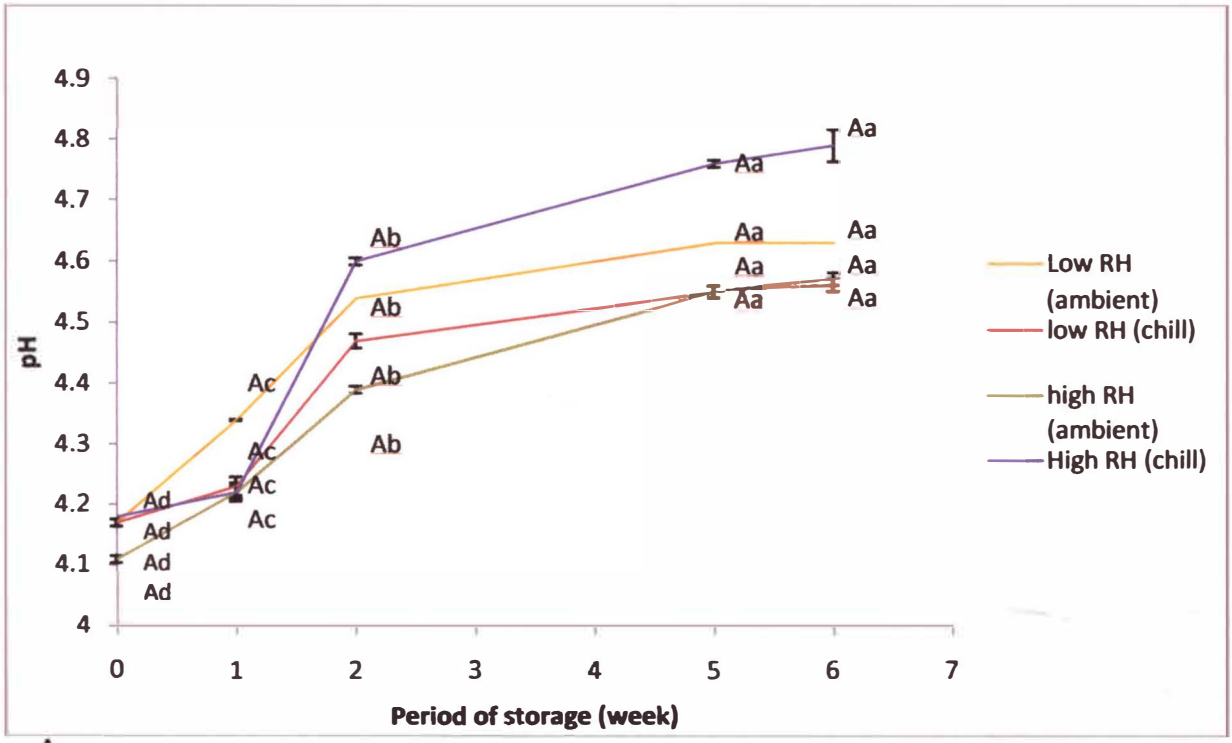
A mean values with different capital letters showed significant difference between samples (P<0.05)

a-c mean values with different capital letters showed significant difference between samples (P<0.05)

Figure 4.3: pH measurement of 'Sambal belacan' during six weeks storage as affected by availability of oxygen in the air

pH usually interacts with other parameters in the food to inhibit the growth of microbes. Because of that, there were no rapid growth of microbes in of the samples and the samples where safe to be consumed until several weeks. The pH can interact with factors such as water activity (a_w), salt, temperature, redox potential, and preservatives to inhibit the growth of pathogens and other organisms (USFDA, 2001). The pH of the food was also significantly influenced the lethality of heat treatment of the food. Less heat is needed to inactivate microbes when the pH is reduced (Mossel et al., 1995).

Organic acid is effective in order to lower the pH of the food. However, according to Debevere (1988), pathogenic bacteria still can survive and even grow in acidic medium of food.



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.4: pH measurement of 'Sambal belacan' during six weeks storage as affected RH factor

According to Holcroft and Kader (1999), high CO_2 concentrations could affect pH values due to the changing of organic acid metabolism and buffering capacity. The high pH will allow a range of Gram-negative bacteria to grow, but spoilage is specifically caused by organisms capable of degrading the vegetable polymer, pectin (Liao, 1989; Liao et al., 1997). Increasing the preservation by a decreasing the pH (below 5), increasing the NaCl concentration (above 6%) and adding sorbate and or benzoate can eliminate the Gram-negative microflora (Gram et al, 2002).

4.4.2 Colour measurement of 'Sambal belacan' as affected by oxygen and relative humidity

Changes of colour in 'Sambal belacan' with different storage conditions for both factors; availability of oxygen in air and relative humidity were compared in Table 4. 2 (a) and (b),

Table 4.2 (a): Colour measurement of 'Sambal belacan' as affected by the availability of O_2 in the air

Temperature (°C)	Storage condition	color measurement for availability of O_2 in the air				
		*L**	*a**	*b**	*H°*	*C°*
Chiller (5 ± 2)	Anaerobic	47.71 ± 3.63^{a}	16.01± 3.04^{a}	14.87± 4.77^{a}	38.66± 7.35^{b}	23.52± 4.85^{a}
	Aerobic	46.11± 3.83^{bc}	16.01± 3.05^{ab}	16.26± 5.68^{a}	44.39± 7.06^{ab}	22.98± 5.81^{a}
Ambient (25 ± 2)	Anaerobic	40.7± 2.82^{ab}	16.76± 2.97^{a}	18.91± 3.55^{a}	48.27± 8.16^{a}	25.3± 3.05^{a}
	Aerobic	43.31± 4.25^{c}	14.02± 1.52^{b}	17.55± 5.09^{a}	50.21± 8.46^{a}	22.68± 4.18^{a}

Values were mean ± SD of three replicates storage for 6 weeks, values followed by difference letters in same column differed significantly ($P < 0.05$)

From Table 4.2 (a), there were no significant difference (P> 0.05) on b^* and $C°$ values but from the Table 4.2 (b), there were significant difference detected for a^* and $C°$ values. L^* value indicated the lightness, a^* indicated the intensity of red colour and b^* described the yellowness. $H°$ referred to the degree which a stimulus can be described as similar to or different from stimuli that were described as red, green, blue, and yellow while $C°$ described how much the colour differ from gray. Excessive accumulation of CO_2 (12 kPa) inside the food package can also prevent the opening of the cap, but resulted in severe browning (Nichols and Hammond, 1973; Lopez-Briones et al., 1992).

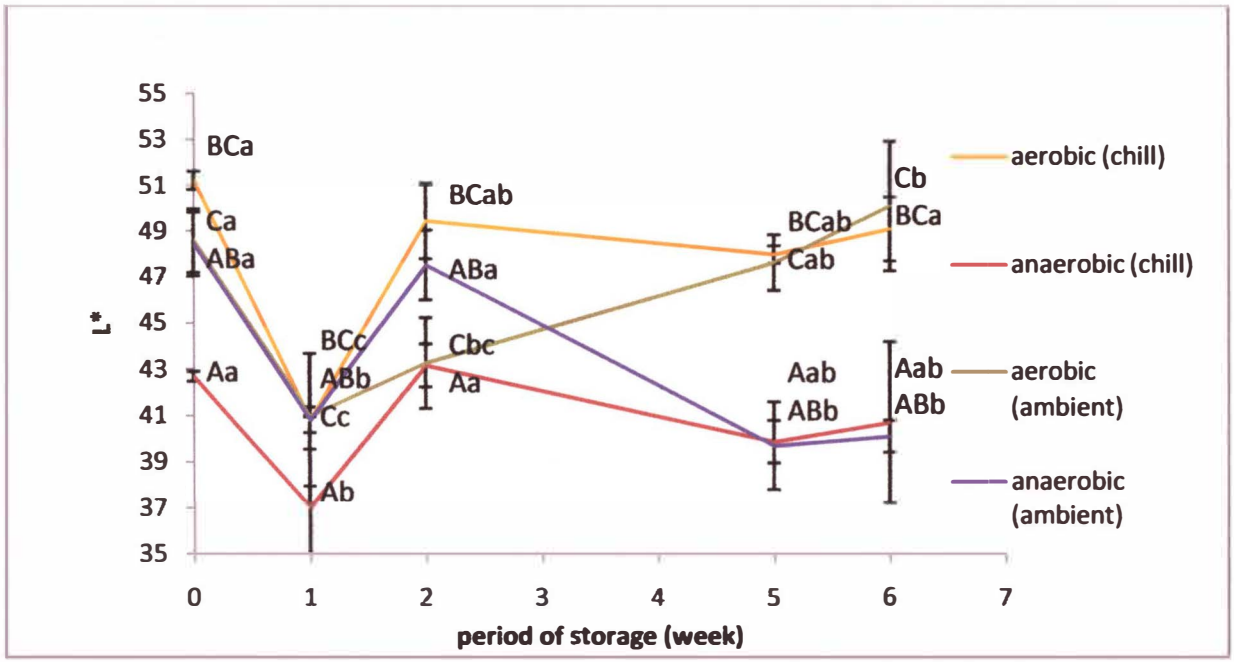
Table 4.2 (b): Colour measurement of 'Sambal belacan' as affected by RH factor

Temperature (°C)	Storage condition	Colour measurement for RH factor				
		L^*	a^*	b^*	$H°$	$C°$
Chiller (5 ± 2)	Low RH	39.11 ±1.93[a]	18.62 ± 2.86[b]	27.74 ± 6.13[a]	55.58 ± 4.93[a]	33.51 ± 6.17[ab]
	High RH	39.22 ± 3.734[a]	19.06 ± 1.71[a]	25.88 ± 5.9[a]	52.64 ± 7.74[a]	32.36 ± 4.69[b]
Ambient (25 ± 2)	Low RH	40.03 ± 3.44[a]	23.9 ± 2.83[a]	29.41 ± 8.39[a]	49.57 ± 8.44[a]	38.22 ± 7.23[a]
	High RH	40.03 ± 3.61[a]	21.89 ± 1.61[b]	28.74 ± 6.79[a]	51.69 ± 8.17[a]	36.42 ± 5.16[ab]

Values were mean ± SD of three replicates storage for 6 weeks, values followed by different letters in same column differs significantly ($P < 0.05$)

Figure 4.5 and Figure 4.6 show the lightness *(L*)* of the 'Sambal belacan' which stored at oxygen and RH factor. 'Sambal belacan' which had stored at different RH showed the low values of L^*. It means that 'Sambal belacan' treated under RH conditions had darker colour compared to those 'Sambal belacan' treated with different availability of oxygen in air. After six weeks storage, there were significant difference (P<0.05)

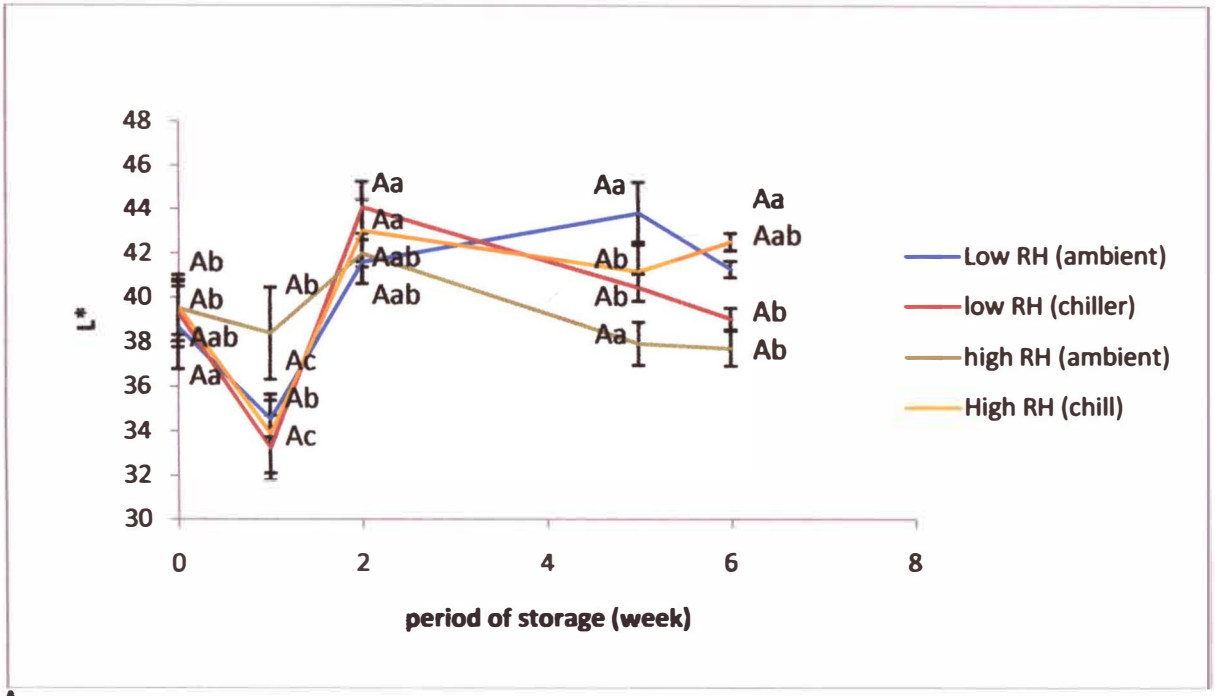
among the samples. From the result, 'Sambal belacan' which stored at high RH at ambient temperature showed the darker colour compare to all samples. Visual observation from the sample that high RH at ambient temperature also contained grey sliminess which showed the possibility of spoilage microorganisms confirmed by the highest count of APC and yeast and mould. According to Gram et al (2002) microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth such sliminess and white spot in foods. The formation of sliminess might become the factor which lower the L^* of the 'Sambal belacan'.



[A] mean values with different capital letters showed significant difference between samples ($p<0.05$)

[a-c] mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.5: Lightness (L^*) of 'Sambal belacan' during storage as affected the availability of O_2 in the air



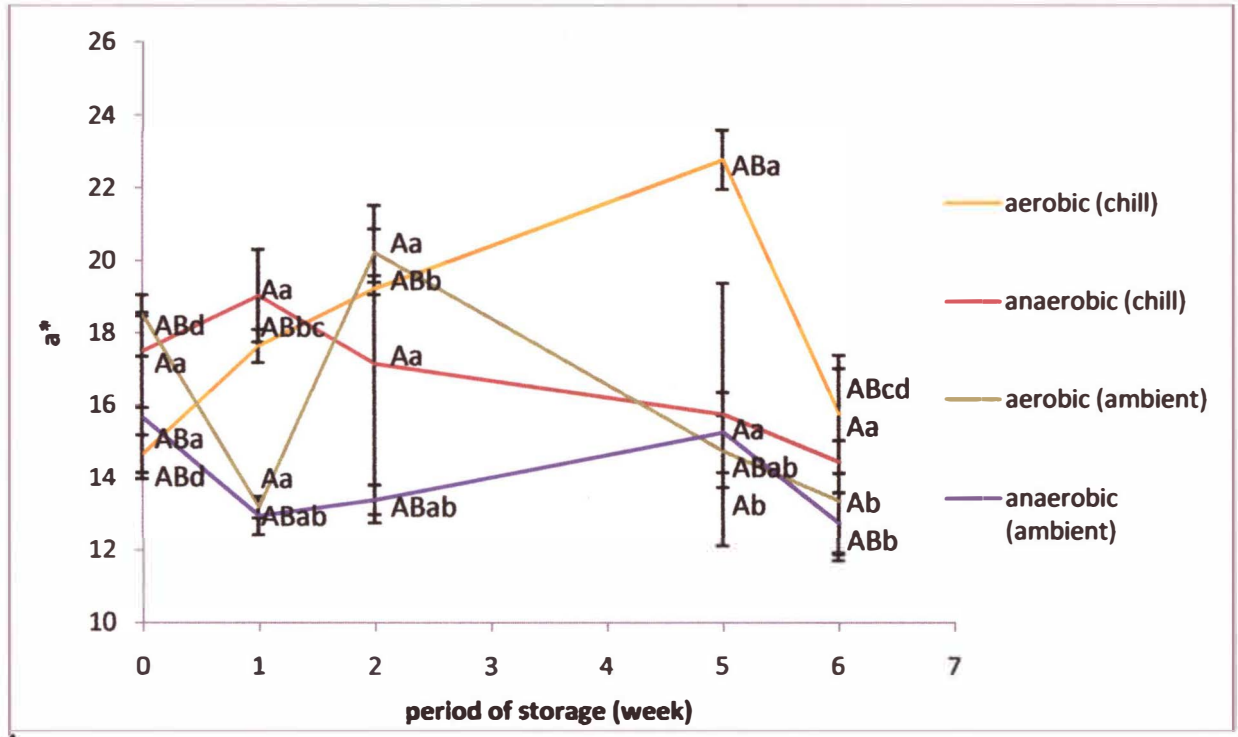
A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.6: Lightness (*L**) of 'Sambal belacan' during storage as affected by RH factor

*a** value described the red intensity of 'Sambal belacan'. Figure 4.7 and Figure 4.8 show the intensity of red colour was higher in 'Sambal belacan' that assessed by RH factor compared to 'Sambal belacan' treated by different availability of oxygen during storage conditions. There were no significant difference ($P>0.05$) was detected in O_2 factor while there was having significant difference ($P<0.05$) between the samples of 'Sambal belacan' during six weeks storage. 'Sambal belacan' that stored at chilled temperature for high and low RH were showed the consistent decreasing of *a** value due to increasing week of storage. The *a** value of 'Sambal belacan' that treated under different availability of O_2 showed the inconsistent decreasing but there significant different ($P<0.05$) among samples from first week to sixth weeks. The lowest *a** value for

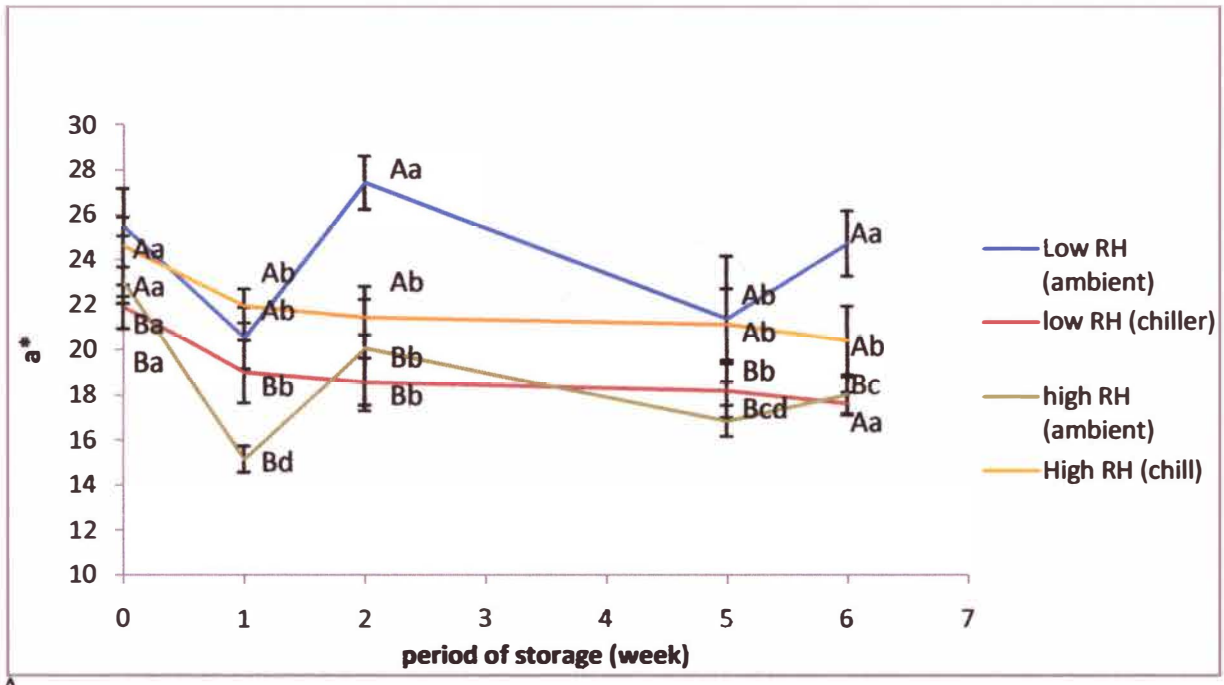
both 'Sambal belacan' showed 'Sambal belacan' without presence of O_2 in the air (anaerobic) at ambient showed the lowest intensity of red colour compared to 'Sambal belacan' which stored with high RH at ambient temperature.



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.7: *a** of 'Sambal belacan' during storage as affected by availability of O_2 in the air

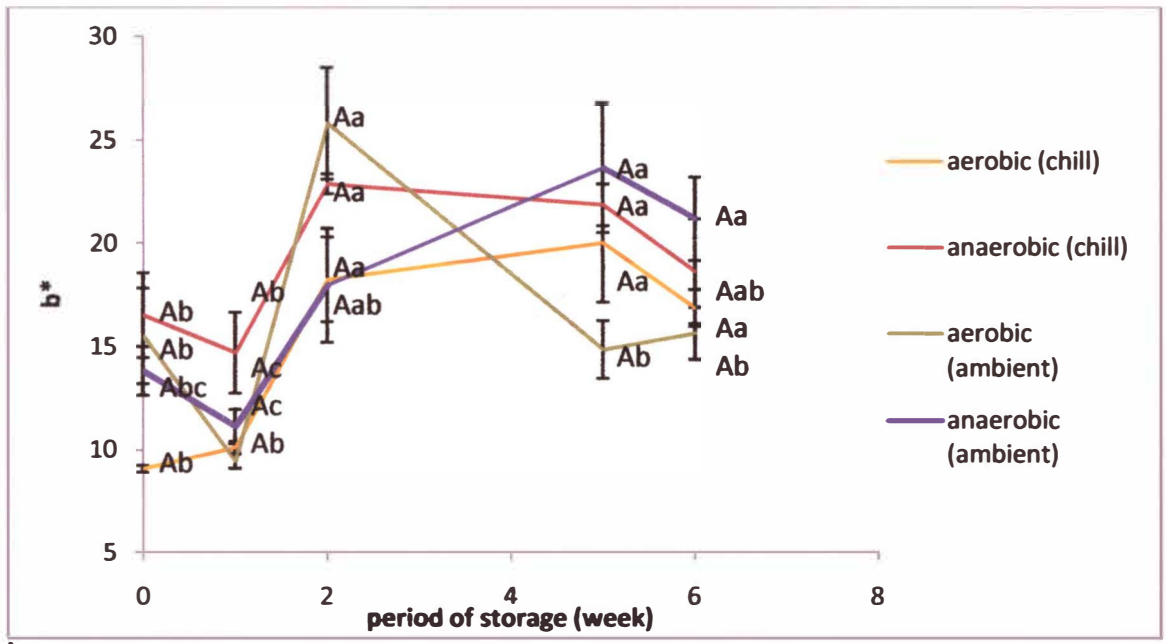


A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.8: *a** of 'Sambal belacan' during storage as affected by RH factor

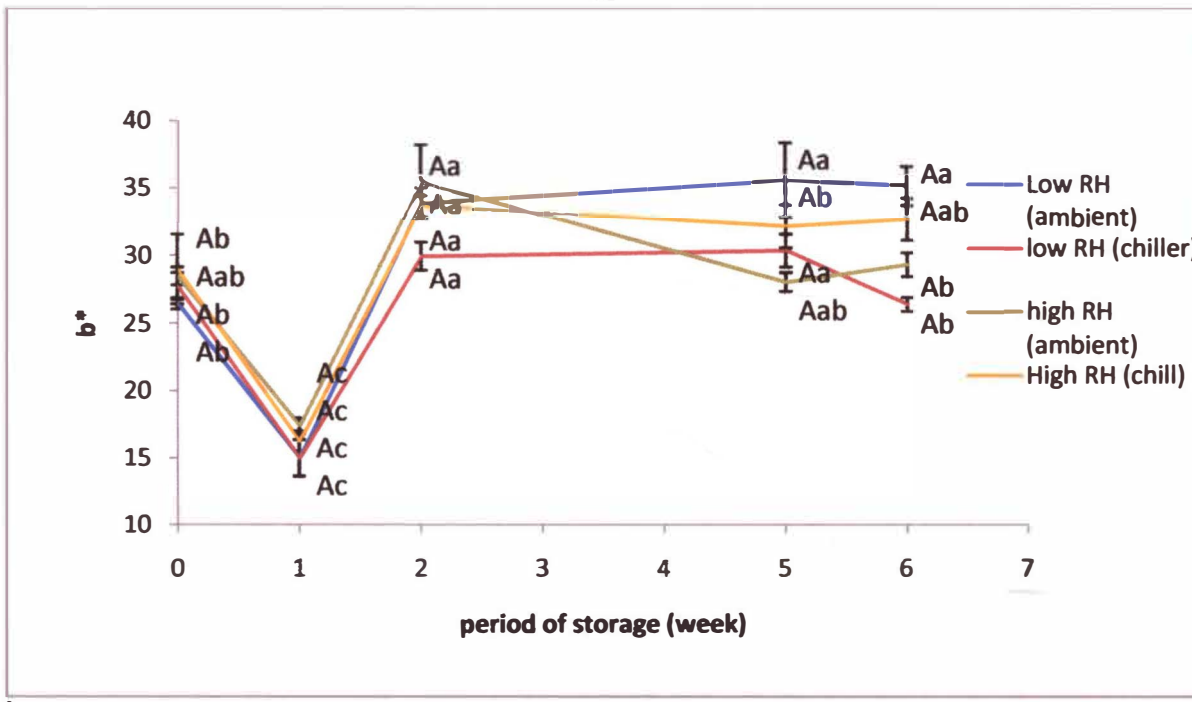
Figure 4.9 and Figure 4.10 showed the *b** value changes during six weeks storage assessed by two different factor; availability of O_2 in the air and RH factor. The *b** values indicated the yellowness of the samples during storages. The yellowness of the 'Sambal belacan' was higher when assessed by RH conditions compared the samples of 'Sambal belacan' treated with different availability of O_2 in the air. There were no significant different ($P>0.05$) detected for all the samples of 'Sambal belacan' at different storage conditions assessed by different factors. The inconsistent increasing of *b**values due to increasing weeks were detected for O_2 factor but for the RH factor, the increasing of *b** value was more stable.



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.9: b^* of 'Sambal belacan' during storage affected by the availability of O_2 in the air

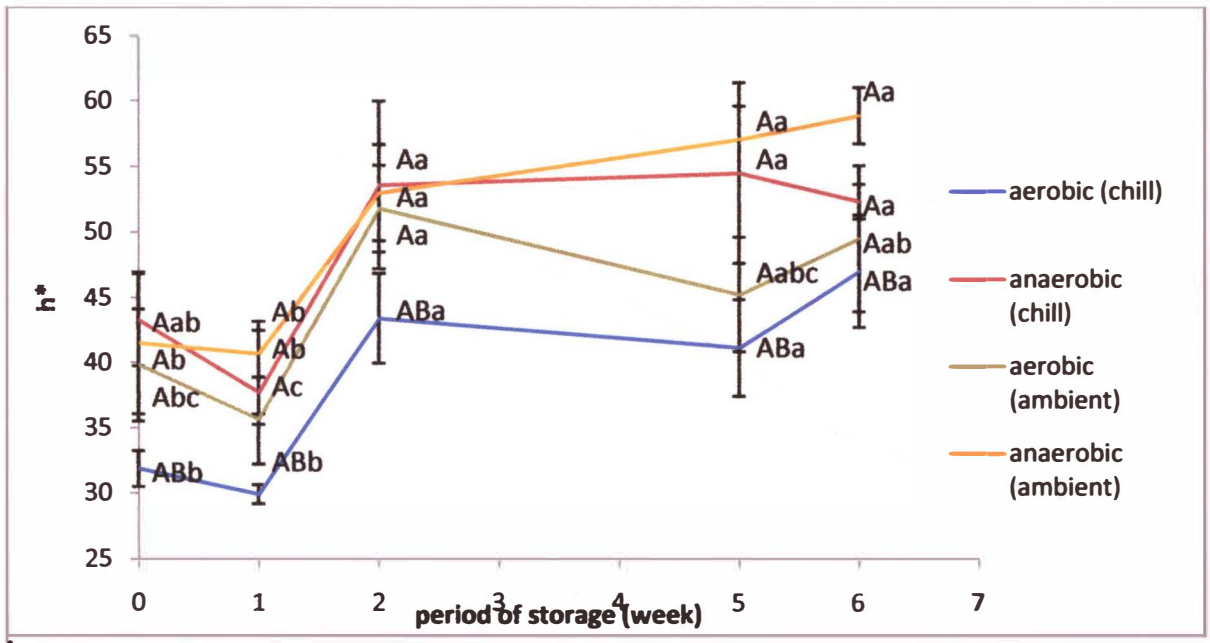


A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.10: b^* of 'Sambal belacan' during storage affected by RH factor

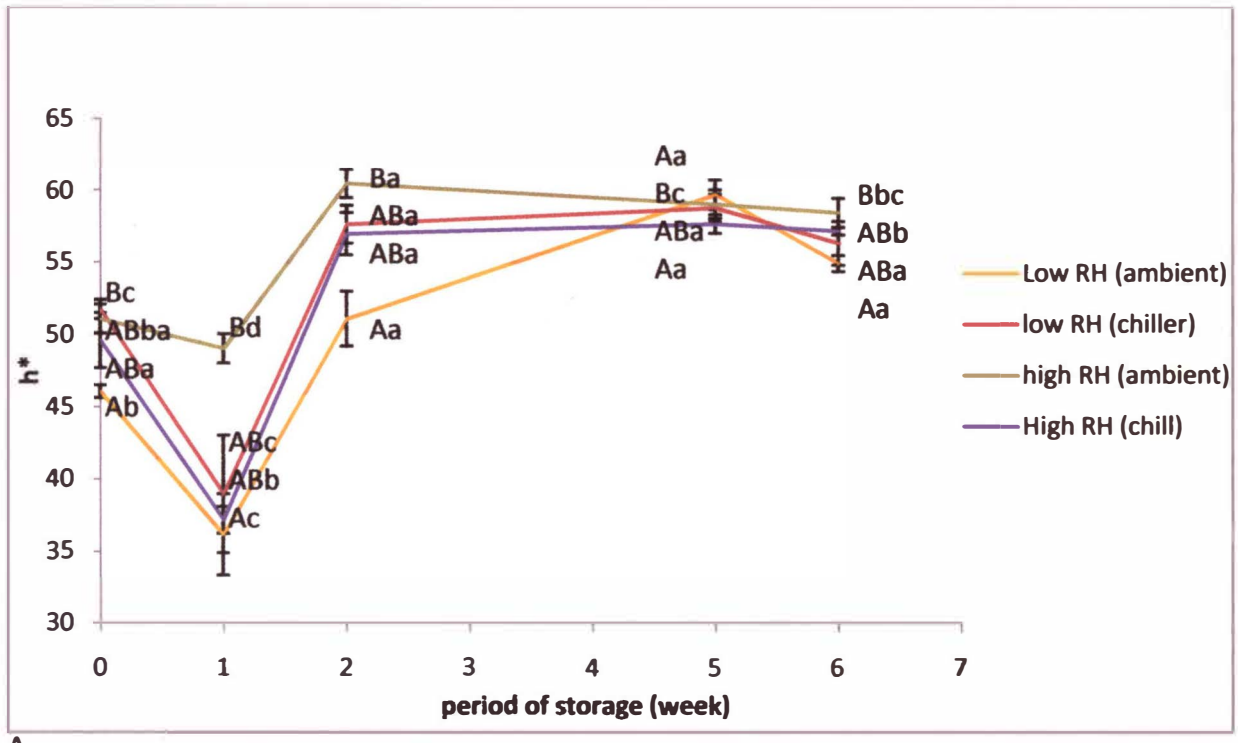
Hue *(H°)* values indicated stimulus can be described as similar or different from stimuli that are described as red, green, blue, and yellow or described the browning index of the samples. Figure 4.11 and Figure 4.12 show the *H°* values for 'Sambal belacan' during six weeks of storage were affected by availability of O_2 in the air and RH factors. Figure 4.11 shows *H°* value was affected by availability of O_2 in the air, 'Sambal belacan' in anaerobic condition at ambient temperature having significant difference ($P<0.05$) from the other samples of 'Sambal belacan'. The significant difference ($P<0.05$) was also detected for 'Sambal belacan' at this storage condition due to increasing weeks of storage. Figure 4.12 shows the *H°* value for 'Sambal belacan' for RH factor. There were no significant different ($P>0.05$) was detected among the samples due to different storage conditions for RH factor. The increasing of *H°* values due to the different period of storages was similar among the samples.



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.11: Hue *(H°)* of 'Sambal belacan' during storage affected by the availability of O_2 in the air



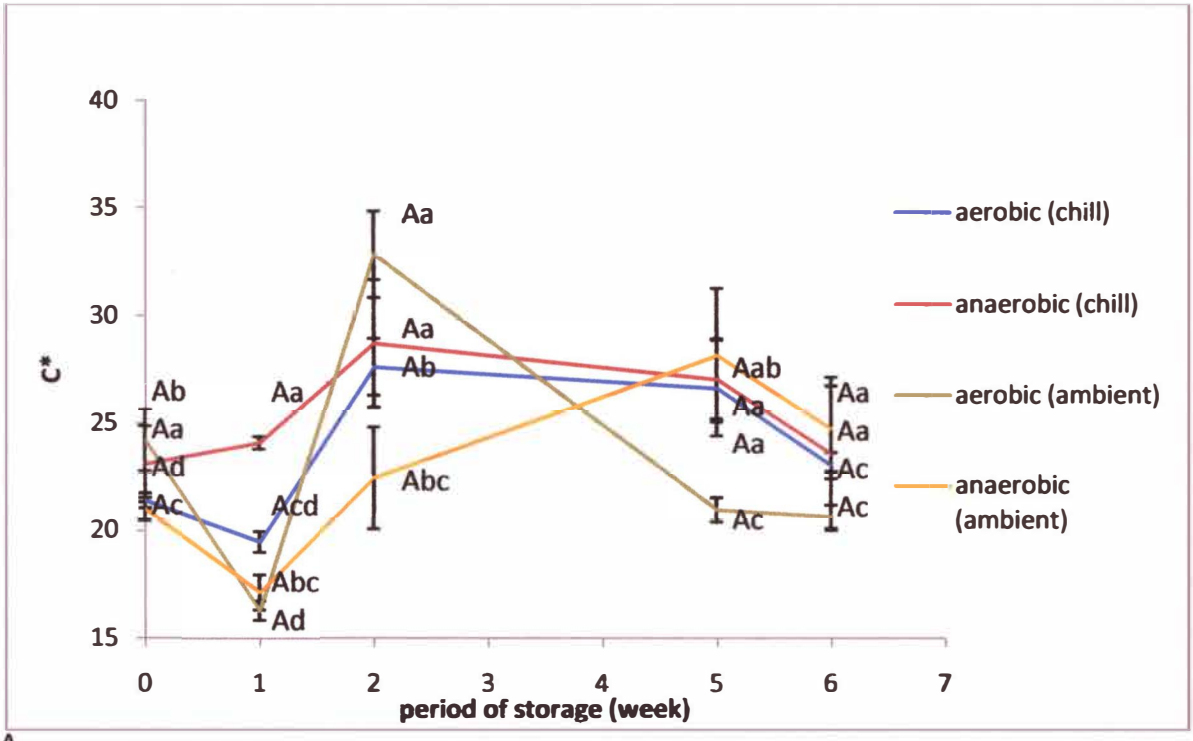
A mean values with different capital letters showed significant difference between samples (p<0.05)

a-c mean values with different capital letters showed significant difference between samples (p<0.05)

Figure 4.12: Hue (H°) of 'Sambal belacan' during storage affected by RH factor

Figure 4.13 and Figure 4.14 show the changes of chroma *(C°)* value during six weeks storage of 'Sambal belacan'. *C°* is indicated how much the colour differ with gray. There were no significant difference (P>0.05) were detected among the samples at different storage conditions as affected by the availability of O_2 in the air. The *C°* value was higher for RH factor compared to the availability of O_2 in the air factor. There were no significant different (P>0.05) showed for 'Sambal belacan' in anaerobic condition at chilled temperature following the increasing period of storage. This result indicated that there were no changes of *C°* values from 0 weeks to six week of storage. Besides, for other type of storage affected by the availability of O_2 in the air, the significant difference (P<0.05) were detected among the samples due to increasing weeks. Figure 4.14 shows there was significant different (P>0.05) detected for *C°* value affected by RH factor

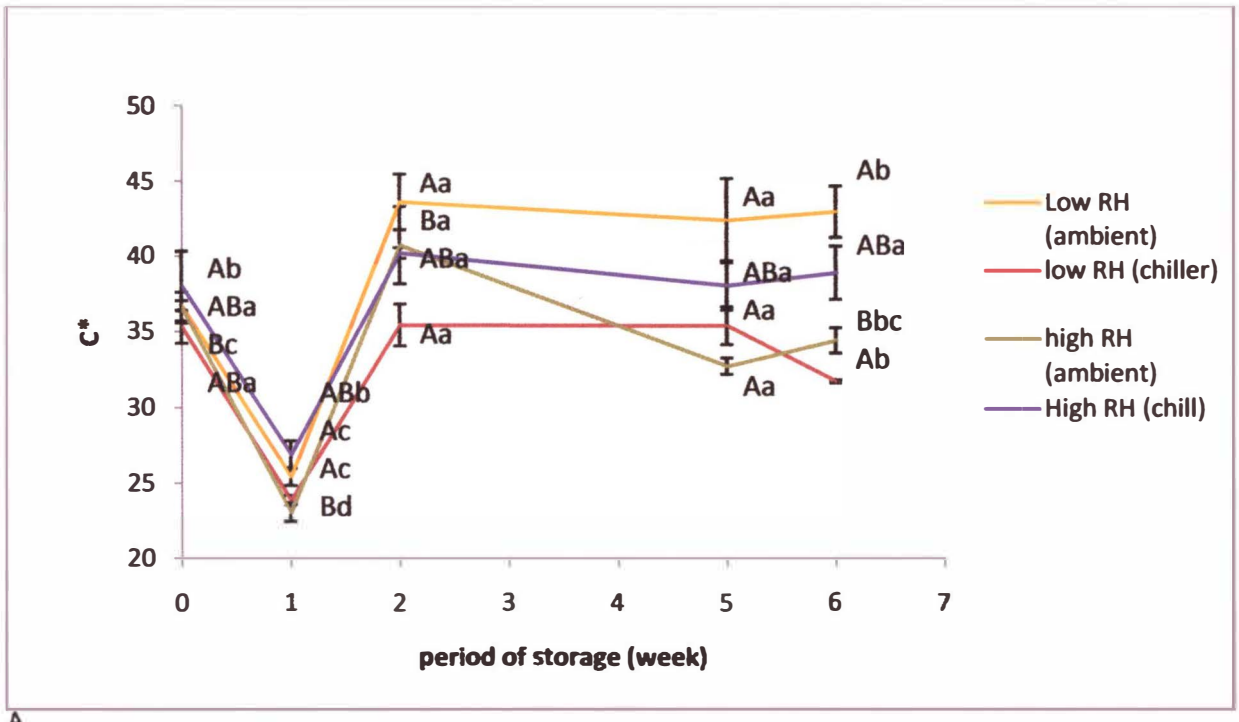
during 6 weeks storage in different conditions. 'Sambal belacan' with high RH that stored at ambient temperature was significantly different (P>0.05) with other storage conditions. There were small changes of $C°$ values detected for both factors but the $C°$ values for RH factor showed higher value compared to the availability of O_2 in the air factors.



A mean values with different capital letters showed significant difference between samples (p<0.05)

a-c mean values with different capital letters showed significant difference between samples (p<0.05)

Figure 4.13: Chroma *(C°)* of 'Sambal belacan' during storage affected by the availability of O_2 in the air



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.14: Chroma *(C°)* of 'Sambal belacan' during storage as affected by RH factor

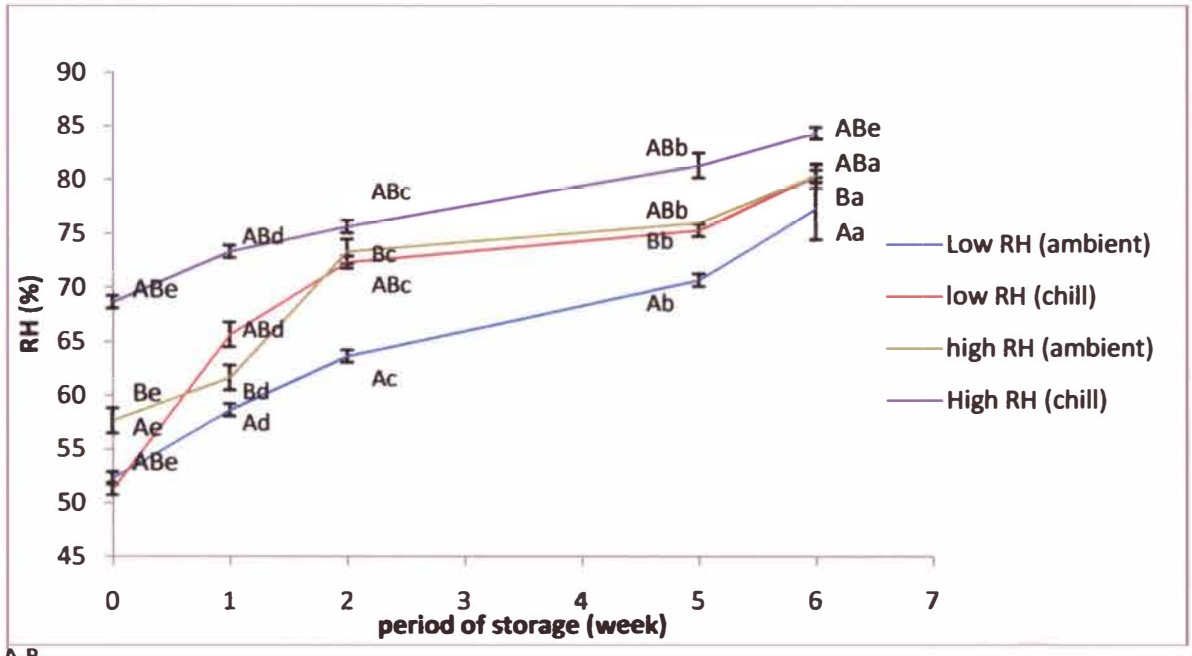
Overall from the colour measurement results, L^*, a^*, b^*, $H°$ and $C°$ values for RH factor were higher compared the O_2 factors although there were made from similar formulation. It showed that relative humidity conditions serve the good medium for the growth of spoilage and pathogenic microbial.

4.4.3 RH measurements during shelf life studies of 'Sambal belacan'

The changes of relative humidity, RH values are showed in Table 4.1 (a) in the section 4.4. After 6 weeks of storage, the results showed that 'Sambal belacan' stored at ambient temperature; low and high RH were significant different ($P<0.05$). From the result, 'Sambal belacan' with high RH at ambient temperature had higher values of RH

which was 76.667 ± 5.827 %. According to Paull (1999), high RH will not prevent moisture loss if the product temperature is not near the air temperature. Besides that, high RH will increase the growth of yeast and mould. It can be seen in Figure 4.15. It showed that the growth of yeast and mould was increased due to the increase of RH (%). The growth of the yeast and mould in 'Sambal belacan' stored in high RH at ambient temperature more rapidly compared to others.

Figure 4.15 shows the changes of RH (%) measurement in package of 'Sambal belacan' for RH factor due to the six weeks of storage. There were significant difference ($P<0.05$) of increasing RH (%) detected during increasing period of storage.



A-B mean values with different capital letters showed significant difference between samples ($p<0.05$)

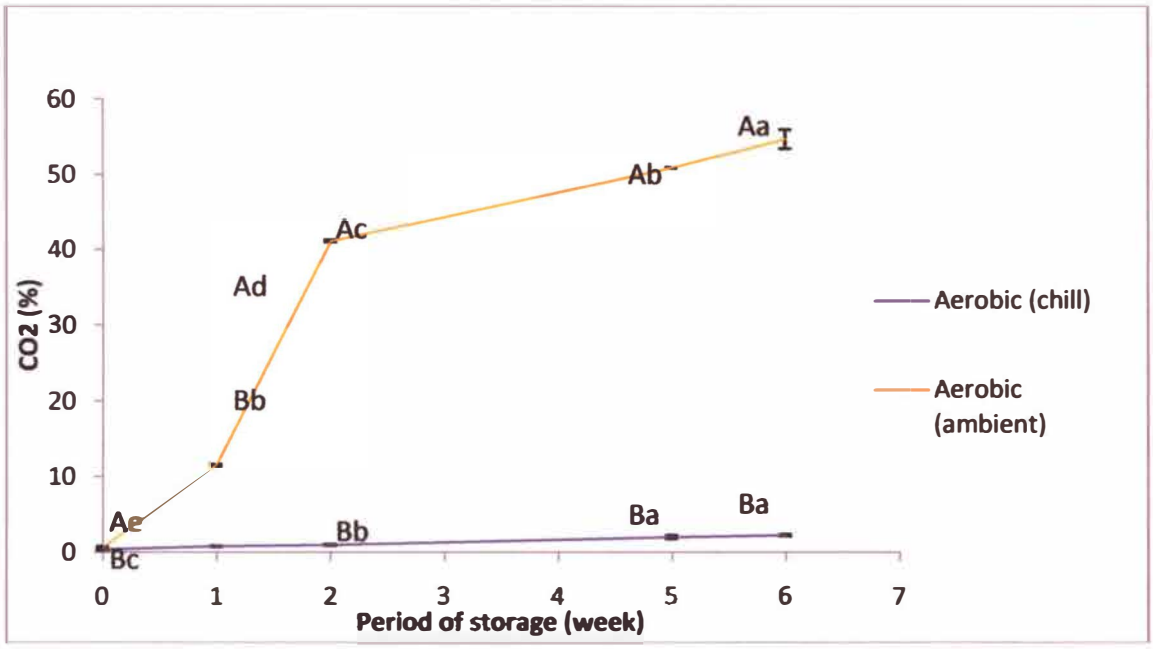
a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.15: RH (%) measurement inside package of 'Sambal belacan' during storage as affected by RH factor

4.4.4 Carbon dioxide measurement during shelf-life studies of 'Sambal belacan'

The results for CO_2 measurement is shown in Figure 4.16. This analysis was chosen due to the availability of the equipment to detect the presence of oxygen inside the packaging. According to Mujica-Paz and Gontard (1997), the natural process of food respiration will reduce O_2 and increase CO_2 in conjunction with adequate gas exchange through a package. From the results, there were significant different ($P<0.05$) among the samples of 'Sambal belacan' affected by availability of O_2 inside the packaging at chilled and ambient temperatures. The difference between both samples may by the temperature of storage condition. The concentration of CO_2 inside the package at chilled temperature of 'Sambal belacan' was 1.307 ± 1.307 % where CO_2 concentration was the lower than 'Sambal belacan' stored at ambient temperature which was 31.8 ± 22.4 % of CO_2. The reason this phenomenon is explained by the study from Mujica-Paz and Gontard, (1997), that temperature was a significant factor in CO_2 permeability and selectivity models and it showed there were interaction between temperature and RH CO_2 for selectivity models.

Figure 4.16 shows the measurement the concentration of CO_2 inside the package for 6-weeks of storage of 'Sambal belacan' with using the available O_2 in the air due to its storage. The increasing of CO_2 concentration was showed significant difference ($P<0.05$) due to increasing period of storage at different temperatures. The mechanism of inhibition is not well understood but it is thought to involve a combination of decreased intracellular pH, inhibition of enzymatic reactions by a mass action effect and interaction with the cell membrane to disrupt solute transport (ICMSF, 1980). This interaction was detected in Figure 4.3, the pH measurement for O_2 at chilled and ambient temperature were lower than the pH measurement for RH factor.



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.16: CO_2 concentration inside the package during 6 weeks storage of 'Sambal belacan'

Since both samples were stored without O_2 or vacuum conditions, it showed this technique was very effective in extending the shelf-life of perishable foods such as meat products (Church and Parsons, 1995).Under these conditions, the oxygen supply will be restricted, the gas phase being determined by the rate of gas permeation through the film and the rate of oxygen consumption in the package. These changes gave a selective effect on the microbial population (Farber, 1991; Labadie, 1999).

4.3 Microbial analyses

There were eight samples from different storage of 'Sambal belacan' undergone microbial analysis for in six weeks. The 'Sambal belacan' were sampled and analyzed for

Aerobic plate count (APC), yeast and mould count, Lactic acid bacteria (LAB), *psychrotrophic* bacteria, *Enterobactriaceae* count, Coliform count, *Escherichia coli* counts and detection of *Salmonella spp.* following the procedure of the United States Department of Agriculture (USDA) (2010).

From the earlier study, Fitri Nurdiyana (2008) on microbial count in 'sambal belacan' had revealed that there were absent of *Lactobacillus* and coliform in 'Sambal belacan'. In different study, there are previous studies that showed the presence of *Salmonella* in 'belacan' and chilies (Steinkraus, 2005; Arumugaswamy et al 1995). In other study done by Faridah (2009), stated that there was no detection of *Salmonella, Lactobacillus*, coliform and *Escherichia coli* in prepared 'Sambal belacan'. When comparing the results studied by Fitri Nurdiyana (2008) and Faridah (2009), this study also showed there was no detection of psychrotrophic bacteria, *E.coli,* coliform and *Salmonella* in 25 gram of samples. This finding was comparable with Faridah (2009). However, results from Fitri Nurdiyana (2008) slightly different. This is because the samples of 'Sambal belacan' were purchased from several premises in Kuala Terengganu. In her study, 'Sambal belacan' was not prepared by hershelf. While the reason for lower microbial load detected by Faridah (2009) was the 'Sambal belacan' prepared in controlled and hygienic condition.

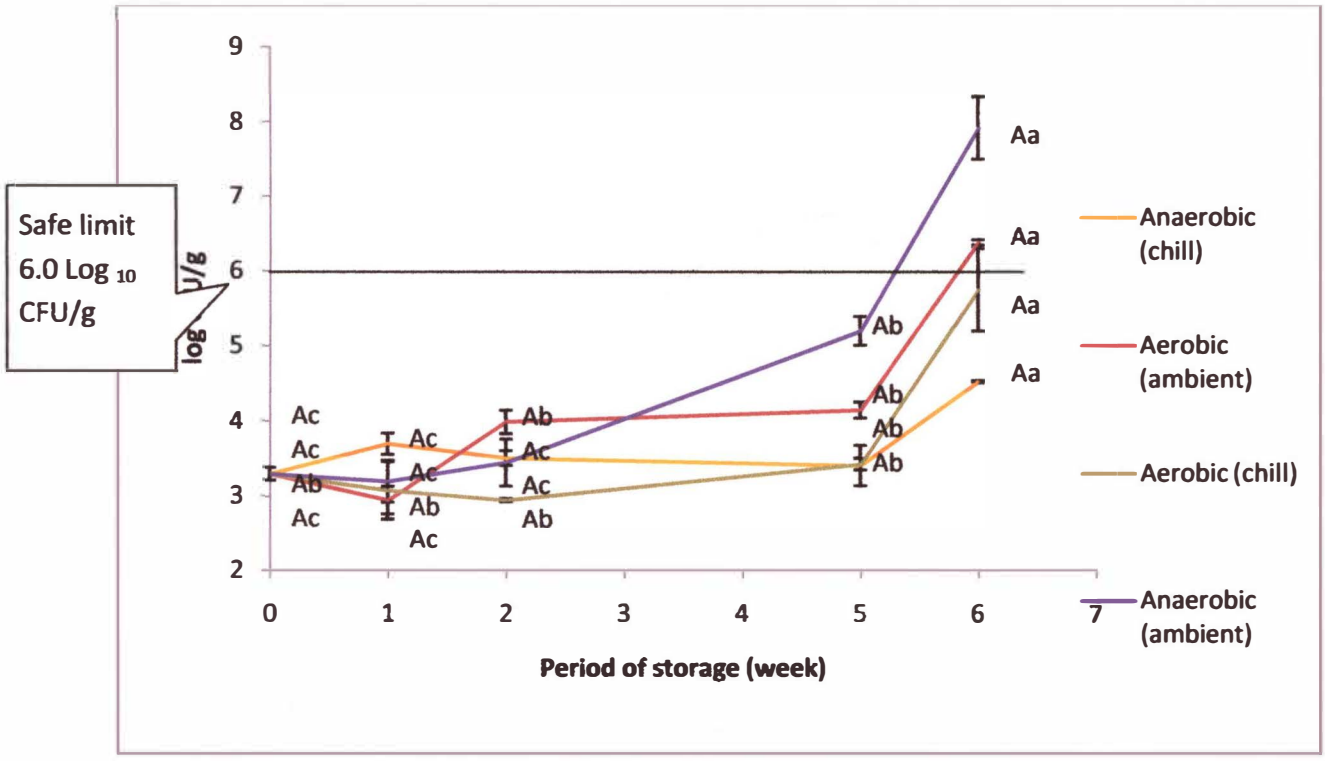
According to the result, 'Sambal belacan' with O_2 and higher RH storage condition that stored at ambient temperature (25 ± 2°C) showed the higher count of microbial load compared to other treatment of storage. It was followed by 'Sambal belacan' which stored in anaerobic condition at ambient temperature (25 ± 2°C), anaerobic condition at chilled temperature (5 ± 2°C) and aerobic condition at chilled

temperature (5 ± 2°C), whereas for RH factor, after high RH with ambient temperature, it was followed by 'Sambal belacan' stored with high RH at chilled temperature (5 ± 2°C), low RH at ambient temperature (25 ± 2°C), and low RH at chilled temperature (5 ± 2°C). When comparing for both factors of storage for 'Sambal belacan', samples of 'Sambal belacan' with high RH which stored at ambient temperature (25 ± 2°C) showed higher APC. The main reason the growth of microbial count was higher in high RH because it can cause moisture condensation in food. In addition, condensation causes moist surfaces, which is conducive to microbial growth and spoilage (Norman and Robert, 2006).

The contamination of 'Sambal belacan' was easily influenced by keeping the samples at prolonged ambient temperature and high relative humidity during point if sale were ideal for multiplication of the vegetative cells that already present the 'Sambal belacan' (Umoh and Odoba, 1999). To avoid the spoilage of microbes become worse, the temperature of storage should be avoided from fluctuation (Umoh and Odoba, 1999). A good sanitation technique of preparation and handling can reduce the growth of *psychrotrophic* bacteria, *E. coli* and coliform bacteria since they were not predominant microbes in this study.

4.3.1 Aerobic plate count (APC)

The APC results from this study showed that all main effects and interactions were not significant difference ($P > 0.05$) among the 24 treatments for the availability of O_2 in the air and 24 treatments for relative humidity factors.



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

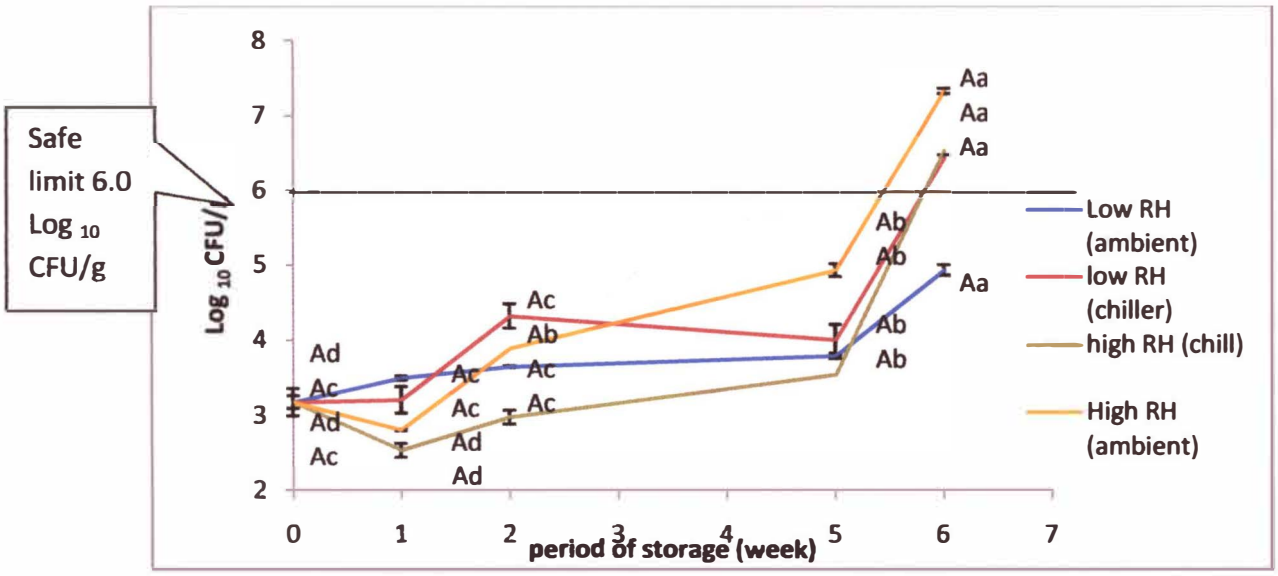
Figure 4.17: Shelf-life of 'Sambal belacan' determined by Aerobic Plate Count as affected by availability of oxygen in the air

According to Food Standards Australia, (2001) the guideline for determining the microbiological quality of APC is cannot be more than 1 x 10^6 CFU/g (6.0 $\log_{10}$ CFU/g). Figures 4.16 and Figure 4.17 show the shelf-life of 'Sambal belacan' for Aerobic plate count (APC) determined by PCA. Both figures show that there were increasing of APC significantly in six weeks storage but between the samples were not shown significant difference at all. 'Sambal belacan' which stored with O_2 at ambient (25 ± 2°C) temperature had higher growth of APC but the growth became the lowest when it was stored at chiller (5 ± 2°C). From the result, it can conclude that storage at chilled temperature may slow the growth microorganisms. Therefore, according to Faridah

(2009), storage at chilled temperature will help to improve the quality of food from spoilage and it may prolong the shelf-life of 'Sambal belacan'. Umoh and Odoba (1999) says, that most of the microorganisms grow much faster if there are availability of O_2, but restricting the presence of O_2 will increase the level of other gases such as carbon dioxide, CO_2. In addition, they also said that by lowering the presence of O_2 supply it may a useful way to preserve the food since many of the normal spoilage organisms will not grow under these conditions.

Figure 4.17 shows after six weeks of storage, 'Sambal belacan' which stored at high RH at ambient temperature (25 ± 2°C) showed the highest growth of microbes compared to the others. Higher growth of microbial spoilage in those samples was contributed by high RH which provides adequate moisture for microbes to grow. When compare the growth of microbes by both factors, microbial load is higher in high RH.

For the result of O_2 factor, the 'Sambal belacan' without O_2 at chilled temperature showed the longest shelf life compared to other samples which only can be consumed until six weeks of storage. While for RH factor, 'Sambal belacan' with low RH at chilled temperature showed the longest shelf life.



[A] mean values with different capital letters showed significant difference between samples (p<0.05)

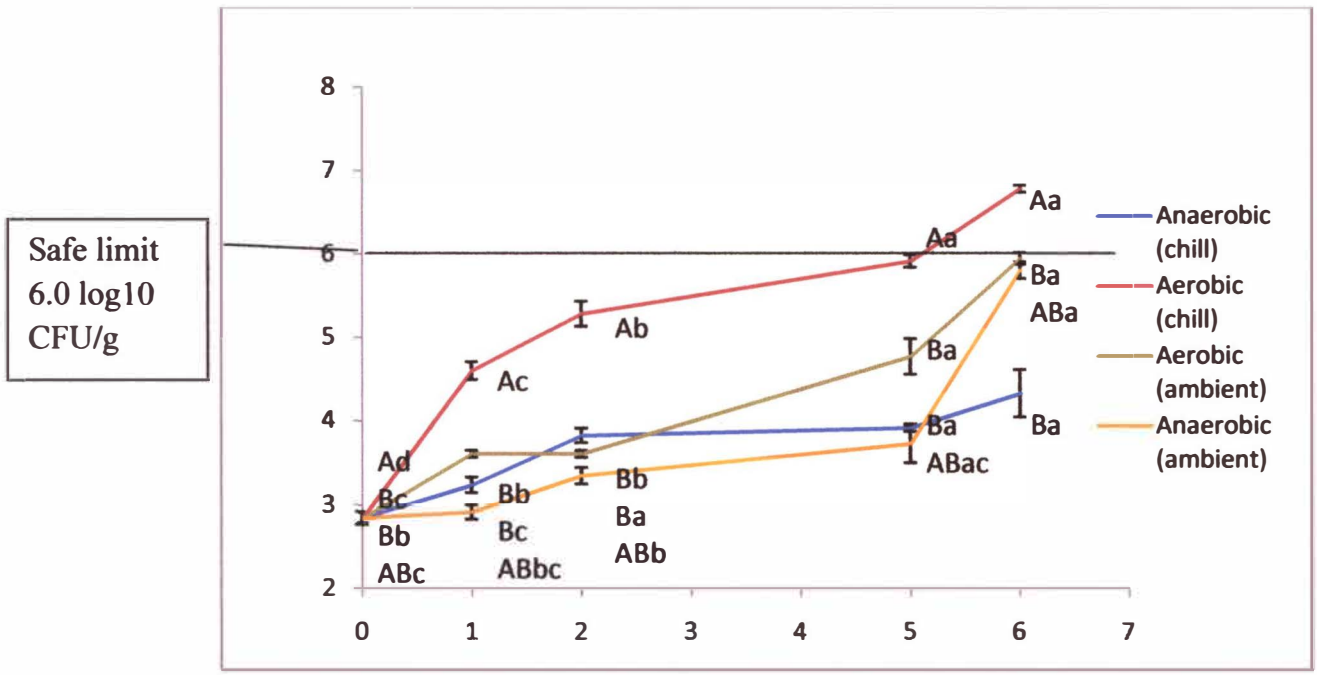
[a-c] mean values with different capital letters showed significant difference between samples (p<0.05)

Figure 4.18: Shelf-life of 'Sambal belacan' determined by Aerobic Plate Count as affected by relative humidity, RH factor

4.3.2 Yeast and mould count

Figure 4.19 shows that there was significant difference (P<0.05) among the samples for oxygen factor but there was not significant difference (P>0.05) for RH factors as shown in Figure 4.20. During the six weeks of storage, there were significant difference (P<0.05) detected among the samples for both factors. 'Sambal belacan' in aerobic condition at ambient temperature showed the highest yeast and mould count while for the RH factor of 'Sambal belacan' showed the highest yeast and mould count stored using high RH at chilled temperature. Based on Guynot et al, (2003) the presence of oxygen may encourage the growth of aerobic bacteria and moulds. The presence of O_2 in packaging and high temperature of storage may promote the growth of yeast and mould

in the 'Sambal belacan'. According to Curiale (1998), the enumeration of yeast and mould in Microbial shelf life testing for ready-to-eat food should lower than 1.0 x 10^6 CFU/mL (6.00 $\log_{10}$ CFU/g).

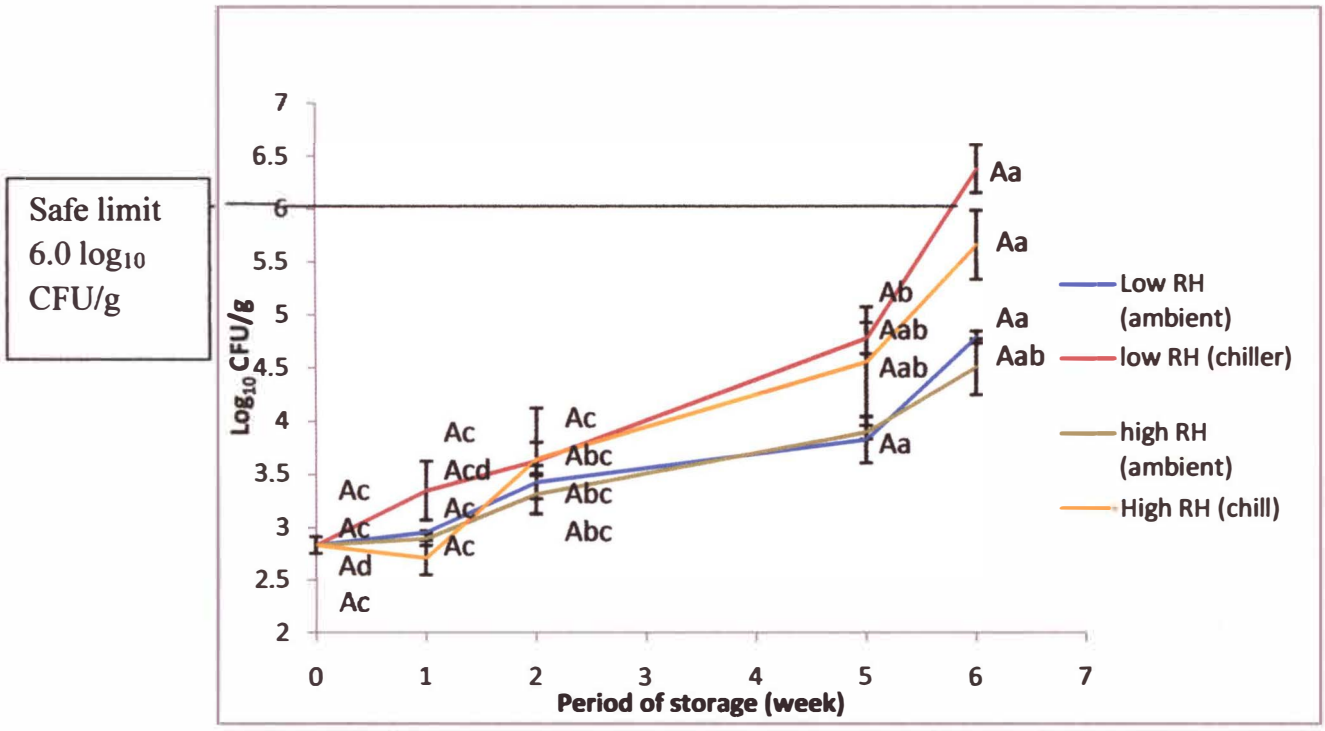


[A] mean values with different capital letters showed significant difference between samples ($p<0.05$)

[a-c] mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.19: Shelf-life of 'Sambal belacan' determined by yeast and mould count as affected by availability of Oxygen, O_2 in the air

The lower of relative humidity can reduce the growth of yeast and mould and spoilage bacteria on food with higher water activity (a_w), such as ready-to-eat meals (Vermeiren et al, 1999). But from the results, all main effects and interactions were not significant difference (P> 0.05) among the different storage conditions of 'Sambal belacan' which were stored at low and high temperatures.



[A] mean values with different capital letters showed significant difference between samples ($p<0.05$)

[a-c] mean values with different capital letters showed significant difference between samples ($p<0.05$)

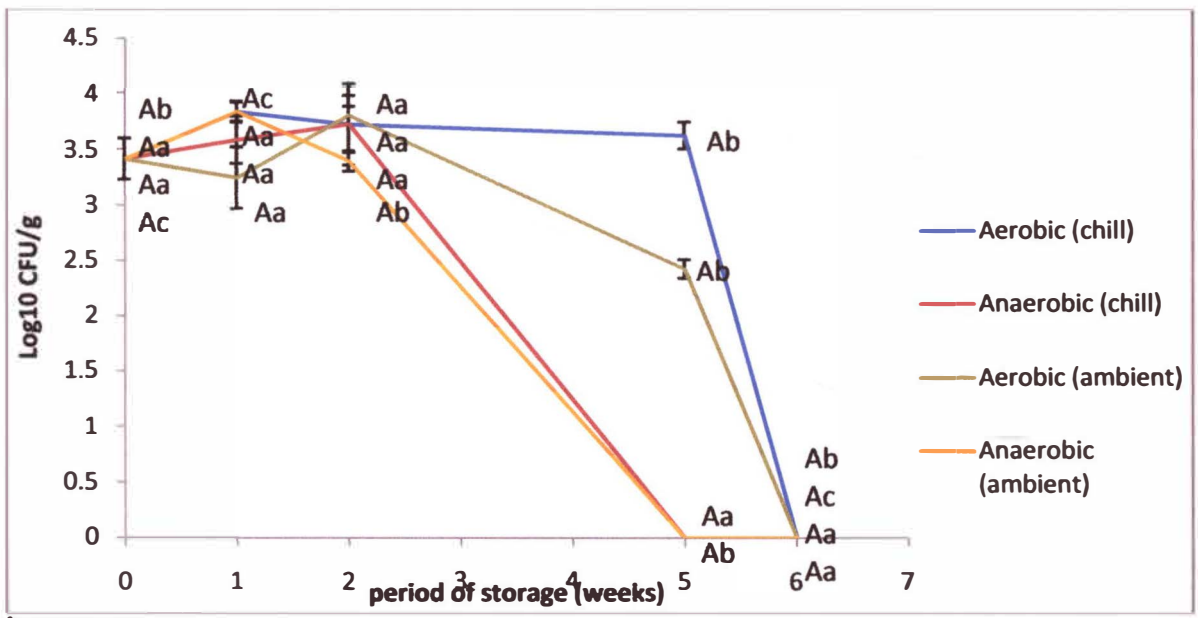
Figure 4.20: Shelf-life of 'Sambal belacan' determined by yeast and mould count affected by relative humidity, RH factor

The growth of yeast and mould in high temperature showed that the growth at ambient temperature (25 ± 2°C) was higher than at chilled temperature (5 ± 2°C). But, when compare with both temperatures, 'Sambal belacan' which stored in high RH at ambient temperature showed the highest growth of yeast and mould. It caused by the accelerated condensation process that happen in those packages. However, according to Vilaesuca et al, (2008), silica gel reduced condensation in those package areas of the film where the sacks were located, particularly when it was placed on the top of the packaging. High RH at chilled temperature gave the high moisture compare to the other storage conditions. Yeast and mould are favourable to multiply with plentiful of water or moisture (Umoh and Odoba, 1999). In addition, yeast and mould can grow at ordinary

temperature which was between 25 to 30°C and they also grow well in presence of oxygen.

4.3.3 Lactic acid bacteria (LAB)

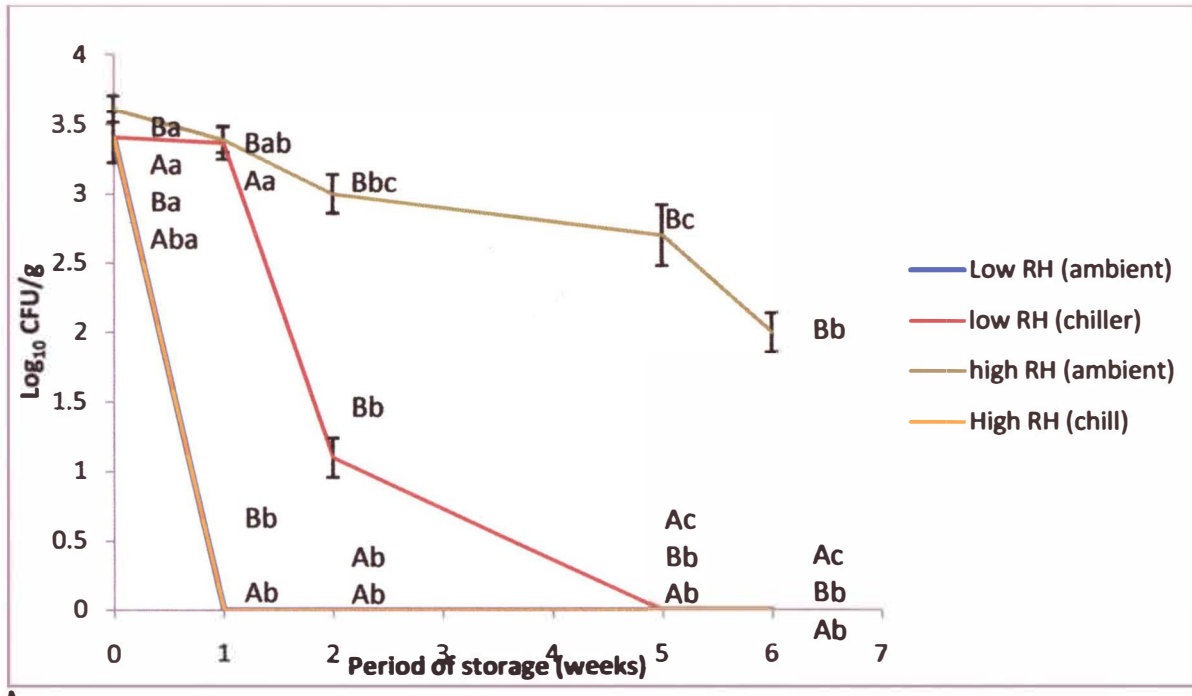
Lactobacillus is type of non- pathogenic bacteria which can inhibit the growth of most foodborne bacterial pathogens (Adams and Nicolaides, 1997). Figure 4.21 exhibits that 'Sambal belacan' without oxygen at chilled temperature showed the highest growth of lactic acid bacteria. Unfortunately, there were no significant difference (P>0.05) among both samples. However, 'Sambal belacan' that treated with difference by RH factor, there was significant difference (P<0.05) detected among the treated samples.



A mean values with different capital letters showed significant difference between samples (p<0.05)

a-c mean values with different capital letters showed significant difference between samples (p<0.05)

Figure 4.21: Lactic acid bacteria count as affected by availability of O_2 in the air



A mean values with different capital letters showed significant difference between samples ($p<0.05$)

a-c mean values with different capital letters showed significant difference between samples ($p<0.05$)

Figure 4.22: Lactic acid bacteria count as affected by relative humidity, RH factor

'Sambal belacan' stored at high RH at chilled temperature show significant difference ($P<0.05$) compared to the 'Sambal belacan' treated with other conditions that affected by RH factor. Both of factors; oxygen and RH were showed the decrease count of lactic acid bacteria due to six weeks of storage. The increasing count in TPC and yeast and mould count in both storage conditions showed the decreasing of lactic acid bacteria count. This condition shows the competition between pathogenic and nonpathogenic bacteria to grow. Due to low count of lactic acid bacteria in 'Sambal belacan', the benefit of lactic acid bacteria in 'Sambal belacan' is not clearly profound.

4.3.4 Other microbial analyses

There were no detection of *Enterobactericea, E. coli, Salmonella,* psychotropic bacteria and coliform bacteria in 25 gram of all samples. Earlier study by Faridah (2009) who studied the effect of different addition of acidic ingredient on microbial quality and shelf-life of 'Sambal belacan' showed *Salmonella,* coliform and *Escherichia coli* were absent in all samples by 'Sambal belacan'.

One factor that inhibits the growth of this microbe is cell injury phenomena. According to Bhaswar (2011), microorganisms live in dynamic environment and they were constantly exposed to variable conditions. During adaptive phases of survival in such environments, microbial cell is prone to injuries at cellular and genetic level. Microbial damage or injury is caused by external natural or artificial sources. Damage can occur by many means such as sudden exposure to physicochemical parameters or by various antimicrobial agents. The extent of injury depends on the susceptibility of microbes to environmental conditions such as temperature, pressure or pH and physiological state (old, growing or resting) of the cell. On the basis of structural components and metabolic processes, various mechanisms of cell injury are known in microorganisms. Injury to microbes generally results in death but still some types of damages are repairable.

The second factor that may lead to inhibition of microbes is the use of heat treatment on shrimp paste during the early stage of 'Sambal belacan' preparation. *Salmonella* was not detected on this study because the shrimp paste was roasted 1at 80°C

for 25 minutes (Adams and Nocolaides, 1997). The use of organic acid also becomes one of the factors that retard the growth of some microbes.

4.3.5 Generation Time

The time needed for a cell to divide and its population to become double is known as generation time. The growth of bacteria is due to the increasing of number of bacteria not due to increasing size of bacteria. It might be varies according to different storage conditions that apply to 'Sambal belacan'. According to Tortora et al. (2001), most of bacteria have a generation time of 1- 3 hours, but others required more than 24 hours per generation. The results of generation time for total plate count, yeast and mould of 'Sambal belacan' are shown in Table 4.3.

Table 4.3: Generation time of microflora in 'Sambal belacan' during shelf-life study

Storage condition of 'sambal belacan'	Generation time (weeks)	
	Aerobic plate count	Yeast and mould count
Oxygen factor		
Anaerobic (chill)	1.04 ± 0.446^{a}	1.44 ± 0.139^{a}
Anaerobic (ambient)	0.70 ± 0.028^{a}	0.57 ± 0.009^{b}
Aerobic (chill)	0.78 ± 0.205^{a}	0.67 ± 0.057^{b}
Aerobic (ambient)	0.45 ± 0.035^{a}	0.74 ± 0.025^{b}
Relative humidity, RH factor		
Low RH (ambient)	$0.56 \pm 0.001b^{c}$	1.05 ± 0.041^{ab}
Low RH (chill)	0.78 ± 0.049^{a}	0.58 ± 0.03^{c}
High RH (chill)	0.61 ± 0.014^{b}	1.14 ± 0.19^{a}
High RH (ambient)	0.48 ± 0.002^{c}	0.65 ± 0.025^{bc}

Values were mean± SD of two replicates, values followed by differences letter in same column differs significantly ($P<0.05$)

Table 4.3 shows the generation time for Aerobic plate count (APC), yeast and moulds counts per weeks at two different factors of storage conditions. Generation time for samples at RH factor was faster than the O_2 factor. Compared with microbial analyses, generation time for APC was faster than yeast and mould count. 'Sambal belacan' in anaerobic condition at chilled temperature showed the slowest generation time for APC and yeast and mould compared to all the samples including for RH factors. It showed that 'Sambal belacan' having longer shelf-life and had prolonged their quality compared to others. The table also shows that APC was taken 1.04 ± 0.446 weeks to doubles, while for yeast and mould count it took 1.44 ± 0.139 weeks for 'Sambal belacan' in anaerobic condition at chilled temperature.

'Sambal belacan' stored with high RH at ambient temperature showed the faster rate to double. There were no significant differences ($P>0.05$) showed for O_2 factor while significant different ($P<0.05$) was detected among the samples of 'Sambal belacan' at RH factor for APC. The faster duplication of microbes at high RH at ambient temperature might caused by the higher moisture content that serve good medium for microbes to grow well.

CHAPTER 5

CONCLUSION AND SUGGESTIONS

5.1 Conclusion

The safety and microbiological quality of the food for public requirement can be achieved by adjusting the environment in order to slow down the growth of microorganisms. The physical properties, microbiological quality and shelf-life of 'Sambal belacan' are the important factors that can used to identify the quality of the product due to the period of storage.

Ambient temperature showed that there had rapidly increasing of CO_2 concentration and percentase of RH in package of 'Sambal belacan'. The colour changes were due to L^*, a^*, b^*, $C°$ and H ° values of the 'Sambal belacan' according to production of sliminess that might cause by the higher growth of spoilage microorganisms. The acidity of 'Sambal belacan' stored that affected by oxygen and relative humidity did not show significant ($P>0.05$) different values except for 'Sambal belacan' with high RH at ambient temperature.

The storage condition of 'Sambal belacan' with low RH at ambient temperature had reduced the microbial load and increase the shelf- life of product compared to the other samples for RH factors, while for the oxygen factor storage condition for 'Sambal belacan' in anaerobic condition at chiller temperature had longer shelf-life. All storage

condition shown that there were significantly inhibited the presence of coliform, *Salmonella, Escherichia coli* and *psychrotropic* bacteria. Since the samples were prepared the same way but all the samples of 'Sambal belacan' were stored at different storage condition.

Lower relative humidity and chilled temperature showed the best storage condition in order to prolong the shelf-life of 'Sambal belacan' due to the growth of microorganisms that assessed by APC and yeast and mould count. This study was clearly shown that pathogenic bacteria were less growing in controlled and hygienic condition.

5.2 Suggestions for further study

More researches could be done for 'Sambal belacan' in order to commercialize this traditional condiment. Shelf-life of 'Sambal belacan' is important factor that needed to understand to achieve the objective to commercialize. Ingredients, packaging and environment of distribution are important factors that need to be studied.

In term of ingredients, there were several preservatives that allowed to be added in 'Sambal belacan' by Food Act 1983 and Food Regulation 1985. Besides that, the use of antimicrobial agents that can be added in 'Sambal belacan' can reduce or inhibit the growth of microorganisms.

Furthermore, packaging is also important factor that can give significant effect on extrinsic factor that lead to deterioration of 'Sambal belacan'. The suitable types of

packaging of 'Sambal belacan' need to be studied in order to understand the reaction between the packaging and 'Sambal belacan'.

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APPENDICES

Appendix A: Preparation of 'Sambal belacan'

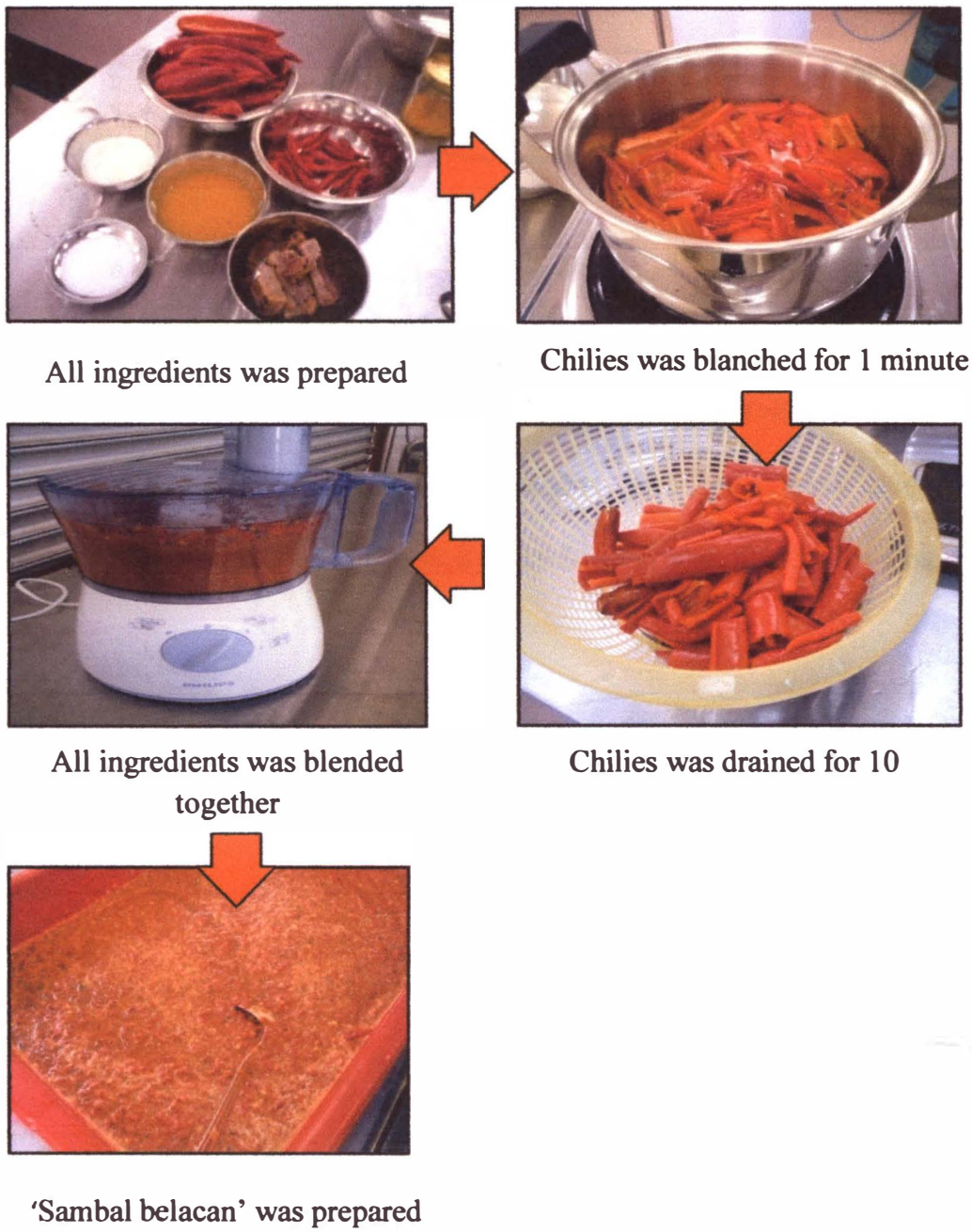

Figure 6.1: Outline of the 'Sambal belacan' preparation

Appendix B: Calculation of generation time

From the separated shelf life of 'Sambal belacan' (log_{10} CFU/g) graf, linear graf was plotted to get the formula of y = mx + C and the R-squared as showed Figure 6.2;

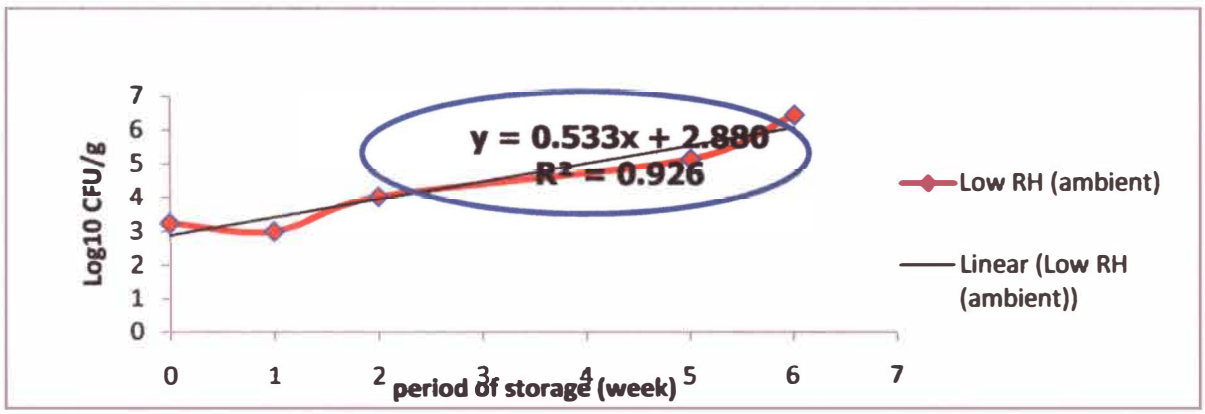

Figure 6.2: Shelf-life of 'Sambal belacan' due to APC in low RH condition and stored at ambient temperature during six weeks storage

From the equation, slope of generation time can directly take to dissolve the equation of generation time suggested by McMeekin et al. (2003). For example;

Calculation;

$$\text{Slope} = \frac{\log_{10} 2}{gt}$$

$$gt = \frac{\log_{10} 2}{\text{slope}}$$

From graph, y =0.533x + 2.880. So, slope is 0.926

$$gt = \frac{\log_{10} 2}{\text{slope}} = \frac{0.301}{0.533} = 0.56 \text{ weeks}$$

Appendix C: Result on One-way ANOVA and Tukey procedure for pH measurement for availability of oxygen in the air among sample.

Samples symbol;

Table 6.1: Symbols for storage condition of 'Sambal belacan'

Symbol	Storage condition of 'Sambal belacan'
A	Anaerobic (chill)
B	Anaerobic (ambient)
C	Aerobic (chill)
D	Aerobic (ambient)
E	Low RH (ambient)
F	Low RH (chill)
G	High RH (chill)
H	High RH (ambient)

One-way ANOVA: pH-availability of oxygen in the air versus Sample

```
Source  DF      SS      MS     F      P
Sample   3  0.0980  0.0327  0.73  0.538
Error   56  2.5047  0.0447
Total   59  2.6027

S = 0.2115   R-Sq = 3.76%   R-Sq(adj) = 0.00%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  -+---------+---------+---------+--------
A      15  4.4747  0.2078          (---------*---------)a
B      15  4.4307  0.2246     (----------*----------)a
C      15  4.5093  0.2189               (----------*----------)a
D      15  4.4040  0.1933   (----------*----------)a
                           -+---------+---------+---------+--------
                          4.30      4.40      4.50      4.60

Pooled StDev = 0.2115

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Sample
```

```
Individual confidence level = 98.94%
Sample = A subtracted from:

Sample    Lower   Center   Upper  ---------+---------+---------+---------+
B       -0.2482  -0.0440  0.1602     (-----------*-----------)
C       -0.1696   0.0347  0.2389          (-----------*-----------)
D       -0.2749  -0.0707  0.1336    (-----------*-----------)
                                   ---------+---------+---------+---------+
                                         -0.16      0.00      0.16      0.32

Sample = B subtracted from:

Sample    Lower   Center   Upper  ---------+---------+---------+---------+
C       -0.1256   0.0787  0.2829             (-----------*-----------)
D       -0.2309  -0.0267  0.1776       (-----------*-----------)
                                   ---------+---------+---------+---------+
                                         -0.16      0.00      0.16      0.32

Sample = C subtracted from:

Sample    Lower   Center   Upper  ---------+---------+---------+---------+
D       -0.3096  -0.1053  0.0989  (-----------*-----------)
                                   ---------+---------+---------+---------+
                                         -0.16      0.00      0.16      0.3
```

Appendix D: Result on One-way ANOVA and Tukey procedure for pH measurement for availability of oxygen in the air through week of storage.

One-way ANOVA: pH-Oxygen factor versus Week sample A

```
Source  DF        SS        MS       F      P
Week     4  0.602773  0.150693  837.19  0.000
Error   10  0.001800  0.000180
Total   14  0.604573

S = 0.01342   R-Sq = 99.70%   R-Sq(adj) = 99.58

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ----+---------+---------+---------+-----
0      3  4.1500  0.0100  (*)e
1      3  4.3367  0.0058            (*)d
2      3  4.5833  0.0058                          (-*)c
5      3  4.6200  0.0265                             (*)b
6      3  4.6833  0.0058                                 (*)a
                          ----+---------+---------+---------+-----
                            4.20      4.35      4.50      4.65
Pooled StDev = 0.0134

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Week

Individual confidence level = 99.18%

Week = 0 subtracted from:

Week    Lower   Center    Upper  -----+---------+---------+---------+----
1     0.15065  0.18667  0.22269                  (*-)
2     0.39731  0.43333  0.46935                        (*-)
5     0.43398  0.47000  0.50602                         (-*)
6     0.49731  0.53333  0.56935                            (*-)
                                -----+---------+---------+---------+----
                                  -0.25      0.00      0.25      0.50
Week = 1 subtracted from:

Week    Lower   Center    Upper  -----+---------+---------+---------+----
2     0.21065  0.24667  0.28269                    (-*)
5     0.24731  0.28333  0.31935                      (*-)
6     0.31065  0.34667  0.38269                        (-*)
                                -----+---------+---------+---------+----
                                  -0.25      0.00      0.25      0.50
Week = 2 subtracted from:

Week    Lower   Center    Upper  -----+---------+---------+---------+----
5     0.00065  0.03667  0.07269              (*-)
6     0.06398  0.10000  0.13602                 (*)
                                -----+---------+---------+---------+----
                                  -0.25      0.00      0.25      0.50
Week = 5 subtracted from:

Week    Lower   Center    Upper  -----+---------+---------+---------+----
6     0.02731  0.06333  0.09935                (-*)
                                -----+---------+---------+---------+----
                                  -0.25      0.00      0.25      0.50
```

Appendix E: Result on t-test for CO_2 measurement

Two-Sample T-Test and CI: CO2, Sample

```
Two-sample T for CO2

Sample   N  Mean  StDev  SE Mean
C       15  1.69   1.10     0.28
D       15  17.8   15.0      3.9


Difference = mu (C) - mu (D)
Estimate for difference:  -16.11
95% CI for difference:  (-24.42, -7.80)
T-Test of difference = 0 (vs not =): T-Value = -4.16  P-Value = 0.001
DF = 14
```

Two-Sample T-Test and CI: CO2, Sample

```
Two-sample T for CO2

Sample   N   Mean  StDev  SE Mean
C       15  1.307  0.769     0.20
D       15   31.8   22.4      5.8


Difference = mu (C) - mu (D)
Estimate for difference:  -30.49
95% CI for difference:  (-42.91, -18.06)
T-Test of difference = 0 (vs not =): T-Value = -5.26  P-Value = 0.000
DF = 14
```

:: From the calculation T calculated is more than t_{table} which is $T_{calculated}$ is 5.26 while t_{table} is -42.91. So the result is significance.

$T_{cal} > t_{table}$ = significance

CURRICULUM VITAE

Name	: Wan Halimah bt Wan Omar
Address	: No. 1A, Kampung Renek, 22020 Jerteh, Terengganu.
Telephone number	: 013-9586210
E-mail	: wan.ima88@gmail.com
Date of birth	: 30 October 1988
Place of birth	: Kampung Renek
Nationality	: Malaysia
Race	: Malay
Gender	: Female
Religion	: Islam

EDUCATIONAL BACKGROUND:

2008-2012	:	University Malaysia Terengganu
2006-2008	:	Kedah Matriculation College (KMK), Kedah
2004- 2005	:	SMK Tengku Mahmod
2001- 2003	:	SMK Renek
1995- 2000	:	SK Renek

HONOUR AND AWARD:

June 2011	:	Accredentials of ***Inspektor Sukarelawan Polis*** from Polis Di-Raja Malaysia (PDRM)

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