ANTIBACTERIAL ACTIVITIES OF LIPID AND PIGMENT EXTRACT OF *Scenedesmus* sp. ISOLATED FROM THE TEMPORARY WATERS OF ENDAU ROMPIN

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A thesis submitted in fulfillment of the requirement for the award of the Degree of Master of Science

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DEDICATION

I dedicate this work with the deepest sense of gratitude to Almighty Allah for giving me Strength and Inspiration in making my dreams come true.
ACKNOWLEDGEMENTS

All Praises and thanks are to ALLAH Subhanahu wa ta’ala for His infinite blessings and mercies. May the peace and blessings of ALLAH Subhanahu wa ta’ala be upon Prophet Muhammad (Sallallahu alayhi wa sallam).

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I am especially indebted to my parents, who are and will always be there for me at every point of my life. Thank you for your wonderful upbringing, love, sacrifices, and support. May Allah continue to be with you and bless you abundantly. I would also like to extend my profound gratitude to all my in- Laws who took me in as their true daughter, for their encouragement and support. And to all my siblings and nieces Farida and Ummi who have remained my anchor in terms of love, support, and prayers. I will forever remain grateful to you all and wish you all success in your various endeavours.

To my colleagues and friends, thank you all for your invaluable support, prayers and encouragement during my study.
The search for alternatives that could circumvent the use of synthetic antimicrobial compounds is the result of a worldwide demand for novel antimicrobial agents from natural sources. This study covered the screening and investigation of the antibacterial potential of freshwater green microalga *Scenedesmus* sp. isolated from temporary rock pools of Taman Negara Johor Endau Rompin, Malaysia. Crude pigment and lipid extracts from the microalga were tested using the agar well diffusion method for their antibacterial activity against foodborne pathogens, *Staphylococcus aureus* (ATCC 25923) and *Salmonella* sp. (ATCC 14028). Following that, antibacterial activities of the extracts were evaluated in food spiked with *Staphylococcus aureus* (ATCC 25923) through bacterial colony count. The minimum inhibitory concentrations (MIC) of both extracts were assessed using macro broth dilution assay. The results of this study showed the pigment extract at concentrations ranging from 0.35 mg/ml – 3.48 mg/ml exhibited antibacterial activity against *Staphylococcus aureus* (ATCC 25923) with mean zones of inhibition from 4.67 mm – 17.33 mm. Likewise, the lipid extract at concentrations ranging from 0.085 mg/ml - 0.85 mg/ml exhibited antibacterial activity against *Staphylococcus aureus* (ATCC 25923) with mean zones of inhibition from 1.33 mm – 15.67 mm. However, both extracts did not show any activity against *Salmonella* sp. (ATCC 14028). The MIC of the pigment and lipid extracts were 80 μg/ml and 21 μg/ml respectively. In food spiked with *S. aureus* (ATCC 25923), outnumbered bacterial colonies were observed from both samples of lipid (0.1 mg/ml - 0.2 mg/ml) and pigment (0.41 mg/ml - 0.81 mg/ml). However, there was no growth of bacterial colonies in both samples of lipid at 0.8 mg/ml - 1 mg/ml and pigment at 2.83 mg/ml - 4.05 mg/ml. The results of the antibacterial activity indicates that *Scenedesmus* sp. extracts of either lipid or pigment contains secondary metabolites with great potential as a food additive.
Pencarian alternatif yang dapat menghalang penggunaan antimikrob sintetik merupakan hasil permintaan dunia terhadap antimikrob baharu berasaskan sumber semula jadi. Kajian ini meliputi penyaringan dan penyiasatan potensi antibakteria bagi mikroalga air tawar hijau *Scenedesmus* sp. yang diasingkan daripada kolam batu sementara di Taman Negara Johor Endau Rompin, Malaysia. Ekstrak kasar bagi pigmen dan lipid daripada mikroalga telah diuji dengan kaedah resapan telaga agar bagi aktiviti antibakteria terhadap patogen bawaan makanan, *Staphylococcus aureus* (ATCC 25923) dan *Salmonella* sp. (ATCC 14028). Berikutnya, aktiviti antibakteria daripada ekstrak tersebut telah dinilai dalam makanan yang dicemari dengan *Staphylococcus aureus* (ATCC 25923) melalui kiraan koloni bakteria. Kepekatan minimum perencatan (MIC) kedua-dua ekstrak telah dinilai menggunakan assai makro pencairan. Keputusan kajian ini menunjukkan ekstrak pigmen pada kepekatan daripada 0.35 mg/ml - 3.48 mg/ml mempunyai aktiviti antibakteria terhadap *Staphylococcus aureus* (ATCC 25923) dengan zon minimum perencatan daripada 4.67 mm - 17.33 mm. Selain itu, ekstrak lipid pada kepekatan antara 0.085 mg/ml - 0.85 mg/ml menunjukkan aktiviti antibakteria terhadap *Staphylococcus aureus* (ATCC 25923) dengan zon minimum perencatan daripada 1.33 mm - 15.67 mm. Walau bagaimanapun, kedua-dua ekstrak tidak menunjukkan sebarang aktiviti terhadap *Salmonella* sp. (ATCC 14028). MIC daripada ekstrak pigmen dan lipid adalah masing-masing 80 μg/ml dan 21 μg/ml. Dalam makanan tercemar dengan *S. aureus* (ATCC 25923), pertumbuhan koloni bakteria yang dapat diperhatikan dari kedua-dua sampel lipid ialah (0.1 mg/ml - 0.2 mg/ml) dan pigmen (0.41 mg/ml - 0.81 mg/ml). Walau bagaimanapun, tidak ada pertumbuhan koloni bakteria dalam kedua-dua sampel lipid pada 0.8 mg/ml - 1 mg/ml dan pigmen pada 2.83 mg/ml - 4.05 mg/ml. Keputusan aktiviti antibakteria menunjukkan bahawa ekstrak lipid atau pigmen *Scenedesmus* sp. mengandungi metabolit sekunder yang mempunyai potensi besar sebagai bahan tambahan makanan.
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LIST OF SYMBOLS

nm - Nanometer

% - Percentage

CFU/ml - Colony forming unit per milliliter

μl - Microliter

μg/ml - Microgram per milliliter

v/v - Volume per volume

℃ - Degree Celsius

mg/ml - Milligram per milliliter

α - Alpha

β - Beta

γ - Gamma
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<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<td>SE</td>
<td>Staphylococcal enterotoxin</td>
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<tr>
<td>sp.</td>
<td>Specie</td>
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<tr>
<td>PUFA</td>
<td>Poly unsaturated fatty acid</td>
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<tr>
<td>aa</td>
<td>Amino acid</td>
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<tr>
<td>MH</td>
<td>Mueller Hinton</td>
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<tr>
<td>BBM</td>
<td>Bold basal medium</td>
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<td>TSB</td>
<td>Tryptic soy broth</td>
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CHAPTER 1

INTRODUCTION

1.1 Background of study

The potentials of microalgae and its products to be used as nutraceuticals or functional foods has been researched, reviewed and accepted for some years now. Several high-value products from microalgae have been used for human or animal nutrition and other uses. Because of the unique diversity of microalgae, it has the capability to be a valuable source of products that supports food (for both human and animal nutrition), cosmetics, pharmaceuticals and biofuel industries (Olaizola, 2003; Pulz & Gross, 2004).

Arrays of biologically active substances have been extracted from several microalgal species both as cell extracts and extracellular products which have been found to possess antimicrobial activities. These activities can be antibacterial, antifungal, antialgal and antiprotozoal. Developing commercial products from microalgae is not a new concept and annually, about 75 % of microalgal biomass produced is used for the manufacture of powders, tablets, capsules, or pastilles (Nasseri et al., 2011).

Globally, the incidence of food borne diseases has a significant level of morbidity. It has affected people’s health as many people become sick and even die as a result of ingesting certain microorganisms or their toxins through unsafe foods. A wide variety of etiological agents such as bacteria, viruses and parasites has also been associated with food poisoning (Gündüz et al., 2011). A significant source of food borne infection and illnesses worldwide has been reported to be *Staphylococcus*
*aureus (S. aureus)*, this is due to its ability to produce several enterotoxins that are heat resistant (Shimizu et al., 2000; Reiser et al. 1984). Annually, about 30% of people fall ill due to foodborne diseases in industrialized nations and it is more likely to be higher in developing nations. It is estimated that each year roughly 1 in 6 Americans (or 48 million people) get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases (CDC, 2014). A survey carried out in late 2010 found that the Chinese consider food safety the second greatest risk they face in daily life after earthquakes (Alcorn and Ouyang, 2012). In Malaysia, foodborne diseases are not rare, and outbreaks occur mainly due to insanitary food handling procedures. This is usually the cause of more than 50% of all poisoning episodes (Abdul-Mutalib et al., 2015; Sharifa Ezat et al., 2013).

According to the Ministry of Health Malaysia (2010), there has been a steady increase in the number of food poisoning cases as evident by the incident rate of 36.17 in 2009 and 44.18 in 2010 per 100,000 population. There was a slight increase by the end of 2013 as the incident rate of food poisoning was 47.79 per 100,000 population (Ministry of Health Malaysia, 2014).

1.2 Problem statement

The safety aspects of chemical or synthetic food additives are being increasingly questioned thereby leading to a demand for naturally occurring food preservatives worldwide. The potential of *Scenedesmus* sp. extracts inhibiting the growth of *S. aureus* and *Salmonella* sp. will be a great feat in defeating bacteria that are constant threats to food formulations.

1.3 Objectives of study

(i) To extract pigment and lipid from *Scenedesmus* sp.
(ii) To determine whether both the lipid and pigment extracts of *Scenedesmus* sp. has antibacterial potential against *S. aureus* and *Salmonella* sp.
(iii) To evaluate the activity of both lipid and pigment extracts of *Scenedesmus* sp. in food spiked with foodborne *S. aureus*. 
1.4 **Scope of study**

This investigation was carried out to determine the antibacterial potentials of lipid and pigment extracts of *Scenedesmus* sp. The study covered the collection and isolation of *Scenedesmus* sp. from the rocky pools of Endau rompin, Johor, Malaysia. The microalga was mass cultured in bold basal medium (BBM) at a temperature of 25 °C with illumination from sunlight. Pigment and lipid were extracted from the cultured microalga and were used to test inhibitory action on foodborne *S. aureus* and *Salmonella* sp. using the agar well diffusion and pour plate method.

1.5 **Significance of study**

Bioactive compounds from microalgae are regarded as functional ingredients of natural origin which can be used to replace synthetic compounds that are currently used in food formulations. This growing interest in microalgae and its products has led to the extraction of many important bioactive compounds with promising biological activities including inhibiting bacterial growth. The significance of this study therefore, is to investigate the antibacterial potential of *Scenedesmus* sp. in inhibiting foodborne pathogen *Staphylococcus aureus*. 
CHAPTER 2

LITERATURE REVIEW

2.1 Microalgae

Microalgae are among the oldest living organisms that does not have roots, stems, and leaves and have chlorophyll a as their primary photosynthetic pigment (Brennan & Owende, 2010). Although the mechanism of photosynthesis in these microorganisms is very similar to that of higher plants, they are generally known to be more efficient converters of solar energy because of their simple cellular structure. Because microalgae can grow in aqueous suspension, they have easier access to water, CO₂ and other essential nutrients than higher plants (Chisti, 2007).

Microalgae use inorganic compounds as a source of carbon, and as autotrophs, they can be photoautotrophic by using light as a source of energy, or chemoautotrophic, by oxidizing inorganic compounds for energy. Photosynthesis is a key component for the survival of autotrophic algae, whereby solar radiation and CO₂ absorbed by chloroplasts are being converted into adenosine triphosphate (ATP) and O₂ (Brennan & Owende, 2010).

2.2 Scenedesmus sp.

Freshwater microalgae are widely distributed in rivers, lakes and polar waters and they exhibit a diverse range of cellular, morphological, structural and biochemical
composition (Chu et al., 2004). *Scenedesmus* belongs to the order Sphaeropleales of the family Scenedesmaceae which is frequently dominant in freshwater lakes and rivers (Borowitzka and Borowitzka, 1988; Guiry, 2014). To date, there are 74 taxonomically recognized species of *Scenedesmus* (Guiry, 2015). The species differ exceedingly in their morphologies thereby making identification difficult. However, *Scenedesmus* sp. can exist as unicellular organisms and are also often found in coenobia of four or eight cells (Miquel and Ellen, 2000). Fossil records have dated *Scenedesmus* 70 to 100 million years ago. Many species of this genus are being used worldwide for various purposes due to their ability to adapt to harsh environmental conditions, ability to grow rapidly and ease of cultivation and handling (Miquel, 2003; Pulz and Gross 2004). Likewise *Scenedesmus* sp. have been used in many biotechnological applications due to their high nutritional content and bioactivities (Chacón-Lee and Gonzalez - Marino 2010; Guedes et al., 2011a).

Figure 2.1: *Scenedesmus* sp.
2.2.1 Factors influencing microalgal growth

Algae have several requirements in their environment and many important parameters that affect growth. Important parameters are to be considered when cultivating algae for production purposes, and these include; nutrients, light, pH, temperature and agitation.

2.2.1.1 Nutrients

Microalgae can obtain nourishment through two major ways namely; autotrophy using light and/or heterotrophy using chemical compounds as their sources of energy. When algae are grown photoautotrophically, it will basically need light and inorganic CO$_2$ to fulfill the energy demands and water for O$_2$ production (Grobbeelaar, 2007). As much as water is crucial for algae growth, it does not consume water as much as higher plants (Lan et al., 2008). Major elements needed for the formation of organic compounds within algae include; carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulfur (S), phosphorous (P), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), chlorine (Cl) and trace elements needed for growth are iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), cobalt (Co) and molybdenum (Mo). (Geider & MacIntyre, 2002).

According to Riebesell & Wolf-Gladrow (2002), nutrient uptake by cells occurs primarily by molecular diffusion. Kong et al. (2009) stated that, the rate of diffusion transport depends on three parameters, such as the concentration gradient from bulk medium to cell surface, the diffusion coefficient of the nutrient and the thickness of the diffusive boundary layer and these parameters can be affected by the microalga itself whose cells can control the nutrient concentrations at the cell surface thereby controlling the concentration gradient. HCO$_3^-$ and CO$_2$ are two forms in which microalgae take up and utilize carbon. CO$_2$ is the only form of carbon with electric neutrality that crosses the membrane passively and even when HCO$_3^-$ is utilized as a carbon source it can only occur via an active uptake or by extracellular conversion from HCO$_3^-$ to CO$_2$ through enzymatic activity (Riebesell & Wolf-Gladrow, 2002).
2.2.1.2 Light and photosynthesis

Photosynthetic reaction is a reduction-oxidation reaction driven by light where chlorophylls present in plants harvest the light (Masojidek & Koblizek, 2007) either naturally by the sun or artificially by lamps and is essential for growth of photosynthetic organisms (Lee & Shen, 2007). Photoautotrophs are known to convert energy from sunlight and inorganic compounds, such as CO$_2$, into energy-rich organic compounds (Masojidek & Koblizek, 2007).

In theory, microalgae can reach a 10 % total light energy conversion to primary products or maybe even up to 20 % but this is true only for low light intensities (Benemann, 2007; Lan et al., 2008). When algae are exposed to full sunlight intensity, a light energy conversion of only 3 % can be achieved (Benemann, 2007). The range of possible wavelengths for photosynthesis is the visible light spectrum from purple of 380 nm to red at 750 nm (Mathews et al., 2000). The summary of photosynthesis can be written in a reaction equation below (Geider & MacIntyre, 2002):

Summary equation of photosynthesis;

$$6\text{CO}_2 + 12\text{H}_2\text{O} + \text{light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 + 6\text{H}_2\text{O} \quad (2.1)$$

Photosynthetic reaction is divided into two separate processes, the light and dark reaction (Masojidek & Koblizek, 2007). In the light reaction, light energy is used to produce NADPH, and the energy-rich compound ATP which are further used in the dark reaction (Mathews et al., 2000).

2.2.1.3 Light harvesting pigments

All organisms capable of undergoing photosynthesis contain organic light harvesting pigments. Pigments are classified into three major classes namely; chlorophylls, carotenoids and phycobilins (Masojidek & Koblizek, 2007). Chlorophylls are green pigments and carotenoids are yellow or orange and both are lipophilic while phycobilins on the other hand are hydrophilic. Chlorophyll a, b, c and d exist in all oxygenic photoautotrophs of which chlorophyll c lacks a long chain terpenoid alcohol (Masojidek & Koblizek, 2007). Chlorophyll a exists within all photoautotrophic organisms functioning as the main light harvesting pigment and is a primary part of
the protein pigment complexes (Karl et al., 2002). All chlorophyll molecules have two major absorption wavelengths, at blue-green (450 - 475 nm) and red (630 - 675 nm) visible light spectrum. The chlorophyll b, c and d extend the absorption range of chlorophyll a thereby making the absorbance spectrum for different species unique but it also depends on the ratio between chlorophyll types (Mathews et al., 2000). The total pigment content varies from 0.1 – 9.7 % of wet biomass and chlorophyll a can vary a 30-fold depending on changes within the species, temperature, light or level and type of nutrients (Harrison et al., 2011).

Carotenoids are biological chromophores which exist in different variants within algae with different roles in the cells, but all functions are connected to light harvesting (Mathews et al., 2000). The absorption spectrum of carotenoids lies within the interval of 400 to 550 nm (Masojidek & Koblizek, 2007). Carotenoids such as carotenes (e.g. α-carotene and β-carotene) and xanthophylls (e.g. lutein and zeaxanthin) could be present in algae. Their various functions includes; facilitation of photosynthesis by aiding the excitation of chlorophyll a, which is a structural part in the pigment-protein complexes and as a defense mechanism to excess irradiance, i.e. carotenoids are produced by cells under irradiance stress (Masojidek and Koblizek, 2007).

2.2.1.4 Agitation

During the culturing process of algal cells, some sort of agitation is necessary to assist nutrient and gas exchange to increase the contact between cell surface and water (Lee and Shen, 2007). Agitation keeps the cells dispersed preventing the settling and sedimentation of growing cells on the walls of the cultivation equipment, thereby minimizing the diffusion gradients at the cell surface and also importantly maximizing high biomass yields (Grobbelaar, 2007). When there is a high cell density in suspension, availability of light is usually limited but can be increased by mixing, which creates minor photic zones that are sufficient for photosynthesis thereby enhancing the light/dark frequency leading to increased photosynthetic effectiveness (Tredici, 2007).
2.2.1.5 Temperature

There is a strong correlation between temperature and biochemical reactions which affect microalgal growth as maximal productivity can only be achieved when nutritional needs are met at correct optimal temperatures (Hu, 2007; Richmond, 2007). Optimum temperature for growth of microalgae is usually between 20 °C to 30 °C, while temperatures lower than 16 °C decrease growth rate, many microalgae die at temperatures above 35 °C (Oilgae, 2009). There is a relationship between temperature and light intensity since lamps and sunlight emits heat (Richmond, 2007).

Polyunsaturated fatty acids within the membranes and fluidity of the membrane system is increased at low temperature. This is essential in protecting the thylakoids and the photosynthetic machinery of microalgal cells from photo inhibition. Therefore, lipid classes and composition is affected by temperature rather than the total lipid content (Hu, 2007).

2.2.1.6 pH

The pH is another factor known to affect the growth of microalgae with the fastest growth achieved for different species and strains at different optima (Kong et al., 2009). The general pH optimum for most freshwater species is roughly between 7-9 and the pH optimum for *Scenedesmus* sp. is around 7.5 (Whitacre, 2010). Failure to maintain the correct pH can lead to slow growth of microalgae or eventual culture collapse, because pH can affect the availability and solubility of CO₂ and minerals in the medium (Oilgae, 2009; Mathias, 2012).

2.2.2 Nutritional Composition of *Scenedesmus* sp.

Similar to higher plants, the chemical composition of algae is not constant as it is determined by factors like environmental, temperature, pH value, mineral contents, CO₂ supply, population, density, growth phase and algae physiology which can modify its chemical composition (Gouveia et al., 2008). According to Yamaguchi (1997), microalgae have the ability to biosynthesize, metabolize, store and also secrete a diverse range of primary and secondary metabolites. Microalgal biomass is made up
of different nutritional components of which the main three are: proteins, carbohydrates and lipids (oil) (Indira & Biswajit, 2012). The following are some of the reasons why microalgae came to be of such commercial importance due to its nutritional composition; (1) the presence of high protein content in microalgae is the main reason it should be considered as a conventional source of protein, (2) its amino acid pattern compares favorably with other foods, (3) carbohydrates are obtained in various forms such as starch, glucose, sugars, other polysaccharides and, (4) its total digestibility is extremely high which explains why there are no limitations in its use in food and feed wholly (Cornet, 1998; Becker, 2004; Solletto et al., 2005).

Microalgal biomass consists of carbohydrates, proteins and oils (Sheenan et al., 1998). Scenedesmus sp. are particularly found to contain all essential amino acids and a good amount of protein, lipid and essential minerals (Geldenhuys et al., 1988). According to (Becker, 2004; Batista et al., 2007) Scenedesmus contains lipids, proteins, and carbohydrates which can compare favorably with other food protein (Table 2.1).

**Table 2.1: Nutrient Composition of Different Scenedesmus sp. (% dry matter)**

<table>
<thead>
<tr>
<th>Scenedesmus sp./Food products</th>
<th>Protein</th>
<th>Carbohydrate</th>
<th>Lipid</th>
<th>Ash</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybeans</td>
<td>37</td>
<td>30</td>
<td>20</td>
<td>-</td>
<td>Becker, 2004</td>
</tr>
<tr>
<td>Meat</td>
<td>43</td>
<td>1</td>
<td>34</td>
<td>-</td>
<td>Becker, 2004</td>
</tr>
<tr>
<td>Milk</td>
<td>26</td>
<td>38</td>
<td>28</td>
<td>-</td>
<td>Becker, 2004</td>
</tr>
<tr>
<td><em>S. dimorphus</em></td>
<td>8-18</td>
<td>21-52</td>
<td>16-40</td>
<td>-</td>
<td>Um &amp; Kim 2009; Sydney et al., 2010</td>
</tr>
<tr>
<td><em>S. obliquus</em></td>
<td>50-56</td>
<td>10-17</td>
<td>12-14</td>
<td>-</td>
<td>Becker, 2004</td>
</tr>
<tr>
<td><em>S. acutus</em></td>
<td>50-60</td>
<td>10-17</td>
<td>12-14</td>
<td>6-10</td>
<td>Soeder &amp; Prabst, 1970</td>
</tr>
<tr>
<td><em>S. quadricauda</em></td>
<td>47</td>
<td>-</td>
<td>1.9</td>
<td>-</td>
<td>Um &amp; Kim 2009; Sydney et al., 2010</td>
</tr>
<tr>
<td><em>S. obliquus</em></td>
<td>6-12</td>
<td>33-64</td>
<td>11-21</td>
<td>-</td>
<td>Batista et al., 2007</td>
</tr>
<tr>
<td><em>S. dimorphus</em></td>
<td>60-70</td>
<td>13-16</td>
<td>6-7</td>
<td>-</td>
<td>Batista et al., 2007</td>
</tr>
<tr>
<td><em>S. obliquus</em></td>
<td>34.5</td>
<td>-</td>
<td>16.13</td>
<td>12.0</td>
<td>Toyub et al., 2008</td>
</tr>
</tbody>
</table>
2.2.3 Antibacterial Activities of *Scenedesmus* sp.

*Scenedesmus* sp. have been reported to produce antimicrobial substances which from the pharmaceutical’s point of view, are a good source of new bioactive compounds. The potential of fatty acids to inhibit the growth and survival of pathogenic bacteria has been recognized for several years. They enhance membrane damage that eventually enables cell leakage. Recently, studies of its structure-function relationship make it more evident that these antimicrobial activities rely on both the chain length and the degree of unsaturation of the fatty acids (Guedes *et al.*, 2011b). Patented application of astaxanthin is also available for preventing bacterial infections (Jouni and Makhoul, 2012).

Desbois and Smith (2009) claimed that *Scenedesmus costatum* exhibited antibacterial activity against aquaculture bacteria as a result of their more than 10 carbon atoms in chain length of fatty acids. Furthermore, Guedes *et al.* (2011a) reported that *Scenedesmus* sp. is among the few members of the green algae to produce antimicrobial substances and have active and prominent antibacterial properties that inhibited the growth of several pathogenic strains of bacteria when tested against them (Table 2.2). These include; *Salmonella* sp., *Escherichia coli* and *Staphylococcus aureus*.

Table 2.2: Antibacterial Inhibition of Various *Scenedesmus* sp.

<table>
<thead>
<tr>
<th><em>Staphylococcus aureus</em></th>
<th><em>Escherichia coli</em></th>
<th><em>Salmonella typhi</em></th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 mm</td>
<td>-</td>
<td>9 mm</td>
<td>Ghasemi <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>16 mm</td>
<td>-</td>
<td>-</td>
<td>Najdenski <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>18 mm</td>
<td>-</td>
<td>-</td>
<td>Najdenski <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>17 mm</td>
<td>-</td>
<td>-</td>
<td>Najdenski <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>10 mm</td>
<td>-</td>
<td>-</td>
<td>Abedin &amp; Taha (2008)</td>
</tr>
<tr>
<td>15 mm</td>
<td>R</td>
<td>R</td>
<td>Abedin &amp; Taha (2008)</td>
</tr>
<tr>
<td>12 mm</td>
<td>15 mm</td>
<td>R</td>
<td>Abedin &amp; Taha (2008)</td>
</tr>
<tr>
<td>5 mm</td>
<td>-</td>
<td>-</td>
<td>Ördög <em>et al.</em> (2004)</td>
</tr>
</tbody>
</table>

Note: R- Resistant
2.2.4 Mechanism of action of antibacterial agents

Antimicrobial agents can be described as either bacteriostatic or bactericidal (Salvador et al., 2007). Several proposed mechanisms responsible for antimicrobial action of bioactive compounds include membrane damage, changes in intracellular pH, membrane potential, and ATP synthesis (Lambert et al., 2001; Leuko et al., 2004). Mechanism of action of antimicrobial agents can also be based on the structure of the bacteria or the function that is affected by the agents (Pradhan et al., 2014). Cucco et al. (2007) reported carotenoids to be responsible for digestion of cell wall by lysozyme enzymes. Flavonoids are said to increase the permeability of the inner bacterial membrane and a dissipation of the membrane potential (Mirzoeva et al., 1997).

Though the exact mechanism of action by fatty acids and lipids remains largely unknown, they are thought to act upon multiple cellular targets, and membrane damage will lead to cell leakage, reduction of nutrient intake and cellular respiration (Lampe et al., 1998; Debois et al., 2009).

2.2.5 Extraction of Biologically Active Compounds from Scenedesmus sp.

This is an important aspect to be considered in order to extract and isolate compounds of interest effectively. The general techniques of plant and algae extraction include maceration, hot continuous extraction (soxhlet), microwave assisted extraction, sonication, supercritical fluid extraction, ultrasound assisted extraction, pressurized liquid extraction and hand grinding with pestle and mortar (Herrero et al., 2013). All of these have also been employed in the extraction of bioactive compounds from Scenedesmus algae. According to Herrero et al., (2013), successful determination of biologically active compounds from plants is also largely dependent on the type of solvent used in the extraction process. The choice of a solvent used is influenced by what the extract is intended for and the targeted compounds.

In extracting different bioactive compounds from Scenedesmus sp. one of the techniques used is solvent extraction. Some important bioactive compounds found in Scenedesmus include; pigments, lipids, vitamins and others. Some of the bioactive compounds are briefly discussed below;
2.2.5.1 Lipids

The term “lipids” is a vague concept, which is defined as biochemical compounds that are not soluble in water but are soluble in organic solvents (Christie, 2005). Lipids and fatty acids are found to exist within algal cells in the membrane, as storage products, or metabolites (Mathews et al., 2000; Griffiths and Harrison, 2009). Primarily, lipid droplets exist in the cytoplasm and not in any other organelles within the cell, making it easy to extract from the cells (Oilgae, 2009). The most important structure among lipids are the fatty acids which possess a long hydrophobic hydrocarbon tail attached to a hydrophilic carboxyl group and are built in other types of lipids (Mathews et al., 2000). Growth phase and environmental conditions affect the composition and productivity of lipids. The lowest yield of lipid formation is achieved during the logarithmic growth phase and increased lipid formation is achieved at the end of logarithmic growth phase and it is highest in the stationary phase where growth is limited (Huerlimann, 2010). All plant cells have lipid and fatty acids as their constituents, where they function as membrane components, storage products, metabolites, and as sources of energy.

Lipids extracted with lipophilic organic solvents are commonly called total lipids. They can be classified according to their polarity, which depends on the non-polar (lipophilic) carbon chains (fatty acids) and the polar (hydrophilic) moieties (carboxylic groups, alcohols, sugars, etc.). The major part of the non-polar lipids (neutral lipids) of microalgae are triglycerides and free fatty acids, whereas the polar lipids are essentially glycerides in which one or more of the fatty acids has been replaced with a polar group, for instance phospholipids and glycolipids. The average lipid content varies between 1 and 40%, and under certain conditions it may be as high as 85% of the dry weight (Becker, 2004). Algal lipids are known to be composed of sugars or bases that are esterified to saturated fatty acids or unsaturated fatty acids with carbon of 12-22 atoms (Pradhan et al., 2012). Fatty acids of the omega 3 and omega 6 families have been of particular interest for some time now. Previous studies have shown that the antibacterial potentials of microalgae are due to fatty acids, particularly saturated and unsaturated long chain fatty acids of more than 10 chain length which induce the lysis of bacterial protoplasts (Pradhan et al., 2012; Spolaore et al., 2006; Kellam et al., 1988). Fatty acids and hydroxyl unsaturated fatty acids, glycolipids, terpenoids, lauric acid, linolenic acid, palmitic acid, steroids, phenolics, oleic and...
stearic acids are all known antibiotics (MacMillan et al., 2002; Shanab, 2007; Tan, 2007). Algal lipids are known to contain a relatively high amount of long chain, especially omega 3- and 6- of FA series such as Eicosapentaenoic, Docosahexaenoic, \( \gamma \)-linolenic and \( \alpha \)-linolenic acid. These long chains PUFAs in algae have profound benefits and functions in dietetics and therapeutic uses (Herrero et al., 2006; Cardozo et al., 2007).

### 2.2.5.2 Pigment

Chlorophylls are the principal pigments in photosynthesis, capable of absorbing light energy and converting it to “chemical energy”. This is done by the formation of chemical compounds rich in energy which are needed for the biosynthesis of carbohydrates and other compounds in photosynthetic organisms such as plants, algae and photosynthetic bacteria (Scheer, 2006; Scheer, 1991). The Chlorophyta, or green algae, have chlorophyll a, b and several carotenoids (Tomaselli, 2004). Chlorophyll is one of the most valuable bioactive compounds that are being extracted from microalgal biomass. Due to its strong green pigment content and consumers growing preference for natural foods, since many foods tend to lose their original colors due to the chemical processes they undergo, chlorophyll is gaining importance as a food additive (Humphrey, 2004). Changes in market demands and legislation resulting from the preference of natural colouring agents used in food products have spiked interest in chlorophyll (Spears, 1988). Studies have shown that it has antioxidant as well as antimicrobial properties (Humphrey, 2004). This is very encouraging and useful information to food manufacturers to switch from artificial pigments to chlorophyll-based natural coloring as consumers prefer products with original appearance. Astaxanthin is considered to be one of the best carotenoid compounds that can successfully protect cells, lipids and membrane lipoproteins from oxidative damage (Ranga Rao et al., 2014). It is a sought after compound as it is used in food, cosmetics and pharmaceutical applications (Kim et al., 2011). Astaxanthin products are commonly found in the form of soft gel, capsules, powder, tablet, oil, energy drink, creams and in combination with other herbal extracts from other sources (Ranga Rao et al., 2014). Its other biological functions include; immune enhancer, strong coloring agent, protects against UV light and is a strong potent antioxidant.
(Guerin et al., 2003). Other carotenoids of importance include β-carotene and lutein whose most important applications are as natural food colorants and in animal feed (Vilchez et al., 1997; Del Campo et al., 2000).

2.2.5.3 Fatty acids

Helena et al. (2011) reported that Pratt et al. (1944) isolated the first antibacterial compound from a freshwater green microalga, Chlorella. The compound chlorellin which is a mixture of fatty acids was found to be responsible for the inhibitory activity against both Gram positive (+) and Gram negative (-) bacteria. From then on, researches aimed at identifying active antibacterial compounds by microalgae has been on the rise (Ghasemi et al., 2004). Antimicrobial activity has been more attributed to long-chain unsaturated fatty acids (C₁₆ to C₂₀) such as palmitoleic, oleic and linolenic acids. Likewise, long-chain saturated fatty acids which includes palmitic and stearic acids are known to have the same effect although to a lesser extent (Plaza et al., 2010). The fatty acids reduce nutrient uptake, inhibit cellular respiration and promote membrane damage that will eventually permit cell leakage (Smith et al., 2010).

2.2.5.4 Vitamins

Microalgae are known to be a non-conventional source of vitamins because they possess several lipid-soluble and water vitamins in much higher concentrations over known conventional food (Kay, 1991; Zhang & Lee, 1997). According to Abd El Baky & El Baroty (2013), the vitamins contents in microalgae such as Vitamin C, B₁ and B₂ are significantly higher than those contained in higher plants. Biological functions of Vitamin C include; strengthening the immune system, trapping free radicals, regenerating Vitamin E, activating intestinal absorption of iron (Burtin, 2003). B Vitamins (B₁, B₂, B₁₂) are used in the treatment of anemia, effect of aging and chronic fatigue syndrome (Herrero et al., 2013).
2.2.5.5 Polysaccharides

Studies carried out with *Scenedesmus* spp. and other microalgae have shown that certain polysaccharides have medical effects (Skjanes *et al.*, 2013). These polysaccharides function as a protection against oxidative stress, have efficacy on gastric ulcers, wounds and constipation (Iwamoto, 2004; Spolaore *et al.*, 2006). Borowitzka (1995) also reported microalgal polysaccharides as having antibacterial properties.

2.2.6 Other Applications of *Scenedesmus* spp.

Some applications of *Scenedesmus* sp. in industries are discussed briefly below;

2.2.6.1 Bio hydrogen (BioH\(_2\)) production

*Scenedesmus* sp. has been used as a feedstock for BioH\(_2\) production as a source of biofuel to power both light and heavy – duty vehicles, as well as jet and marine engines (Gouveia *et al.*, 2012). Currently all major car producers offer cars running on hydrogen as fuel. It was discovered that *Scenedesmus* sp. was able to produce hydrogen and this discovery has led to the search for a way to use this algae to convert solar energy into this useful energy carrier (Skjanes *et al.*, 2013).

2.2.6.2 Potential source of biodiesel

Due to global warming and exhaustion of fossil fuels which has become a worldwide problem resulting from the emission of greenhouse gasses (GHG), attempts have been made to find alternative source of energy from various biological materials such as plants, animal fat and microalgae (Pandian and David 2012). In a study carried out by Pandian and David (2012), they showed that *Scenedesmus* sp. had a high oleic acid content of about 52.8 % making it most suitable for the production of good quality biodiesel.
2.2.6.3 Wastewater treatment

Treatment of waste is an important problem in the world due to the increase in population and industrial activities. Algal technology is an important alternative source of solution to this problem as algae biovolume is used to remove unwanted materials such as heavy metals, textile dyes and excess fertilizer. Agricultural and municipal wastes contain all the macro and micro-nutrients that are needed for algal growth; therefore it is economical to grow algae in wastewater (Toyoub et al., 2008). Because of the special ability of *Scenedesmus* sp. to adapt to different environmental conditions, it is being used in domestic and industrial wastewater treatment. It has recently been used in removing heavy metals and in the production of oxygen and in converting waste products into beneficial substances (Abuzer et al., 2008).

2.2.6.4 Feed for aquaculture organisms

Various microalgal species have become a preferred natural feed for many aquaculture organisms including oysters, mussels, brine shrimp and fish larvae. In a study reported by Yamaguchi (1997), it was shown that a diet of dried microalgae biomass or extracts could improve the quality of cultured fish, particularly in terms of enhancing resistance to diseases and improvement of flesh quality. *Scenedesmus* sp. is one of the most popular food sources used in experiments with herbivorous zooplankton (Natrah et al., 2007).

2.2.6.5 Medicine

All algae contain one or more type of Chlorophyll (a, b or c) and so does *Scenedesmus* sp. (Karen et al., 2000; Guedes et al., 2013). Apart from their use in food and pharmaceuticals as colorants, chlorophyll derivatives exhibit health promoting features which have been used traditionally in wound healing and anti-inflammatory conditions as well as control of calcium oxalate crystals and internal deodorization (Gouveia et al., 2008).
2.2.6.6 Human Nutrition

Microalgae have a prospect of enhancing the nutritional content of conventional food preparations and to also act as probiotic agents that positively affect the health of humans. Today, they are being marketed as health food or food supplement, and are commonly sold in the form of tablets, capsules, and liquids (Becker, 2004). Algae are also incorporated into pastas, snack foods, candy bars or gums, in drink mixes, and beverages, either as nutritious supplement, or as a source of natural food colorant. (Spolaore et al., 2006; Gouveia et al., 2008).

*Scenedesmus* is among the most used microalgae that has attracted the attention of manufacturers in the food and health-food market (Chacón-Lee and Gonzalez Marino, 2010). Compared to casein, *Scenedesmus* has a very high nutritional quality and several toxicological assessments on it have not revealed any toxic impacts or abnormalities in experiments with test animals (Becker, 1984). Gross et al. (1978) carried out a nutritional study by incorporating *Scenedesmus* sp. into the diet of malnourished children (5 g/daily) and adults (10 g/daily) and a slight increase in weight was discovered. Subsequently, there was a significant improvement in the weight of four year-old children who were fed with microalgae compared to those fed with normal diet. In another study carried out by Natrah et al. (2007), a *Scenedesmus* sp. among other microalgae was shown to possess antioxidant properties and biochemical contents that could be applied in the nutraceutical industry. Other active metabolites have been applied in various industries including; pharmaceutical, food, cosmetics, energy, aquaculture, medicine and others (Table 2.3)
Table 2.3: Some valuable metabolites found in *Scenedesmus* sp.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Applications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycosporine-like amino acids, sporopollenin</td>
<td>Cosmetics (UV – screening compounds)</td>
<td>Indira and Biswajit, 2012; Skjanes <em>et al.</em>, 2013.</td>
</tr>
<tr>
<td>Chlorophyll a, b, c</td>
<td>Food colorants, Pharmaceuticals, cosmetics</td>
<td>Karen <em>et al.</em>, 2000; Gouveia <em>et al.</em>, 2008; Indira and Biswajit, 2012; Guedes <em>et al.</em>, 2013.</td>
</tr>
<tr>
<td>Monounsaturated, polyunsaturated and Saturated fatty acids (Oleic acid, lauric acid, palmitic acid, linoleic acid, α-linoleic acid, stearic acid, and others.)</td>
<td>Biodiesel, Pharmaceuticals, Animal and Human nutrition, Aquaculture.</td>
<td>Ahlgren <em>et al.</em>, 1992; Becker, 2004; Kim <em>et al.</em>, 2007; Gouveia and Oliveira, 2009; Pandian and David, 2012; Mahale and Chaugule, 2013.</td>
</tr>
</tbody>
</table>
2.3 Biotechnology of *Scenedesmus* sp. in food formulations

In the 1950’s, an increase in world population lead to the search for alternatives in case of shortages of protein sources and food, hence algae was proposed (Huntley and Redalje, 2007; Spolaore *et al.*, 2006). Various aquatic algae, including the green microalgae *Scenedesmus* have been studied for their possible use in applications as fish feed, human food supplement, human nutrients and pharmaceutical products (Belay *et al.*, 1993) and also for bioremediation of polluted water (Chong *et al.*, 2000). Microalgae have been used for food by humans thousands of years ago and this was started by the Chinese who used Nostoc to survive a famine (Milledge, 2011). Other well-known microalgae used for food by humans include; *Spirulina* by ancient people of Chad and Mexico (Chisti, 2007; Henrikson, 2008) *Aphanizomenon* (Singh *et al.*, 2005), *Dunaliella* (Chacon-Lee and Gonzalez-Marino, 2010), and *Chlorella* (Spolaore, 2006). With these discoveries, there was an increased interest in the unique bioactive compounds in microalgae which makes them a good candidate as a source of food and other functional molecules (Chacon-Lee and Gonzalez-Marino, 2010). In the past decade, over 75% of microalgae biomass produced have been dedicated for manufacturing tablets and capsules from it (Pulz and Gross, 2004). *Scenedesmus* amongst other more well-known microalgae such as *Chlorella, Dunaliella, Spirulina,* and *Haematococcus* are gradually gaining acceptance in the food and health-food industry (Chacon-Lee & Gonzalez-Marino, 2010).

The food industry seem to be focused on producing food products with low fat or sugar content and a higher fiber content due to consumer demands. With an increased demand for healthy foods, microalgae with its array of unique bioactive compounds with antibacterial properties can be tapped and included in food formulations to protect against food borne diseases. All over the world commercial production of microalgae for human nutrition is already a reality. Numerous combinations of microalgae or mixtures with other health foods can be found in the market in the form of tablets, powders, capsules, pastilles and liquids, as nutritional supplements (Gouviea, 2008). They can also be incorporated into food products (*e.g.* pastas, biscuits, bread, snack foods, candies, yoghurts, soft drinks), providing the health promoting effects that are associated with microalgal biomass, probably related
to a general immune-modulating effect (Belay, 1993). Microalgal pigment has commercial uses as a natural food coloring and cosmetic ingredient. Some microalgae contain substantial amounts of carotene and chlorophyll which are the main components of microalgal pigments and are both tapped for use as food coloring agents. Beta carotene is also used as a food coloring (a major application of it is in providing the yellow color to margarine), and it also enhances the color of the flesh of fish and the yolk of eggs and to improve the health and fertility of grain-fed cattle when ingested as a food additive (Borowitzka & Borowitzka, 1987).

2.4 Food borne microorganisms

Food borne microorganisms are organisms that cause disease through the ingestion of contaminated food. They include a variety of enteric bacteria, aerobes, anaerobes, viral pathogens, parasites, as well as marine dinoflagellates. Pathogens like *Shigella* spp. or the Norwalk-like viruses, require the human host as part of their life cycle (Tauxe, 2002). Food borne pathogens are always transmitted through food, and food poisoning outbreaks take place due to the ingestion of pathogenic bacteria like *Salmonella* sp., *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholera*, *Campylobacter jejuni*, and *Listeria monocytogenes* (Abdul-Mutalib et al., 2015)

2.4.1 Description of *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram positive, non-motile, non-spore-forming spherical, facultative anaerobic bacterium belonging to the *Staphylococcus* genus. The cells are usually spherically single and often grape-like clusters (Figure 2.1). As a facultative anaerobe it can grow under both aerobic and anaerobic conditions but growth occurs at a much slower rate under anaerobic conditions. The *Staphylococcus* genus is subdivided into 32 species and sub species (Le Loir et al., 2003; Montville & Mathews, 2008; FDA, 2012).
2.4.2  Growth and survival characteristics

The growth and survival of *S. aureus* is dependent on a number of environmental factors such as temperature, water activity ($a_w$), pH, the presence of oxygen and composition of food. These physical parameters vary for different *S. aureus* strains (Stewart, 2003). The staphylococcal cell wall is resistant to lysozyme and sensitive to lysostaphin, which specifically cleaves the pentaglycin bridges of *Staphylococcus* species (Le Loir *et al.*, 2003). *S. aureus* are able to grow in a temperature range of 7 - 48 °C with an optimum of 30 °C - 37 °C, pH of 4.2 - 9.3 with an optimum of 7.0 - 7.5 (Le Loir *et al.*, 2003). These characteristics enable the bacteria to survive in a wide variety of foods especially those requiring manipulation during processing including fermented food products like cheese (Le Loir *et al.*, 2003). *S. aureus* is resistant to freezing and survives well in food stored below - 20°C; It is uniquely resistant to adverse conditions such as low $a_w$, high salt content and osmotic stress. (ICMSF 1996; Stewart 2003; Montville & Mathews 2008). Several chemical preservatives including
sorbates and benzoates inhibit the growth of *S. aureus* but the effectiveness of these preservatives only increases as the pH is reduced (Stewart 2003; Davidson and Taylor 2007).

### 2.4.3 Virulence

Some *S. aureus* strains are able to produce staphylococcal enterotoxins (SEs) and are the causative agents of staphylococcal food poisoning (Le Loir *et al.*, 2003; Ono *et al.*, 2008; Thomas *et al.*, 2007). Staphylococcal food poisoning is an intoxication that is caused by the ingestion of food containing preformed SEs (Argudin *et al.*, 2010). There are several different types of SE and to date, 21 SEs have been described with some of them proven to be emetic (Le Loir *et al.*, 2003; Ono *et al.*, 2008; Thomas *et al.*, 2007). SEs are short proteins (194 - 245 aa) which are soluble in water, highly stable, resist most proteolytic enzymes such as pepsin or trypsin and thus keep their activity in the digestive tract after ingestion (Thomas *et al.*, 2007). Enterotoxin A is commonly associated with staphylococcal food poisoning while enterotoxins D, E and H and to a lesser extent B, G, and I have also been associated with staphylococcal food poisoning (Seo and Bohach, 2007; Pinchuck *et al.*, 2010).

Staphylococcal enterotoxins are produced during the exponential phase of *S. aureus* growth, with the quantity being strain dependent. Typically, doses of SE that cause illness result when at least $10^5$- $10^8$ CFU/g of *S. aureus* are present (Seo and Bohach, 2007; Montville & Mathews, 2008). *S. aureus* produces SEs within the temperature range of 10 - 48 °C with an optimum of 40 - 45 °C. As the temperature decreases, the level of SE production also decreases. However, SEs remains stable under frozen storage. They are extremely resistant to heating and can survive the process used to sterilize low acid canned foods. SE production can occur in a pH range of 4.5 - 9.6, with an optimum of 7 - 8. Production of SE can occur in both aerobic and anaerobic environments; however, toxin production is optimum in aerobic conditions (ICMSF 1996; Stewart, 2003). SEs are resistant to the heat and low pH conditions that can easily destroy *S. aureus* bacteria. They are also resistant to proteolytic enzymes, hence SEs retain their activity in the gastrointestinal tract after ingestion. SEs range in size from 22 - 28 kDa and contain a highly flexible disulphide loop at the top of the...
N-terminal domain that is required for stable conformation and is associated with the ability of the SE to induce vomiting (Argudin et al., 2010).

It has been suggested that SEs stimulate neuroreceptors in the intestinal tract which transmit stimuli to the vomiting center of the brain via the vagus nerve (Montville & Mathews 2008; Argudin et al., 2010). In addition, SEs are able to penetrate the lining of the gut and stimulate host immune response. The release of inflammatory mediators such as histamine causes vomiting. The host immune response also appears to be responsible for the damage to the gastrointestinal stomach and upper part of the small intestine. Diarrhea that can be associated with staphylococcal food poisoning maybe due to the inhibition of water and electrolyte re-absorption in the small intestine (Argudin et al., 2010).

2.4.4 Mode of transmission

Staphylococcal food poisoning occurs when food is consumed that contains SEs produced by S. aureus. It frequently involves foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation (Le Loir et al., 2003). S. aureus colonizes in 30 % - 50 % of healthy human population (Lowy, 1998) and the anterior nares of the nose are the most frequent carriage site for the bacteria (Wertheim et al., 2005). Food handlers carrying enterotoxin-producing S. aureus in their noses or on their hands are regarded as the main source of food contamination via direct contact or through respiratory secretions (Argudin et al., 2010).

2.4.5 Foods susceptible to Staphylococcus aureus poisoning

Foods that are frequently susceptible to Staphylococcus aureus poisoning include; poultry and egg products, milk and dairy products, meat and meat products, vegetables, bakery products, particularly cream-filled pastries and cakes, sandwich fillings, salads and salted food products have all been implicated (Wieneke et al., 1993; Qi & Miller, 2000; Tamarapu et al., 2001). Fast food or restaurant prepared food has become a necessity due to the fast pace of living nowadays. In Malaysia, the limitation of time for most people to prepare meals for themselves has increased the demand for food
REFERENCES


http://faculty.css.edu/lmccahey/web/chm102/solutions/concalc.html


