Breakdown of Food Waste by Anaerobic Fermentation and Non-Oxygen Producing Photosynthesis using a Photosynthetic Bacterium

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Abstract

The objective of this study was to measure breakdown of food waste using the photosynthetic bacterium *Rhodopseudomonas palustris* (CGA 009). Large volumes of food waste are produced by restaurants, food centers, hotels, schools, etc generating problems in its collection, processing and disposal. Disposal as garbage increases the organic matter in landfills and landfill leachates. *R. palustris* produces H_2 under anaerobic conditions and digest a very wide range of organic compound. Tap water was used as the basic medium with the food waste providing carbon sources, electron sources, vitamins and minerals. *R. palustris* reduced BOD by ≈ 70 % and COD by ≈ 33%, but had little effect on reducing sugar (2%) or the total phosphorus (1%) in a 7-day incubation. 43% of the starch, 41% of the ammonia, 44% of the nitrate, 37% of the total solid, 16% of the protein and 12% of the lipid was removed. *R. palustris* produced a maximum of 80 ml H_2/g COD/ day but rapidly stopped after a few days. A two-stage anaerobic digestion using yeast as the first stage, followed by a *R. palustris* digestion was tested. The yeast-step lowered the COD, removed much of the reducing sugar and proteins but digestion of oil and starch very low. Yeast removed little phosphorus and increased the total phosphorus by mobilizing organic phosphorus. In the 2^{nd} stage yeast digested food waste was inoculated with 75 ml of *R. palustris* (375 μg BChl *a*). *R. palustris* removed 78% of BOD, 76% of COD, 66% of total phosphorus, 52% of starch, 24% of the protein and 10% of the reducing sugar and the oil. H_2 production from the second-stage *R. palustris* digestion was very low (<13 ml H_2/g COD) *R. palustris* grows well on food waste after partial digestion by yeast and in breaks down cooking oils and starches that yeast cannot digest quickly. The two stage process is highly successful in digesting food waste but not producing H_2.

Keywords: Anaerobic Fermentation, *Rhodopseudomonas*, Food Waste, Photosynthetic Bacteria.

ชื่อวิทยานิพนธ์ การย่อยสลายของเสียประเภทเศษอาหารโดยการใช้กระบวนการหมักแบบไม่ใช้ออกซิเจนและกระบวนการสังเคราะห์ด้วยแสงชนิดไม่ผลิตออกซิเจนด้วยแบคทีเรีย

ผู้เขียน นางสาวนุชสรา เมฆจินดา

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บทคัดย่อ

งานวิจัยมีวัตถุประสงค์เพื่อย่อยสลายของเสียประเภทเศษอาหาร ด้วยแบคทีเรีย *Rhodopseudomonas palustris* (CGA 009) ซึ่งเป็นแบคทีเรียที่สังเคราะห์แสงได้ ของเสียประเภทเศษอาหารสามารถพบได้จากสถานประกอบการต่างๆ ไม่ว่าจะเป็นร้านอาหาร โรงแรมและโรงเรียน ก่อให้เกิดปัญหาต่อกระบวนการจัดการขยะในขั้นตอนการเก็บ และการกำจัดโดยเฉพาะในขั้นตอนการกำจัดของเสียประเภทนี้จะเพิ่มปริมาณสัดส่วนของสารอินทรีย์ในหลุมฝังกลบและน้ำชะขยะได้ งานวิจัยนี้จึงได้นำแบคทีเรีย *R. palustris* มาใช้ในการย่อยสลายเศษอาหาร เนื่องจากแบคทีเรียประเภทนี้สามารถย่อยสลายสารอินทรีย์ได้หลายชนิด และยังผลิต H_2 ได้อีกด้วย จากผลการทดลองพบได้ว่าน้ำประปาสามารถเป็นแหล่งพลังงานและสารอินทรีย์เมื่อผสมกับเศษอาหารได้ดี โดยมีร้อยละการย่อยสลายของ บีโอดี แป้ง ซีโอดี น้ำตาลรีดิวส์ ฟอสฟอรัสทั้งหมด แอมโมเนียไนเตรท ของแข็งทั้งหมด โปรตีน และน้ำมันเท่ากับ 70, 43,33, 2, 1 41, 44, 37, 16, และ 12 ตามลำดับ นอกจากนี้สามารถผลิตแก๊สไฮโดรเจนได้สูงสุดเท่ากับ 80 มล./กรัม ซีโอดี/วัน และระบบจะหยุดผลิตแก๊สภายในเวลาเพียงไม่กี่วัน ในกระบวนการย่อยสลายอาหารจะประกอบด้วย 2 ขั้นตอนได้แก่ (1) การย่อยสลายแบบไม่ใช้ออกซิเจนโดยยีสต์ (*Saccharomyces cerevisiae*) โดยยีสต์สามารถย่อยสลาย ซีโอดี น้ำตาลรีดิวส์ และโปรตีนได้ดี แต่ย่อยสลายน้ำมัน แป้ง และฟอสฟอรัสได้น้อย และปริมาณฟอสฟอรัสเพิ่มขึ้น (2) กระบวนการย่อยสลายเศษอาหารจากยีสต์ โดยใช้ *R. palustris* 75 มล. (แบคทีเรียคลอโรฟิลล์ เอ 375 ไมโครกรัม) โดยผลการทดลอง *R. palustris* ย่อยสลายร้อยละบีโอดี ซีโอดี ฟอสฟอรัส แป้ง โปรตีน น้ำตาลรีดิวส์ และน้ำมัน เท่ากับ 78, 76, 66, 52, 24, 10 และ 10 ตามลำดับ ผลิตแก๊สไฮโดรเจนได้ต่ำประมาณ 13 มล./กรัม ซีโอดี/วัน จากผลการศึกษาชี้ให้เห็นว่า *R. palus*tris เจริญเติบโตได้ดี สามารถใช้น้ำมัน และแป้งได้ดีกว่ายีสต์ ย่อยสลายของเสียประเภทเศษอาหารได้ แต่ผลิตแก๊สไฮโดรเจนได้ปริมาณที่ต่ำมาก

คำสำคัญ: Anaerobic Fermentation, *Rhodopseudomonas*, Food Waste, Photosynthetic Bacteria.

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Nutsara Mekjamda

Contents

Contents (cont.)

List of Tables

List of Figures

List of Figures (cont.)

List of Figures (cont.)

CHAPTER 1

Introduction

1.1 Statement of the Problem

Food waste is found everywhere such as restaurants, food centers, hotels, schools, etc. Moreover, food waste has large numbers of used toothpicks and some chopsticks and paper as is solid waste. Food waste is the one of the main types of municipal solid waste (Zhang and Jahng, 2012). Food waste has presented many management problems in its collection, processing and disposal. Disposal of food waste is an increasing environmental problem. In many parts of the world found food waste problem. In Thailand had many large tourists such as Phuket etc. Phuket has a large tourist industry that is important economically and as a source of waste. The most popular tourist areas in Phuket are beaches such as Patong Beach, Kata-Karon Beach etc. Phuket has an increasing resident population and tourist numbers increase each year (โชติเศรษฐพันธุ์, 2553). The tourist industry has developed very rapidly bringing income. Consequently, tourist industry has an increasing impact on the environment and generates environmental problems such as proper garbage disposal, water supply air pollution etc. Further, Phuket has a lot of food trolleys selling food and stalls selling food on the roadside and this adds to the already large amounts of food waste in Phuket. There are concerns about disposal of food waste discarded in bins and left to wash into drainpipes. Most food waste was formerly used by pig farms (Westendorf *et al*., 1998). Pig farms in Phuket used most of the restaurant and hotel food waste. Today Phuket has a few pig farms because of increasing price of land and undesirability of pig farms in tourist areas of Phuket. This makes it necessary to develop alternative methods of disposal of food waste discarded in bins. In the past, food waste was collected with other municipal solid waste and sent to landfills or incinerators for final disposal. As landfills have been closed at an alarming rate and fewer incinerators have been under construction in recent years, waste disposal has become a serious problem in many Thai cities.

Since food waste has high moisture content, a high organics-to-ash ratio, and a loose physical structure, composting seems to be an ideal disposal method (Chang and Hsu, 2008). Disposal as garbage increases the organic matter in the landfill and the volume of leachate from landfills endangers primary sources of fresh water and causes ground water spoilage and the leachate is a major source of eutrophication in surface waters (Han and Shin, 2002). However, available sites for landfill are lacking and incineration of high-water-containing food wastes requires a large amount of energy. Some researchers have mentioned that treating food wastes in a waste water treatment plant may be more effective due to the high water content instead of it being treated as solid waste (Gonzales *et al*., 2005).

Many different methods have been investigated for dealing with food waste including, anaerobic digestion, on-site composting, fermentation by continuous-flow reactor, hybrid anaerobic solid-liquid, anaerobic digestion using bacteria, digestion using yeast, biological solubilization and mineralization etc (Crine *et al*., 2006; Hwang *et al*., 2002; Han and Shin, 2002; Stabikova *et al*., 2008; Zhang *et al*., 2007; Gonzales *et al*., 2005). However, the treatment system needs to be very easy and very cheap. Microorganisms can be used to modify waste food to breakdown chemical compounds, reduce COD, BOD, the physical and chemical of characteristics of food waste (Cirne *et al*., 2006). Proposals have been made to feed food waste from cafeteria and canteens at PSU-Phuket into anaerobic digesters or anaerobic fermentation using methanogenic bacteria in sewage plants. Unfortunately, sewage is usually heavily contaminated with heavy metals and dangerous organic compounds and so the digest sludge is not very useful. Hence treating the food waste stream separately has the advantage that the digested sludge derived from food waste would have a very low heavy metal content and so would be potentially recyclable. Purple non-sulfur bacteria have been used very successfully in treating swine waste water from piggeries (Kim and Lee, 2000; Kim *et al*., 2009). Studies show that these bacteria can remove or detoxify many organic compounds, organic acids, COD and phosphate (Kim *et al*., 2004, Larimer *et al*., 2004). They have a wider range of nutrition than nearly all other microbes and so are able to grow on virtually any organic material with the notable exception of cellulose (Larimer *et al*., 2004, KEGG, 2013). It is proposed in this project to investigate the use of photosynthetic bacteria to break down food waste under anaerobic conditions. Kim and Lee (2000) and Kim, *et al.* (2009) found that *Rhodopseudomonas* grew very well photosynthetically

in an open pond even though the surface was exposed to the atmosphere. The BOD of the piggery waste was so high that anaerobic conditions were maintained in the pond.

It is interesting the break-down of food waste using anaerobic fermentation and non-oxygen producing photosynthesis using *Rhodopseudomonas palustris* because *R. palustris* can produce hydrogen under anaerobic conditions and they can digest vegetable, starch, sugarcane juice and whey to produce hydrogen gas. Sterile food waste was used in this study to ensure that any effects on NH_3, NO_3^-, P, BOD, COD and gas production must have been due to the *Rhodopseudomonas* inoculum and not a result of the metabolism of any organisms already present.Raw food waste would contain many different and highly variable populations of microbes.A particular advantage of *Rhodopseudomonads* is that they are able to readily break down lipids. *R. palustris* can produce hydrogen gas from palm oil milling effluent which has a very high oil and fatty acid content (VFAs-Volatile Fatty Acids) (Suwansaard *et al*., 2009). There are very few organic compounds which *Rhodopseudomonas* cannot use as a carbon source (Larimer *et al*., 2004; KEGG, 2013).

1.2 Objective

Breakdown of food waste using anaerobic fermentation and non-oxygen producing photosynthesis processes using *Rhodopseudomonas palustris.*

1.3 Scope

I did an investigation on using anaerobic fermentation and non-oxygen producing photosynthesis to deal with the food waste from cafeterias (PSU, Phuket). It was found that *Rhodopseudomonas* successfully broke down sugars, carbohydrates, lipids and proteins of the food waste and significantly reduced BOD and COD. The microbe in project was *Rhodopseudomonas palustris* (CGA 009) (Larimer *et al*., 2004; Ritchie and Runcie, 2013). The advantage of using this strain of *Rhodopseudomonas* was that it is the same strain, from the same laboratory as that used by Larimer, *et al.* (2004) for sequencing *Rhodopseudomonas palustris*

1.4 Expected Outcome

Basic information for waste garbage management and the methods can be used to reduce the organic matter in the wastewater and leachate before treatment wastewater in Phuket. Digestion using *Rhodopseudomonas* should produce useable amounts of hydrogen gas and the spent sludge could be safely used in agriculture. Sludge digests from domestic garbage or sewage are often unsafe to use in agriculture because they have unacceptable amounts of heavy metal and toxic organic compounds. Some results of the present study have been presented at two international conferences as refereed conference papers (Mekjinda and Ritchie, 2013a,b).

CHAPTER 2

Literature Review

2.1 Food Waste

Food waste is found everywhere such as restaurants, food centers, hotels, schools, etc. moreover, food waste has large numbers of used toothpicks and some chopsticks and paper that is solid waste. Food waste is the one of the main types of municipal solid waste (Zhang and Jahng, 2012) and represents the bulk of the organic matter in municipal waste. Food waste accounts for 11.7% of municipal solid waste (MSW) in the United States and 20–45% in Asia and European nations (Chang and Hsu, 2008). Food waste is a type of garbage that has a very high BOD and COD and a high nitrogen content (Cirne *et al*., 2006). It is important to point out that food waste is a lipid-rich waste containing large amounts of used cooking oil and fats, this is especially the case in oriental food waste where large amounts of cooking oil are used (Kim *et al*., 2009). Food waste has a complex composition (Han and Shin, 2002): if possible measurements should be made of the physical and chemical characteristics of food waste such as lipid, many different sugars, cellulose, ash, C/N ratio, protein, starch, glycerol, fiber and cooking-oil residues (Hwang *et al*., 2002; Han and Shin, 2002; Davis, 2008). Typical analysis parameters measured on food waste using standard methods are: Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Solids (TS), Volatile Solids (VS), Moisture Content (MC), pH, Volatile Matter (VM) (Han and Shin, 2002; Kastner *et al*., 2012; Hwang *et al*., 2002).

Asian food waste is somewhat different to European food waste, for example Korean food waste has a higher moisture content, high oil content, high salinity and very low pH and is predominantly rice-based (Kwon and Lee, 2004).

European style food waste can be simulated by various mixtures of pork lard, white cabbage, chicken breast and potato flakes, to simulate lipids, cellulose, protein and carbohydrates, respectively (Neves *et al*., 2008). European food waste has been found to have the

following approximate composition on a dry weight basis: Sugars total 34%, Fiber 3%, Glycerol 4%, Starch 26%, Carbohydrates 67%, Protein 9%, Total lipid (fat) 12%, Ash 12%. Ready-to-eat packaged food (PRE) has Ash 5%, Total lipid (fat) 9%, Protein 20%, Carbohydrates 66%, Starch 52%, Glycerol 1%, Fiber 1% and Total Sugars, 12% (Davis, 2008). Representative Asian food waste has been found to have the following composition on dry weight basis: Vegetables (Cabbage18%, Carrot 18%), Fruits (Apple 14%, Banana16%), Meat (Fried Chicken 10%), Rice 10%, Egg 10%, Used Tea 4%) (Gonzales *et al*., 2005). Food waste in Thailand contains substantial amounts of pork and fish and food waste in Thailand has a rice content much higher than the 10% reported by Gonzales, *et al*. (2005). Such data can be used to estimate the character of food waste and the effectiveness of treatment process in a reactor system. Furthermore, analysis of food waste shows that different types of food waste vary considerably in Protein Carbohydrate and Fat content.

The chemical properties of foods are important in considerations about how easily they can be broken down (William and Daphne, 2002). Proteins in food waste are usually in a normal or "native" protein form, the polypeptide chain is folded up, the shape being largely determined by weak bond interactions. In this compact folded form, many of the peptide bonds are hidden away inside the molecule where they are not accessible to hydrolytic enzymes. An important step is to denature the native proteins. This is done by acid hydrolysis of the native proteins. Generally cooking partially hydrolyses food proteins.

The main carbohydrates in the diet are starch and other polysaccharides, and the disaccharides sucrose and lactose. Free glucose and fructose also occur. Sugars are linked together by glycosidic bonds to from glycosides. This is an important bond from several viewpoints and is important in how easily a carbohydrate breaks down. For example, starch has œ and ß glycoside bonds and several amylases of different types are needed to break down starch. The last major components of food waste are fats. The physical properties of fats present problems because most are not readily water soluble and fatty acids are only partially soluble. Fat is physically unwieldy in an aqueous medium. Droplets of fat and oils present a very small surface area for attack by microbes and breakdown is rapid only if the droplets are very small. Lipids and starchy material tend to form large globs called grease balls which are highly resistant to breakdown. Lipase can only attack at the oil/water interface and the area of this in a simple

fat/water mixture is insufficient for the required rate of digestion, the surface area/volume ratio is even more unfavourable in the case of a grease ball. The available surface area is increased by emulsifying the fat. In the human digestive system, the monoacylglycerol and free fatty acid produced by lipase action together with bile salts, help to disperse the oily liquid into droplets (emulsifying) and phospholipids in the food also help this process (William and Daphne, 2002). In bioreactors, biodegradable detergents can be added to emulsify fats. *Rhodopseudomonads* are not readily killed by most detergents and emulsifiers and will metabolise them as carbon sources. Depending upon its vegetable content food waste has a substantial amount of cellulose from the cell walls of plants but also has a substantial component derived from food handling such as tooth picks, chopsticks and paper. Cellulose is a carbohydrate that is not readily hydrolyzed. *Rhodopseuodomonas* cannot break down cellulose (KEGG, 2013).

Food waste presents many management problems in its collection, processing and disposal. Disposal is a major problem. Hard decisions have to be made about some way of disposing of it because little land is available for garbage dump landfills, incinerators etc. Landfills and incinerators affect quality of the environment around the area (and hence land values) and leachates from landfills and garbage piles awaiting incineration are generally contaminated with heavy metal, inorganic compounds and toxic organic compounds. Garbage dump leachates are a world-wide problem, typically they have very high salinities, very high ammonia content and high but variable levels of heavy metals (Cameron and Kock, 1980; Bull *et al*., 1983; Chueng *et al*., 1993; Devare and Bahadir, 1994; Xu *et al*., 2006). Food waste is the main source of decay, odor and the organic components leachate in the collection and transportation of food waste due to its high volatile solids (VS; 85–95%) and high moisture content (75–85%) (Kim *et al*., 2009).

Many different methods have been investigated for dealing with food waste including, anaerobic digestion, on-site composting, fermentation by continuous-flow reactor, hybrid anaerobic solid-liquid, anaerobic digestion using bacteria, digestion using yeast, biological solubilization and mineralization (Crine *et al*., 2006; Hwang *et al*., 2002; Han and Shin, 2002; Stabikova *et al*., 2008; Zhang *et al*., 2007; Gonzales *et al*., 2005). There are some low technology methods for recycling food waste such as direct use as fertilizer or feeding it to pigs or poultry. The Ministry of Environment (MOE) of Korea has recommended recycling methods for food

waste for animal feeds, fertilizers and for energy recovery (Kwon and Lee, 2004). Such approaches can be applied in Thailand but as stated above feeding food waste to pigs is not a practical solution in tourist areas such as Phuket. In some situations, however, more sophisticated approaches are needed, for example limited land and competition with tourism has largely removed the pig farm industry from islands like Phuket and Koh Samui. Anaerobic fermentation offers a much better opportunity because this method is easy, cheap and requires very little land. An anaerobic pond for breaking down food waste could be incorporated into existing sewage treatment plants. The digest sludges from digestion would be useable as fertilizer with little danger from heavy metals because food waste if collected separately from garbage will have inherently low heavy metal content.

2.2 Anaerobic Fermentation and Photosynthetic Bacteria

Anaerobic treatment systems have been used to deal with garbage dump leachates, food waste and similar wastes such as piggery waste effluent (Kim and Lee, 2000; Kim *et al*., 2004). In general terms, restaurant waste most closely resembles piggery waste in composition (Forster-Carneiro *et al*., 2007). Methods used for processing piggery waste, kitchen garbage, lipid-rich (cooking oil waste) waste, sago starch processing industry, restaurant waste, chicken faeces, etc. (Stabnikova *et al*., 2008; Hwan *et al*., 2002; Cirne *et al*., 2000; Awg-Adeni *et al*., 2010; Canakci, 2007; Zhang *et al*., 2002) can be applied to anaerobic digestion of restaurant and tourist food waste. Many different designs can be used such as the pilot-scale plant established and operated at the treatment facility for solid municipal waste in Cheonan, Korea (Lee and Chung, 2010). This pilot-scale plant was suitable for large-scale industry because this experimental plant had a large reactor and the pilot-scale plant can produce a lot of hydrogen gas and methane. Han and Shin (2002) developed a continuous-flow reactor which could produce biogas and was used for enhanced acidogenic fermentation of food waste because the reactor could be used to control pH and temperature. Many different methods have been investigated for dealing with food waste including; hybrid anaerobic solid–liquid (HASL) system, single-stage wet anaerobic digestion, once-a-day feeding and drawing-off bioreactor; CSTR (Continuous Stirred Tank Reactor) have been shown to have good performance at elevated temperature, but

only limited organic loads could be used. The FBR (Fluidized Bed Reactor) has been found to be highly stable in operation at high organic loads and continuous stirred-tank reactors and photo bio-reactor system equipped with external light sources and gas collection device have also been successful (Stabnikovav *et al*., 2008; Nagao *et al*., 2012; Lim *et al*., 2008; Kastner and Schnitzhofer, 2012; Li *et al*., 2009; Chen *et al*., 2007). However, some experimental designs of reactors cannot extract such as hydrogen gas and methane. Comparisons with more bioreactor systems may not be very useful because some methods do not improve the quality treatment of solid waste or useable product and the capability of some fermentation processes are low.

Chen, *et al*. (2007) devised a very simple and interesting methodology for a photo-bio-reactor system equipped with external light sources and a simple gas collection device. This basic setup is appropriate for experiments with photosynthetic bacteria because this method has external light sources and is easy to design and set up (Fig. 2.1).

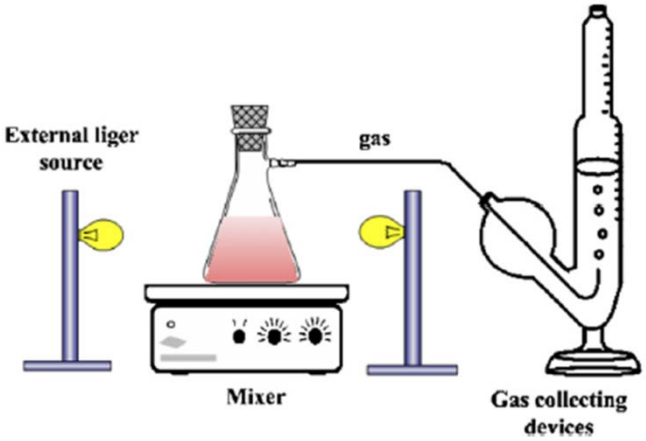


Fig. 2.1 *Photo-bio-reactor system (from Chen et al., 2007).*

Anaerobic fermentation treatment systems can be based on a lot of bacterium species and eukaryotic yeasts. Universally, the major limitations of methanogen bacteria for breaking down food wastes are that they cannot readily break down starches and cannot readily use lipids and fats and do not readily break down large molecular weight organic compounds. There are three other difficulties with methanogen bacteria: (a) many cannot be kept in pure culture, (b) they can be surprisingly sensitive organisms and starter cultures often die

unexpectedly and (c) they are strict anaerobes and readily die if exposed to oxygen and so require specialist anaerobic facilities. Methanogens in sludge digesters are actually a community of many different organisms.

The photosynthetic bacterium *Rhodopseudomonas palustris* has one of the widest ranging metabolisms of any organism (Larimer *et al*., 2004, KEGG, 2013) and is easy to grow and is completely sequenced. It is not only capable of growing aerobically, chemosynthetically and photosynthetically (it uses bacteriochlorophyll and so does not produce oxygen) but it can also grow anaerobically on a huge range of organic compounds including chlorinated hydrocarbons, benzoic acid, oils and recalcitrant organic materials such as lignin. If a culture is started under aerobic conditions and is not aerated, anaerobic conditions rapidly set-in because the *Rhodopseudomonas* will pull the oxygen concentration down to zero. This is a critical advantage in handling over obligate anaerobic organisms. *Rhodopseudomonas* would be able to hydrolyse starch, cellulose, fats and oils by themselves without a complex pre-treatment step. Growing the organism photoheterotrophically requires light, but only low illumination is generally required (Ritchie and Runcie, 2013) and this could be accommodated in a bed-type reactor or pond setup.

Rhodopseudomonas strains are also capable of hydrogen gas production both anaerobically in the dark and photosynthetically using organic compounds as a hydrogen source (Hillmer and Gest, 1977; Mangels *et al*., 1986; Suwansaard *et al*., 2009; Panwichian, *et al*., 2010; Suwansaard *et al*., 2010) *R. palustris* can produce hydrogen gas from palm oil milling effluent which has a very high oil and fatty acid content (VFAs-Volatile Fatty Acids) (Suwansaard *et al*., 2009).

Rhodopseudomonas is not easily killed off as are the strictly anaerobic methanogen bacteria. *Rhodopseudomonas* is tolerant of oxygen and is not a strict anaerobe. Studies on strict anaerobes often require special facilities for handling the organism under strict anaerobic conditions and so a study of methanogen bacteria can be very difficult. *Rhodopseudomonas* is a good choice experimentally for the present project and in later development. Fig. 2.2 shows the general process of fermentation and digestion component of food waste.

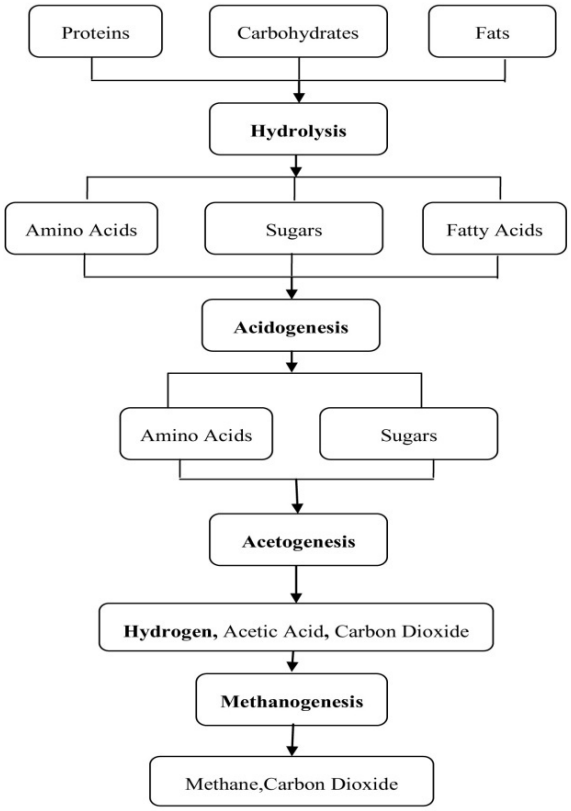


Fig. 2.2 Fermentation Processes. (Adapted ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล, 2554)

2.3 Photosynthetic Bacteria (Purple Non- Sulfur Bacteria)

Purple Non-sulfur Bacteria are prokaryotic cells. Prokaryotes come in only a few shapes and sizes, not rivaling eukaryotes in terms of their structural complexity. They compensate for this by a seemingly unlimited spectrum of physiological specializations. Prokaryotes are literally everywhere: from the upper reaches of the atmosphere to the deepest trenches of the oceans; they live on ice and in boiling acid. Photosynthetic bacteria (Purple Non-sulfur Bacteria) such as *Rhodopseudomonas*are noted for their remarkable biochemical versatility (Larimer *et al*., 2004; KEGG, 2013). The photosynthetic system of *Rhodopseudomonas* has only one photosystem (RC-2) which is very similar in structure to the PSII found in oxygen producing

photosynthetic organisms (Larimer *et al*., 2004; Ritchie and Runcie, 2013). It is based on bacteriochlorophyll *a* as the primary photosynthetic pigment. Water is not used as an electron source; organic compounds, Fe^{2+} and some sulfur compounds are used as electron sources (Ritchie and Runcie 2013). One of the problems in working on photosynthetic bacteria has been that is was difficult to directly measure their photosynthetic rates. Ritchie and Runcie (2013) have shown recently that photosynthesis can be easily estimated in a *Rhodopseudomonas* using the pulse amplitude modulation (PAM) fluorometer method that was originally developed for use in measuring oxygen photosynthesis. The reason why PAM technology works on *Rhodopseudomonas* is that chlorophyll *a* and bacteriochlorophyll *a* both absorb blue light and produce far red/infrared fluorescent light that is detectable using a PAM machine (Fig. 2.3). The response of *Rhodopseudomonas* to various organic compounds can be measured experimentally using a PAM machine.

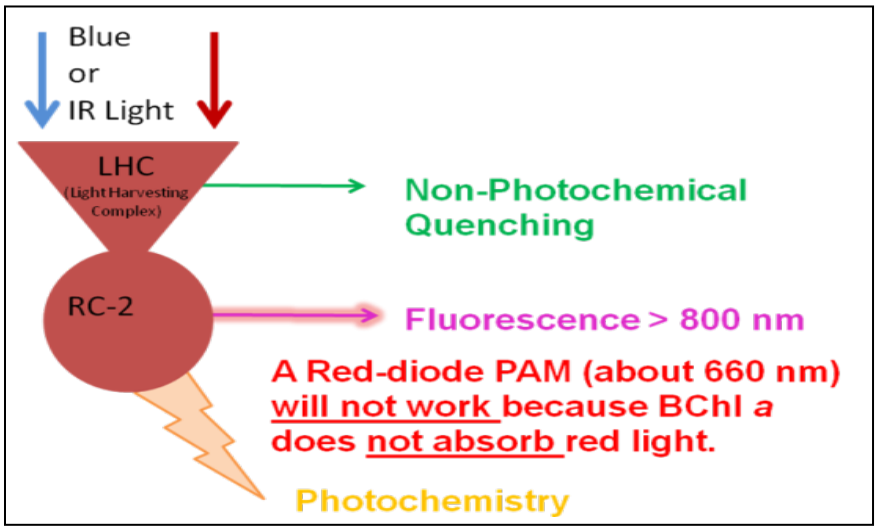


Fig. 2.3 Photosynthesis in *Rhodopseudomonas* (Ritchie and Runcie, 2013).

Purple Non-sulfur Bacteria are *Pseudomonadaceae* family, and all consist of motile bacteria in which the motility is caused by polar flagella and none of them is capable of forming endospores (van Niel, 1944). Bergey's Manual of Determinative Bacteriology classification (van Niel, 1944) has *Rhodospirillum*, *Rhodopila*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodomicrobium*, *Rhodocyclus* and *Afifella* as established genera. Purple Non-Sulfur bacteria are

the most commonly found types of photosynthetic bacteria. Purple Non-sulfur bacteria are characteristically found in lower strata of intact algal mats, but their contributing function in mats survival and function by mediating the chemical environment has not been explored (Mehrabi *et al*., 2001). This present project is on *Rhodopseudomonas palustris* which is the most well known *Rhodopseudomonas* (Larimer *et al*., 2004; KEGG, 2013).

2.3.1 *Rhodopseudomonas palustris.*

Rhodopseudomonas palustris is a *alphaproteobacteria* family, with an outer membrane mainly composed of lipopolysaccharides. They are found in many places in nature such as swine waste lagoons, earthworm droppings, pond water and marine coastal sediment. They are found in both freshwater and saltwater. They are rod-shaped to ovoid, sometimes slightly curved. The typical dimensions are 0.6-0.9 x 1.2-2.0 μm. Their motility is by subpolar flagella. They are very motile and migrate in sediments etc. to find favourable habitats. They are phototactic and migrate to favourable light and redox conditions and often form visible layers in mud and algal mats and in ponds at the aerobic/anaerobic interface (Larimer *et al*., 2004; KEGG, 2013). Genomic Information on *Rhodopseudomonas palustris* (rpa: CGA 009) genes (KEGG, 2014) are glucose, fructose, amylase, lipase, protease, phenol, alcohol, benzoate, acetate, butyrate, glycerol, lipoprotein, and polyphosphatase.

They reproduce themselves by an asymmetrical polar cell division in a budding mode. On the opposite side of the flagella of the mother cell is produced a slender prostheca 1.5 to 2 times the length of the cell. The end of the prostheca swells, and the daughter cell grows. It is then that asymmetrical division takes place (Larimer *et al*., 2004).

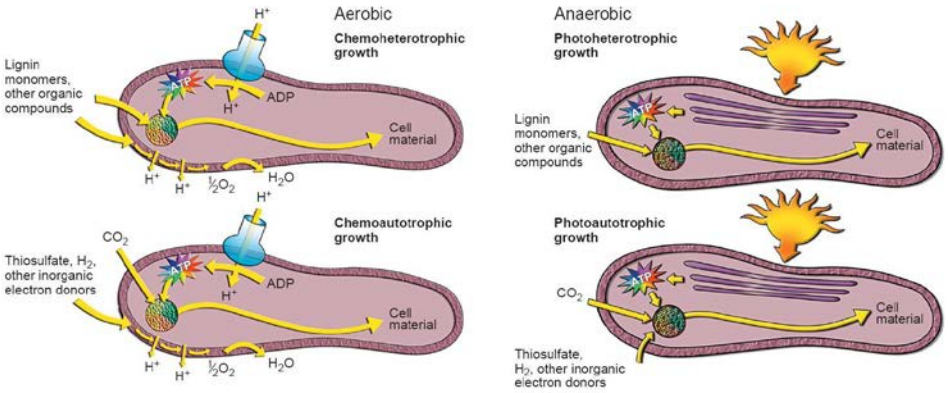


Fig. 2.4 Overview of physiology of *R. palustris*. Representation of the four types of metabolism that support its growth (from Larimer *et al*., 2004).

There are four modes of metabolism that support life in *Rhodopseudomonas* are shown in Fig. 2.4 (Larimer *et al*., 2004):

- Chemoheterotrophic ⟶ Aerobic. Carbon and energy from organic compounds.
- Chemoautotrophic ⟶ Aerobic. Energy from inorganic compound and carbon from carbon dioxide.
- Photoheterotrophic ⟶ Anaerobic. Energy from light and carbon from organic compounds. Light energy is used to rearrange organic compounds that they take up from their environment.
- Photoautotrophic ⟶ Anaerobic. Energy from light and carbon from carbon dioxide. In this study of using *Rhodopseudomonas* to break down organic compounds this 4th mode of nutrition is of less interest.

Chemotrophy: when *Rhodopseudomonas palustris* cannot use light as the energy source they can use reduced compounds as a source of energy. These compounds can be of organic origin (organotroph is organisms using organic molecules for energy production and oxidize a wide array of organic compounds available in their surroundings (William and Daphne, 2002) or inorganic (lithotroph) origin (Larimer *et al*., 2004). *Rhodopseudomonas palustris* can make use of both kinds. Reduced compounds are integrated in the respiration chain and are transformed into chemical energy in the form of ATP. The basic mechanism in the cell membranes is similar to photophosphorylation but cytochrome chain electrons are used instead of light as the driving force.

Phototrophy – Photophosphorylation: there are two basic types of photosynthetic bacteria: those that use sulfur compounds only as a source of electrons and those that can use other electron sources. Purple bacteria are sulfur bacteria that use reduced or oxidized sulfur compounds in their energy transformations (Camacho, 2009). Reduced sulfur compounds, such as sulfide and elemental sulfur, can be used as electron donors for photosynthetic growth by anoxygenic phototrophic sulfur bacteria, or as energy and electron sources by colorless sulfur-oxidizing bacteria. The availability of sulfur compounds in an adequate redox state is consequently a requirement for this group of sulfur bacteria. Most purple sulfur bacteria are obligate photoautotrophs and so are of limited interest in bioremediation projects. *Rhodopseudomonas* (purple non-sulfur bacteria) can use sulfur compounds, Fe^{2+} or manganese and many different organic compounds as electron sources (Larimer *et al*., 2004; Ritchie and Runcie, 2013).

Rhodopseudomonas can use some novel compounds as carbon sources, for example latex. *Rhodopseudomonas* bacteria (strain DK6) can metabolites latex rubber sheet wastewater. DK6 is most closely allied to *Rhodopseudomonas blastica* (Kantachote *et al*., 2005) and removes carbon from the wastewater but needs added fixed nitrogen as ammonia or amino acids. *Rhodopseudomonas* DK6 can reduce very high BOD and COD loads. Moreover, purple non-sulfur bacteria isolated from shrimp pond sediments in Southern Thailand can remove heavy metals and sodium from contaminated shrimp ponds: two strains were isolated, NW16 and KMS24 (Panwichian *et al*., 2010). The isolate NW16 removed a greater percentage of the heavy metals than the isolate KMS24. Furthermore, a strain of *Rhodopseudomonas palustris*, TN1

isolated from Songkhla Lake in Southern Thailand was found to be an effective producer of hydrogen gas (Suwansaard *et al*., 2009; Suwansaard *et al*., 2010) from palm oil mill effluent. *Rhodopseudomonas palustris*, TN1 was also shown to be able to grow on VFAs (Volatile Fatty Acids) and will also grow on added acetate, butyrate, and propionate as carbon sources.

Photosynthetic bacterial digestion of restaurant food waste by photosynthetic bacteria could potentially produce useful amounts of hydrogen gas and would be capable of converting much of the organic matter of a waste effluent into CO_2 and hydrogen gas (Oh *et al*., 2004; Merugu *et al*., 2011).

Nitrogen Fixation – Nitrogenase: nitrogen fixation (biological conversion of N_2 into NH_3) is done by many prokaryote cells by the use of a matell-oenzyme called Nitrogenase. Strains of all 18 species of the family *Rhodospirillaceae* (non-sulfur photosynthetic bacteria) were studied for their comparative nitrogen-fixing abilities (Madigan *et al*., 1984). All species, with the exception of *Rhodocyclus purpureus*, were capable of growth with N_2 as the sole nitrogen source under photosynthetic (anaerobic) conditions (Madigan *et al*., 1984) even though some strains are only able to grow very slowly on N_2 as a nitrogen source. *Rhodopseudomonas palustris* has the capacity to use inorganic nitrogen by breaking the triple bond of the dinitrogen (N_2), hydrogenating the N_2 to form ammonia (NH_3). The catalyzed reaction is as follows:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi$$

This nitrogen fixationcan increase the H_2 production yield from nitrogenase. *Rhodopseudomonas palustris* has the peculiarity of encoding three different nitrogenases in its genome (Larimer *et al*., 2004; Oda *et al*., 2005). A molybdenum-dependent nitrogenase, found in all nitrogen-fixing bacteria, and also a vanadium-dependent and an alternative iron nitrogenase are present (Oda *et al*., 2005)**.** For the simple reason that N_2 fixation is so energetically expensive, *Rhodospeudomonas* like other N_2 fixing organisms do not fix N_2 if a suitable source of fixed N such as ammonia or amino acids are available. This is important in the context of this thesis because food waste contains a large amount of nitrogen sources, in particular amino acids from the breakdown of the protein in the food. Hence, it was not expected that any substantial

N-fixation by *Rhodopseudomonas* would occur when growing on food waste. It was found in the present study that sometimes *Rhodopseudomonas* removed all the available ammonia and so would have switched to N_2-fixation. The linkage of N_2-fixation with H_2 production has the unfortunate consequence that much H_2 production is linked to N_2-fixation and so if photosynthetic bacteria are grown in media with abundant N sources the production of H_2 can be very low.

This survey of the genome of *Rhodopseudomonas palustris* shows that the organism is able to transport and metabolise sugars, starches, alcohols, acetate, glycerol, butyrate, lipids, proteins, lipoproteins etc. *R. palustris* can break down phenols and related compounds such as benzoate. The organism cannot use nitrate (NO_3^-) as a nitrogen source but can use nitrite. *Rhodopseudomonas* is able to use nitrate as an alternative electron acceptor to O_2 in an aerobic respiration (KEGG, 2013). They are able to metabolise polyphosphates and use polyphosphate to store phosphate.

2.4 Hydrogen Gas

Hydrogen gas (H_2) is a clean and renewable energy source. *R. palustris* can produce hydrogen gas from chemoheterotrophically, from CO by water-gas shift reaction or from various sugars by anaerobic fermentation and from organic compounds by an anaerobic light dependent electron transfer process in which nitrogenase functions as the terminal catalyst (Kwon and Lee, 2004; Oh *et al*., 2004; Larimer *et al*., 2004**).** Hydrogen gas can also be produced photosynthetically. Non-sulphur purple bacterial such *Rhodopseudomonas palustris* can use light and both organic carbon compounds and the inorganic electron donor thiosulfate as suitable electron-donating feedstocks and have been intensively studied for photobiological production of hydrogen gas as a potential biofuel (Larimer *et al*., 2004; Huang *et al*., 2010). H_2 is a potential alternative to gasoline for use as a transportation fuel in conjunction with hydrogen fuel cells because it has a high energy content. Furthermore, hydrogen gas can be produced from bacteria in

fermented food waste (Yasin *et al*., 2011). One note of caution should be made about hydrogen production by *Rhodopseudomonas*; it is highly variable from one species or strain to another and also varied greatly according to the conditions under which they are grown (Suwansaarda *et al*., 2009). As pointed out above much of the hydrogen production of Rhodopseudomonads is related to nitrogen fixation.

CHAPTER 3

Materials and Methods

3.1 Microorganism and Culture Conditions.

Apparatus

- Universal bottle size 30 ml
- Duran bottle size 250, 500, and 1,000 ml
- Transfer or Inoculating needle and loop
- Rack
- Alcohol lamp
- Lighter

Procedure

Rhodopseudomonas palustris was a kind donation from Prof Carrie Harwood (University of Washington) and can be grown on PM media (Table 3.1) as described by Ritchie and Runcie (2013). This strain of *Rhodopseudomonas* is completely sequenced (Larimer *et al*., 2004) and is in the KEGG (2013) genomic database.

Table 3.1 Medium for *Rhodopseudomonas palustris*.

PM component	For 1000 ml PM (ml)
Double distilled water	800
0.5M Na_2HPO_4 (MW 142)	25
0.5M KH_2PO_4 (MW 136.1)	25
$CaCl_2 \cdot 2H_2O$ (MW 147)	1
$MgSO_4 \cdot 7H_2O$ (MW 246.5)	1
Concentrated trace element base (Trace elements)	1
2 mg/ml *p*-aminobenzoic acid (PABA)	1
Sodium Acetate (1 M)	10
Sodium Benzoate (1 M)	10
	Bring volume to 1000 ml

- For agar slopes media was poured into 30 ml tubes (about 15 ml of each media/tube).
- Poured agar 0.15g / 1 tube (1% W/v) was added and close tube, shake 1-2 times.
- Autoclaved for 15 min, temperature at 121^0C, and racked up sloping at 45^0 before cooling.

3.2 Preparation of Standard Culture Curve

Apparatus

- Universal bottle size 30 ml
- Transfer or Inoculating needle and loop
- Rack
- Alcohol lamp
- Lighter

Procedure

Standard culture media used for growing *Rhodopseudomonas* contain about 20 mM organic carbon source. Growth conditions were about 25 ^{o}C, pH = 7 and 200 µmol m^{-2} s^{-1} (PPFD). PPFD is Photosynthetic Photon Fluence Density measured using a LiCor Photon Flux Meter (LiCor, Lincoln, Nebraska, USA).

Rhodopseudomonas stock cultures were routinely grown in PM medium which has acetate and benzoate as carbon sources. Culture experiments were also run in a range of culture media with carbon sources similar to those encountered in food waste.

3.2.1 Standard starch culture

1) Carbon-free PM was prepared (No carbon source but with added Thiosulfate)
2) The culture from PM medium was 45 ml and then centrifuged at 5,000 rpm for 10 minute and put into carbon-free PM media.
3) 5g of starch (333.33 mg/ml) was added as a carbon source in 15 ml of carbon- free PM medium.
4) Check Absorbance at 650 nm to follow growth.

3.2.2 Standard vegetable oil (Oleen brand) culture

1) Carbon-free PM was prepared (No carbon but with Thiosulfate)
2) The culture from PM medium was 45 ml and then centrifuged at 5,000 rpm for 10 minute and put into carbon-free PM media.
3) 5 ml of vegetable oil (333.33 mg/ml) was added to every tube (15 ml of carbon- free PM medium).
4) Absorbance at 650 nm (A_{650}) was used to follow growth as A650 vs. time.

3.2.3 Standard glucose culture

1) Carbon-free PM was prepared (No carbon but with added Thiosulfate)

2) The culture from PM medium was 45 ml and then centrifuged at 5,000 rpm for 10 minute and put into carbon-free PM media.

3) Standard glucose 5 ml (333.33 mg/ml) was added to each tube (15 ml of carbon- free PM medium).

4) Check absorbance at 650 nm to follow growth.

3.2.4 Standard BSA (Protein) culture

1) Carbon-free PM was prepared (No carbon but with added Thiosulfate)

2) The culture from PM medium was 45 ml and then centrifuged at 5,000 rpm for 10 minute and put into carbon-free PM media.

3) 11 mg of BSA (0.733 mg/ml) was added to each tube (15 ml of carbon-free PM medium).

4) Check absorbance at 650 nm to follow growth.

3.2.5 Standard glutamate culture

1) Carbon-free PM was prepared (No carbon but with added Thiosulfate)

2) The culture from PM medium was 45 ml and then centrifuged at 5,000 rpm for 10 minute and put into carbon-free PM media.

3) 11 mg every tube of glutamate (0.733 mg/ml) was added to each tube containing 15 ml of carbon-free PM medium.

4) Check absorbance (optical density) at 650 nm to follow growth.

3.3 Characteristics of Food Waste.

Food wastes were collected from the cafeteria in PSU-Phuket Campus, Phuket. Toothpicks, Plastic, Tissues and Chopsticks and bones were first removed and different components of the food: Protein, Carbohydrates, Fat and Oil were analyzed (Table 3.2).

Table 3.2 Characteristics of food waste.

Parameter	Method
Total Solids (TS)	Standard Methods of American Public Health Association (APHA, 1998)
Moisture Content (MC)	
COD (Chemical Oxygen Demand – Closed Reflux Method)	
BOD (Biochemical Oxygen Demand – Azide Method)	
Starch (Iodine Method)	
Reducing sugar (Dinitrosalicylic acid)	
Nitrate (Brucine Colorimetric Method)	
Lipid (petroleum ether as solvent)	
Total phosphorus (Ascorbic Acid Method)	
Ammonia	Standard nitroprusside method (Solozano, 1969)
Protein	Lowry method (Lowry, 1951)

3.4 Experimental Setup for Incubation Experiments

Apparatus

- Two lamps (yellow light)
- Stirrer or magnetic mixer
- Magnetic spin bar

3.4.1 Anaerobic digestion test

1) Preparation cultures for experiment

- The cultures grown in PM medium (45 ml) were centrifuged at 5,000 rpm for 10 minute and the supernatant was poured off.
- Pellet was resuspended in autoclaved tap water (121^{o}C, 15 min).

2) Preparation of food waste

- Food waste was collected from the cafeteria at PSU-Phuket campus, Phuket. Firstly Toothpicks, Plastic, Tissues, Bones and Chopsticks were removed and then homogenized in a blender (2 kilogram of food waste was mixed with ≈ 1,000 milliliter of tap water).
- Mixed food waste was autoclaved after blending at temperature 121^{o}C, 15 min.

3) Experiments were run on experimental medium of various volume of *R.palustris* with food waste.

- 3 times (*R.palustris* 100 ml : food waste 300g)
- 5 times (*R.palustris* 60 ml : food waste 300g)
- 7 times (*R.palustris* 42.85 ml : food waste 300g)
- Control (food waste 300 g)

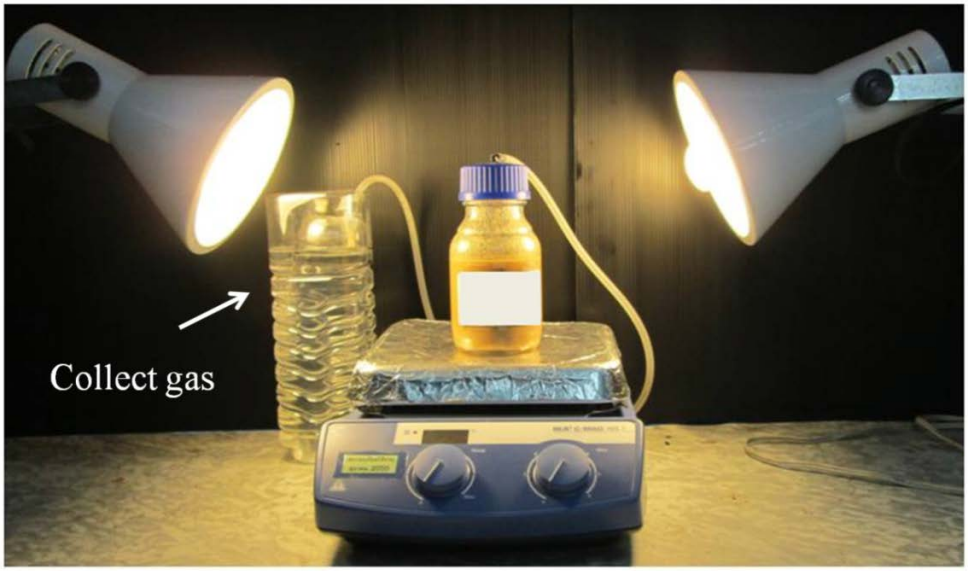


Fig. 3.5 Experimental setup (From Chen *et al*., 2007).

3.4.2 Non-Oxygen Producing Photosynthetic test

A two-stage process was also tried. *Rhodopseudomonas* was grown on spent media from digestion experiments using yeast.

1) First stage incubation was 25 days anaerobic digestion using yeast. The common yeast *Saccharomyces cerevisiae*, in this study was obtained from the Biology Laboratory of Prince of Songkla University, Phuket campus. *S. cerevisiae* stock cultures was grown in liquid medium containing 6 g of peptone, 3 g of yeast extract, 6 g of dextrose, 15 g of agar added to 300 ml of distilled water. Three lots of 125 g FW/l food waste were inoculated with 10 ml of yeast inoculums (about 4.6×10^{6} of cells/ml), under dark conditions, room temperature and shaken all the time.

Fig. 3.6 Experimental setup using yeast.

2) Second stage incubation where the spent yeast culture was inoculated with *Rhodopseudomonas*. The *Rhodopseudomonas* took over the cultures after inoculation.

Table 3.3 Experimental medium of various volumes of *R. palustris* with food waste.

Experiment	**Food waste (g)**	***R.palustris* (ml)**
1	150	75
2		70
3		65
4		60
5		55
6		50

3.5 Hydrogen Gas Analysis by Gas Chromatography

Hydrogen gas (H_2) was quantified using a gas chromatograph Shimadzu 8A Gas Chromatograph. Gas production was measured as volume of gas. The number of moles of gas could be calculated from the universal gas law, PV = nRT where, P is the pressure (101.3 kPa), n is the number of moles of substance (6.023×10^{23}), R is the universal gas constant (8.3143 J K^{-1} mol^{-1}) V is the volume, and T is the absolute temperature (K). At STP 1 mol of an ideal gas occupies 22.4 l or 24.4 l at 25 ^{o}C.

Gas production was measured by collecting the gas in a simple pneumatic trough made out of plastic drink bottles. Centimeters of gas were converted into volume of gas using a calibration curve for the drink bottle used of volume versus cm of water in the bottle (see Fig A2.8). Volume of gas could be converted to mmols of gas based on one mole of an ideal gas at 25 ^{o}C (298K) occupies 24.4 l (Ideal Gas Law, see below).

3.6 Analysis of Cell Concentration during Growth

The relationship between bacteriochlorophyll *a* (BChl *a*) present and cell absorbance at 650 nm was measured using the Shimadzu double-beam spectrophotometer (Oh *et al*., 2004). Optical density of the cells was measured at 650 nm as described by Ritchie and Runcie (2013).For the bacteriochlorophyll *a* assay 5 ml samples of cell suspension were centrifuged at 5,000 rpm for 10 minutes.

Procedure

1) After centrifugation, the liquid was decanted off as much as possible.
2) 2.5 ml of methanol: acetone (3.5:1) was added.
3) Put into freezer for 1 h.
4) After 1 h samples were then centrifuged at 5,000 rpm for 10 minute and the supernatant were used for bacteriochlorophyll *a* determination using the spectrophotometer at 772 and 775 nm and bacteriochlorophyll *a* estimated as described by (Ritchie and Runcie, 2013).

3.7 Statistics

Errorbars shown in this study are standard errors, with at least n = 3 replicates. Linear regressions were fitted using least squares methods.

CHAPTER 4

Results and Discussion

4.1 Preparation of Standard Culture Curve

4.1.1 Preparation *Rhodopseudomonas palustris* calibration curve

Standard culture used 5 ml of cells grew in PM media and bacteriochlorophyll *a* was extracted in methanol: acetone (7:2) and calculation in μg units using the absorbance at 772 nm (Clayton, 1963) (fig. 4.7). Cell absorbance was linearly related to BChl content up to an A 650 nm of 1.4.

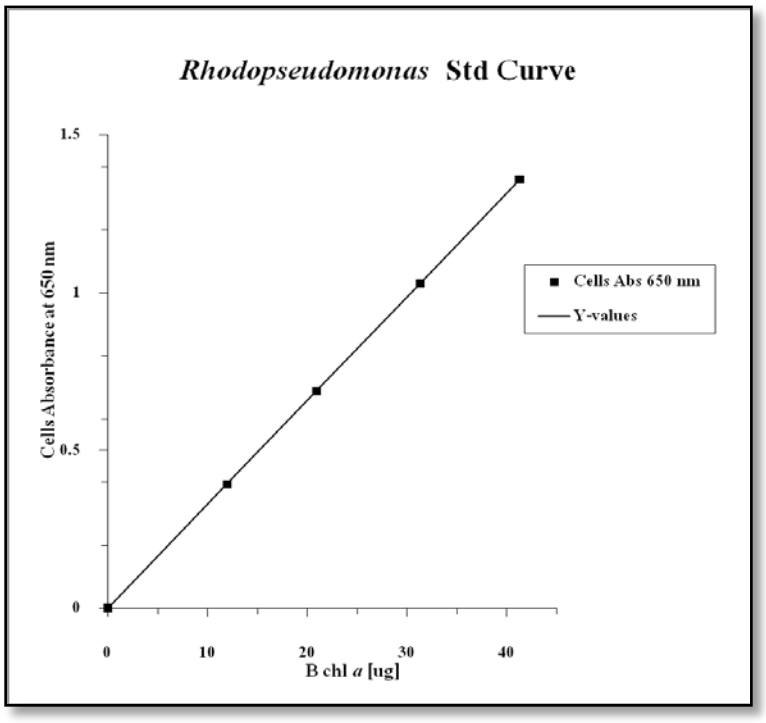
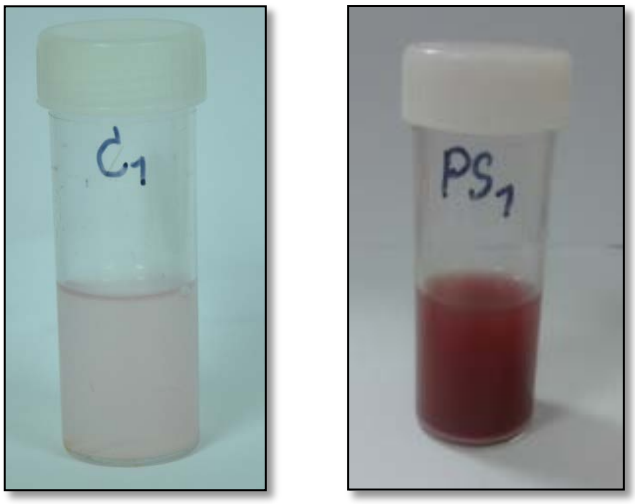


Fig. 4.7 *R.palustris* calibration curve between Cell suspension Abs $_{(650\ nm)}$ and Bchl *a* (μg)**.**

4.1.2 Growth of *Rhodopseudomonas* in some Components of Food Waste.

Rhodopseudomonas can use a very wide range of carbon sources. Preparations of the standard PM media culture including starch, vegetable oil, glucose, BSA and glutamate as carbon sources were incubated for 10 days and bacteriochlorophyll *a* was checked every day. Cells were grown in PM medium with no added carbon source as the control and PM medium with the carbon source to be tested was added.

4.1.2.1 Standard starch culture



(a) (b)

Fig. 4.8 *Rhodopseudomonas palustis* growing on starch (b) and control (a).

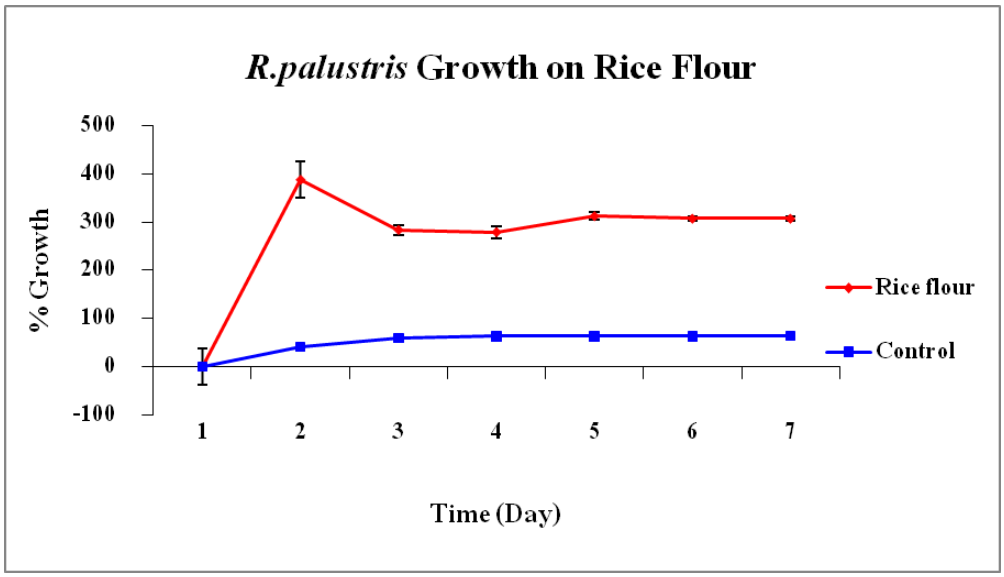
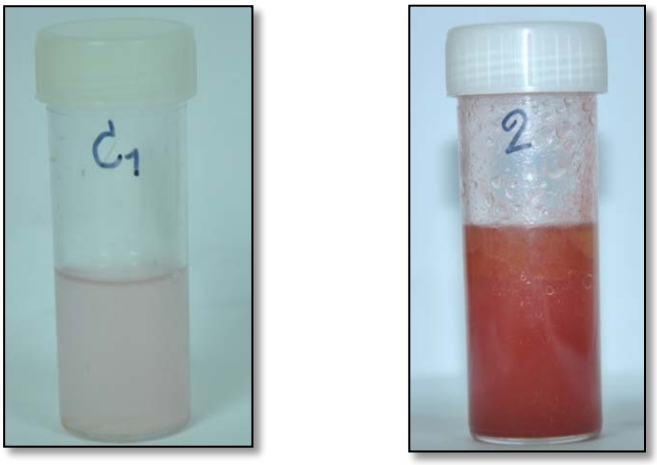


Fig. 4.9 Exponential growth on starch**.**

Fig. 4.8 and fig.4.9 show *R.palustris* can use starch very well. In 2 day % growth is 387 % but in 3 day is decrease of % growth is 283 % they are to growth content in 6 and 7 day % growth is 306 %. The Control was PM media with no added carbon sources (thiosulfate) and the culture did grow to some extent photoautotrophically (fixing CO_2) it grew 61-62 % over the first 4 days. It is known that *Rhodopseudomonas* can readily breakdown starch wastes (Vikineswary *et al*., 1997) and this is consistent with the KEGG genomic data shown in the CHAPTER 2.

4.1.2.2 Standard vegetable oil culture



(a) (b)

Fig. 4.10 *Rhodopseudomonas palustis* growing on vegetable oil (b) and control (a).

Fig. 4.10 shows that *R. palustris* can use lipid (vegetable oil) as a carbon source for growth and grew continuously over the course of the experiment. This is an important result because lipid wastes are a major environmental nuisance.

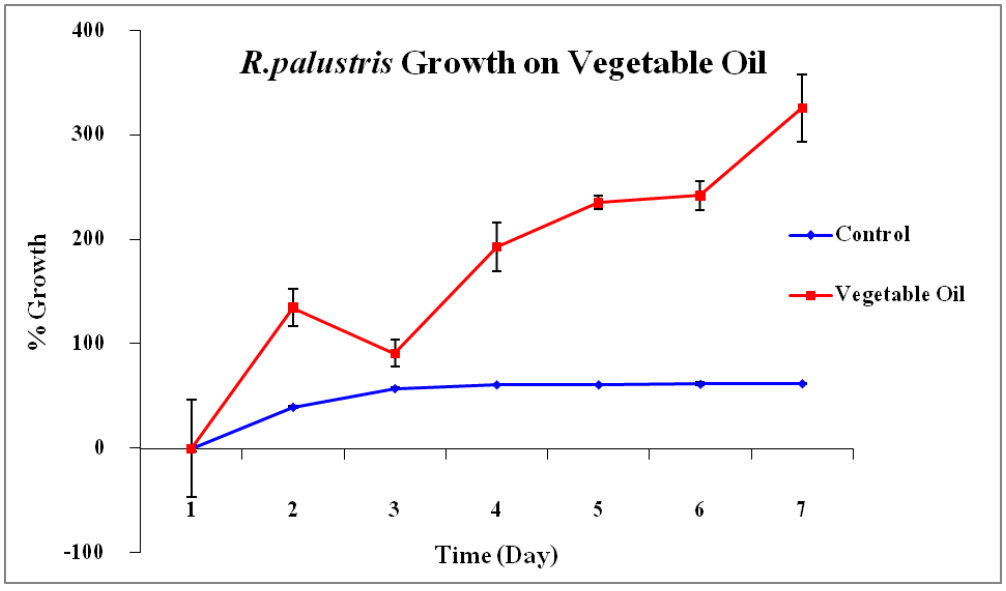
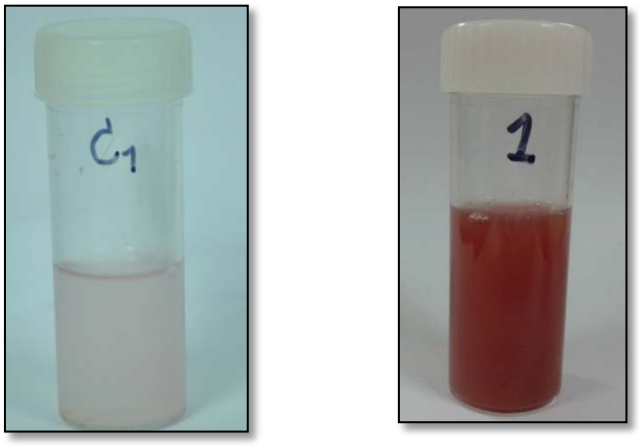


Fig. 4.11 Exponential grew of vegetable oil.

Fig. 4.11 shows standard vegetable oil is *R. palustris* grew very well in 2 day was 135 % growth, 3 day % growth was decrease % growth and growth 91% from 2 day but in 3, 4, 5, 6, and 7 day % growth was increase and then % growth were 193 %, 235 %, 242 %, and 326 % respectively. *R. palustris* grew very well between 1–7 day and after 7 day on day 10 of the experiment the cells had not grown any more than they had on day 7. *R. palustris* can grew on acetate, butyrate and propionate present in anaerobic fermentative treated palm oil milling effluent (Suwansaard *et al*., 2010). This is an important result because breakdown of grease balls and waste fat deposits are a major environmental problem and accumulation of organic matter as grease balls not only slows down breakdown of the oil, fat and grease but other organic matter are bound up with the grease.

4.1.2.3 Standard glucose culture



(a) (b)

Fig. 4.12 *Rhodopseudomonas palustris* grew on glucose (b) and control (a).

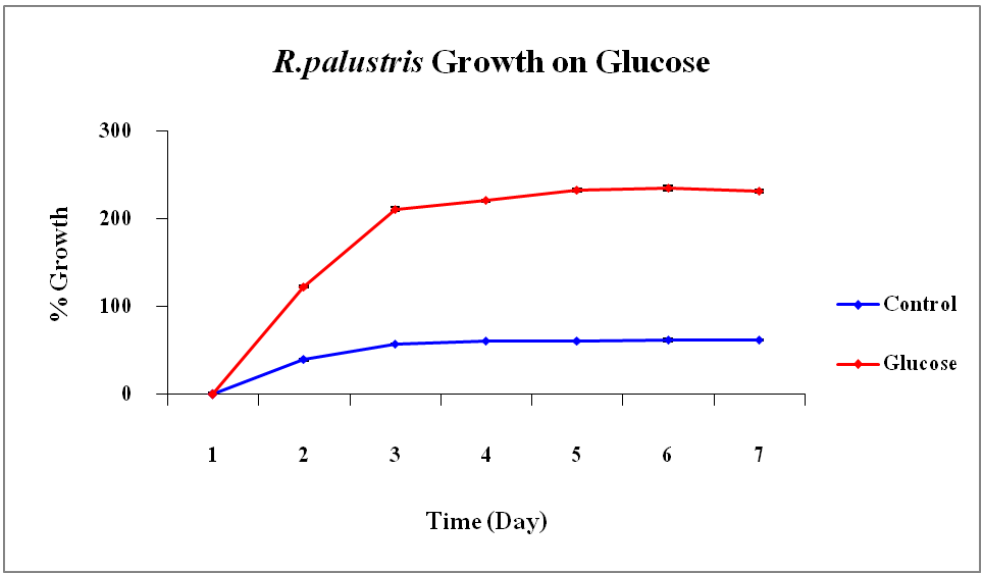
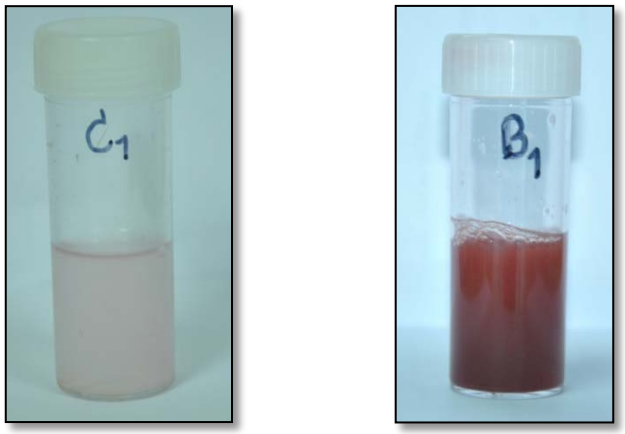


Fig. 4.13 Exponential grew of glucose.

In fig. 4.12 and fig. 4.13 show *Rhodopseudomonas* can grow on glucose. In the first day *R. palustris* grew was 123 % and in 2 day *R. palustris* was 211 % and in 5-7 day growth 233 – 232 % but had reached a stationary phase. Thus *Rhodopseudomonas* can breakdown starch into glucose and use the glucose for growth (Larimer *et al*., 2004).

4.1.2.4 Standard BSA (Protein) culture



(a) (b)

Fig. 4.14 *Rhodopseudomonas palustis* grew on BSA (Protein) (b) and control (a).

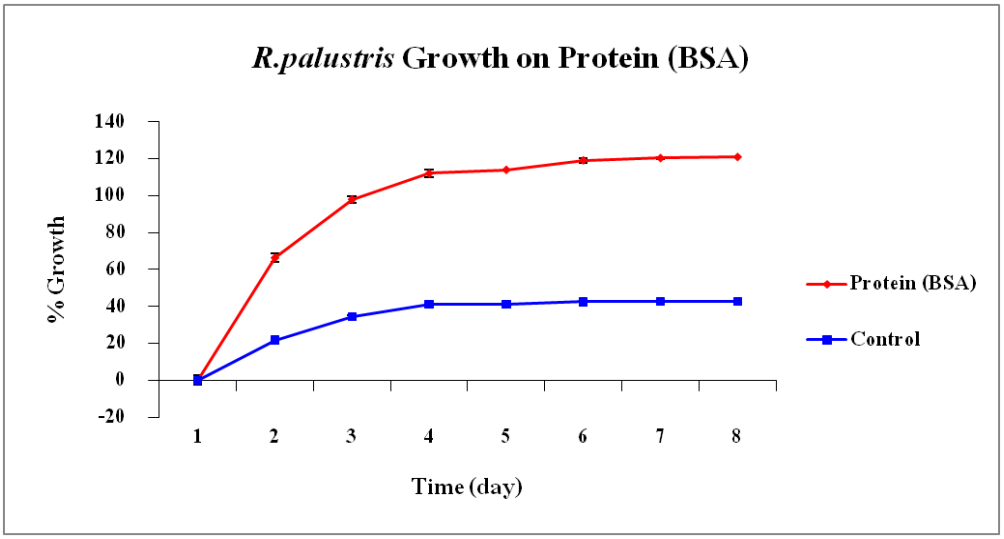
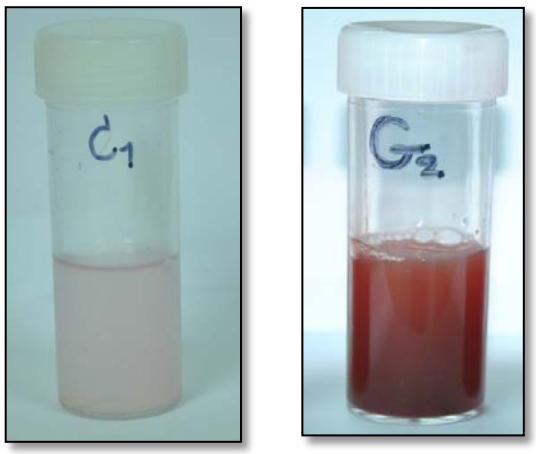


Fig. 4.15 Exponential growth of protein (BSA).

Fig. 4.14 shows growth of *Rhodopseudomonas* on protein (BSA) compare with control (no organic carbon but with thiosulfate). *R. palustis* can use BSA protein very well and after 7 day *Rhodopseudomonas* reached stationary phase and grew of about 120 % (fig. 4.15). This graph is on a different scale to the starch and oil experiments.

4.1.2.5 Standard glutamate culture



(a) (b)

Fig. 4.16 *Rhodopseudomonas palustis* grew on glutamate (b) and control (a).

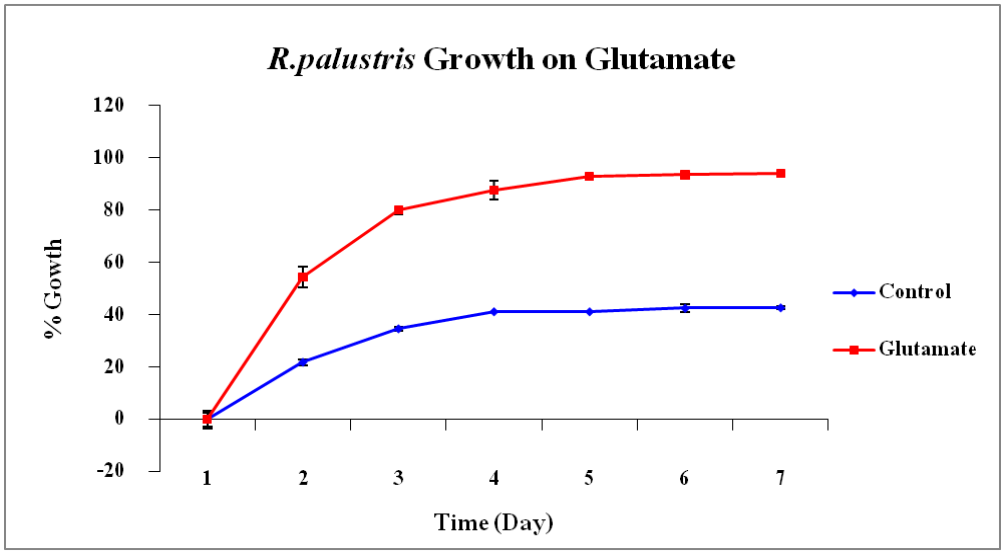


Fig. 4.17 Exponential growth of glutamate.

Glutamate can act as the sole carbon and nitrogen sources for *R. palustris*. The bacterium can grow and use glutamate very well (Fig. 4.17). In 2 day growth is 60 % and in 3 day growth increased by 80%. After 7 day *R. palustris* growth stopped and % total growth was nearly 100 %. *Rhodopseudomonas* grew well on an amino acid source.

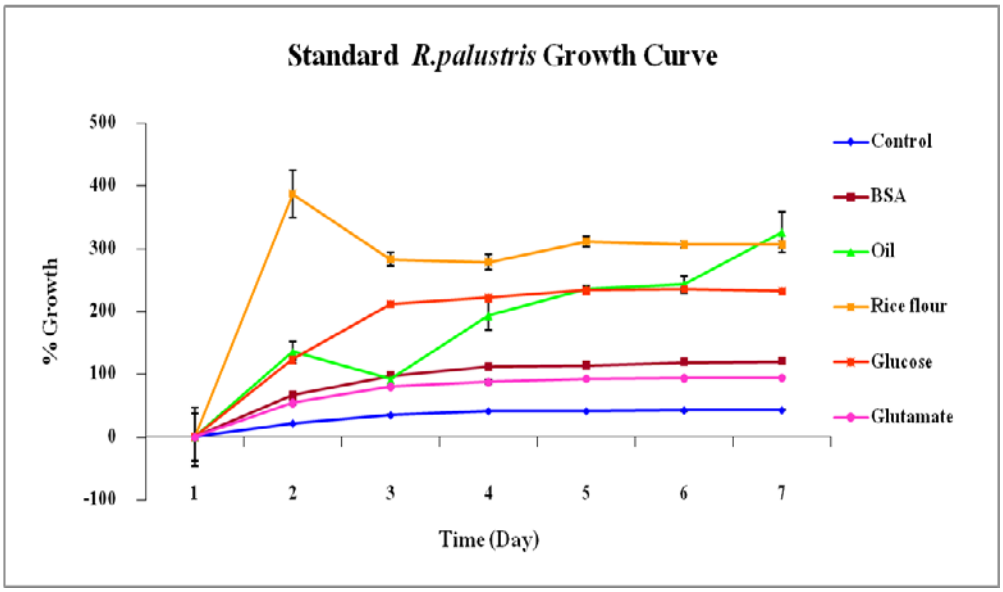


Fig. 4.18 Comparison the total standard *Rhodopseudomonas palustris* growth.

Rhodopseudomonas palustris can grow very well on all the substrates offered to the cells but grows much better on oil, rice flour and glucose than BSA protein and glutamate. After 7 days *R.palustris* growth had reached stationary phase for all the carbon sources except for the cooking oil. The control experiment was PM media with no organic carbon source but provided with thiosulphate as an electron acceptor. The controls grew about 50 % over the course of the experiment. Rolls and Lindstrom (1967) found that 80 to 90% of substrate organic carbon was incorporated into cell material. Very little is lost as CO_2 by the cells because it is fixed photosynthetically if sufficient light is available.

4.2 Characteristics of Food Waste (Before Fermentation)

Food waste was collected from the cafeteria at PSU-Phuket campus, Phuket. Firstly toothpicks, plastic, tissues, bones and chopsticks were removed and then the food waste was homogenized in a blender (Fig. 4.19) (2 kilogram of food waste was mixed with ≈ 1,000 milliliter of tap water). Mixed food waste was autoclaved after blending at temperature 121^{o}C, 15 min. Most of the volume of the waste is cooked rice and the analyzed composition of food waste before fermentation is shown below (table 4.4).

(a) (b)

Fig. 4.19 Mixed food waste before homogenize (a) and after homogenization (b).

Table 4.4 Composition of food waste (before fermentation)

Composition	mg/g DW
Starch	26.96741
Protein	7.1318
Lipid	33.3182
Reducing sugar	5.3565
Total phosphorus	0.1255
Ammonia	2.1381
Nitrate	0.9945
Moisture Content	84 %
COD	18,619 mg/l
BOD	5,372 mg/l
Total solid	177,192 mg/l
FW/DW ratio	5.8314

This table shows that food waste has a great deal of potential as a growth medium for organisms that can readily break down carbohydrates, proteins and lipids. The table shows that the food waste is a lipid-rich waste containing large amount of used cooking oil and fats, this is especially the case in oriental food waste where large amounts of cooking oil are used (Kim *et al*., 2011). The BOD and COD of the food waste was very high. Food waste is a type of garbage that has a very high BOD and COD and a high nitrogen content (Cirne *et al*., 2006). The glycerol content of food waste was not specifically measured but there would have been considerable amounts of glycerol present from hydrolysis of the cooking oils. *R. palustris* is able to readily break down glycerol in biodiesel wastes (Pott *et al*., 2013). The BOD and COD of the food is comparable to industrial effluent from olive mill waste which has been successfully broken down using *Rhodopseudomonas* (Pintucci *et al*., 2013). *Rhodopseudomonas* has also been used successfully to break down palm oil mill waste (Suwansaard *et al*., 2009). *R. palustris* is also very efficient in breaking down fatty acids which would arise from the hydrolysis of cooking oils

(Choorit *et al*., 2011). Breakdown of oils and greases are a major environmental problem and *Rhodopseudomonas* can break down both the glycerol and fatty acid components.

4.3 Anaerobic Fermentation

4.3.1 Comparison of the optimum conditions.

The optimum condition for anaerobic fermentation was using food waste 100 g/FW with 150 ml of *R. palustris* cell suspension (A650 = 0.967 or 29.4 µg BChl a). In the first experiment the culture was mixed with carbon-source-free PM medium (Fig. 4.20) and the second experiment the culture was mixed with tap water (Fig. 4.21) (inoculum A650 = 0.881 or 26.8 µg BChl *a*). Growth of the *Rhodopseudomonas* in food waste was considerable: the dry weight increased by 96%. This was because the cells converted much of the organic matter in the culture medium into cellular dry mass rather than losing it as CO2 (Rolls and Lindstrom, 1967). *Rhodopseudomonas* incubated in food waste in C-free PM media for only 7 days digested 85% of the oil, 78% of the protein and 63% of the starch but they had not reduced the reducing sugars by very much (≈12 %). Starches would have been converted to glucose as it was digested but nevertheless the cells had not reduced the reducing sugar concentration to a low level. It is likely that the reducing sugar content of the medium would have rapidly fallen to zero once the supply of starch had been exhausted. Reduction in the phosphorus concentration was negligible (≈8%) because PM media contains high concentrations of phosphorus. In case of BOD and COD *R. palustris* can digest and can use about 71% of the total COD organic matter for growth on food waste (zero day zero had 110 g/l and last day have 32 g/l) and BOD was reduced by 21 % (the zero day had 880 mg/l and the last day had 693 mg/l). This difference is a result of the anoxygenic photoheterotrophic metabolism of *Rhodopseudomonas* is better at breaking down organicmatter in the food waste than the aerobic bacteria that are responsible for breakdown of organic matter in the BOD test.

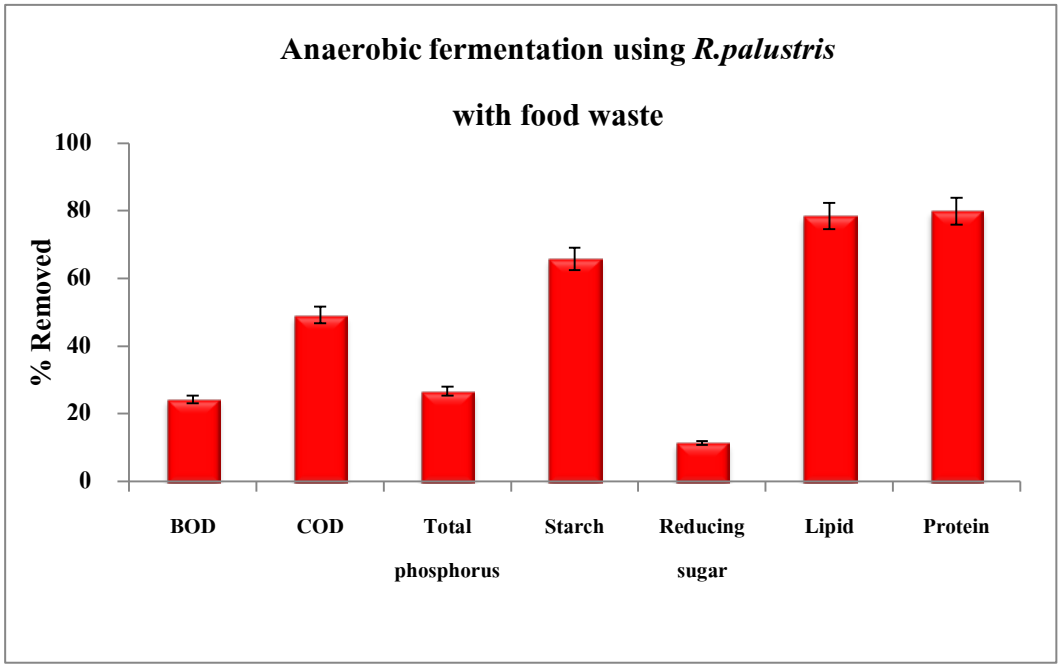


Fig. 4.20 Percent of removal of *R. palustris* in anaerobic fermentation of food waste mixed PM medium.

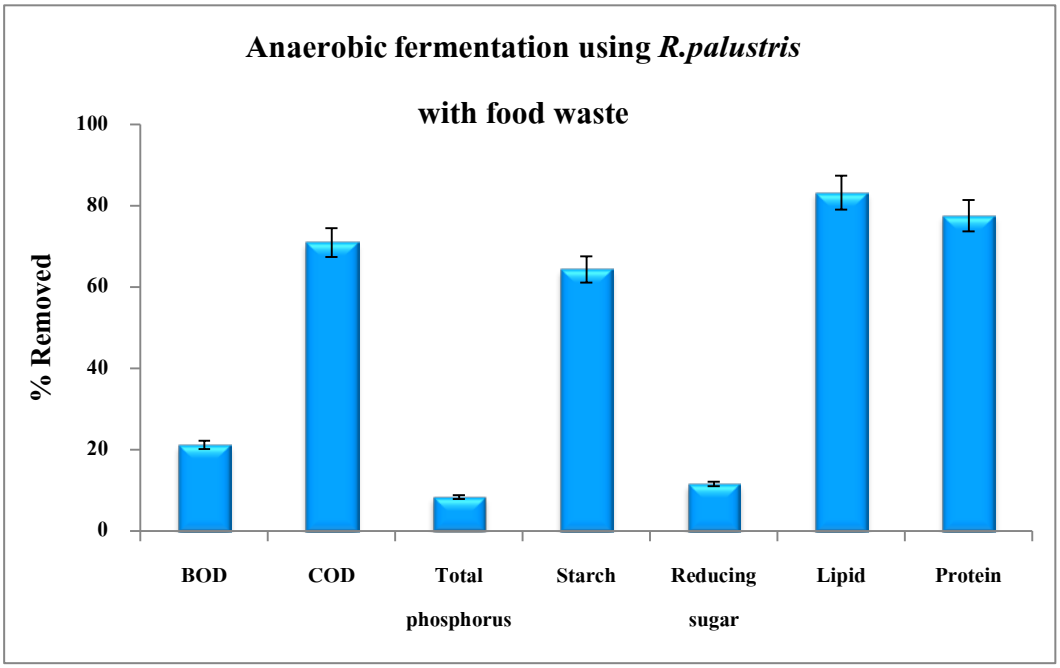


Fig. 4.21 Percent of removal of *R. palustris* in anaerobic fermentation of food waste mixed tap water.

The second experiment involved growing *Rhodopseudomonas* in food waste diluted with tap water and so in practice the sole source of nutrients for the cells was the food waste. *Rhodopseudomonas* grew well in the diluted food waste (Fig. 4.21) and the results were closely comparable to the results using carbon-free PM as the base culture medium with food waste added. In the 7 day incubation *R. palustris* digested 80 % of the protein, 78 % of the lipid, 66 % of the starch, 27 % of the total phosphorus, 11 % of the reducing sugar and reduced the COD by 49 % (before fermentation had COD of 110 g/l and after fermentation had 56 g/l) and the BOD by 24 % (before fermentation had 880 mg/l and after fermentation had 667 mg/l). Comparisons of the two experiments show that in the second experiment *R. palustris* can digest much of the available carbon sources and since there was no added phosphate in the second experiment, the cells removed a larger proportion of the total phosphorus in the incubation medium. In both cases the reducing sugar in the medium did not fall to very low levels even though the cells were using starch which would have to have been broken down first to glucose to be metabolized. Tap water was sufficient for incubating *Rhodopseudomonas* and no extra minerals or vitamins are needed. Tap water could be routinely used for incubating *Rhodopseudomonas* in food waste. Water is not used as an electron source; organic compounds, Fe^{2+} and some sulfur compounds are used as electron sources (Ritchie and Runcie, 2013).

4.3.2 Determination of Suitable Inoculation volume of *R. palustris* to grow on food waste.

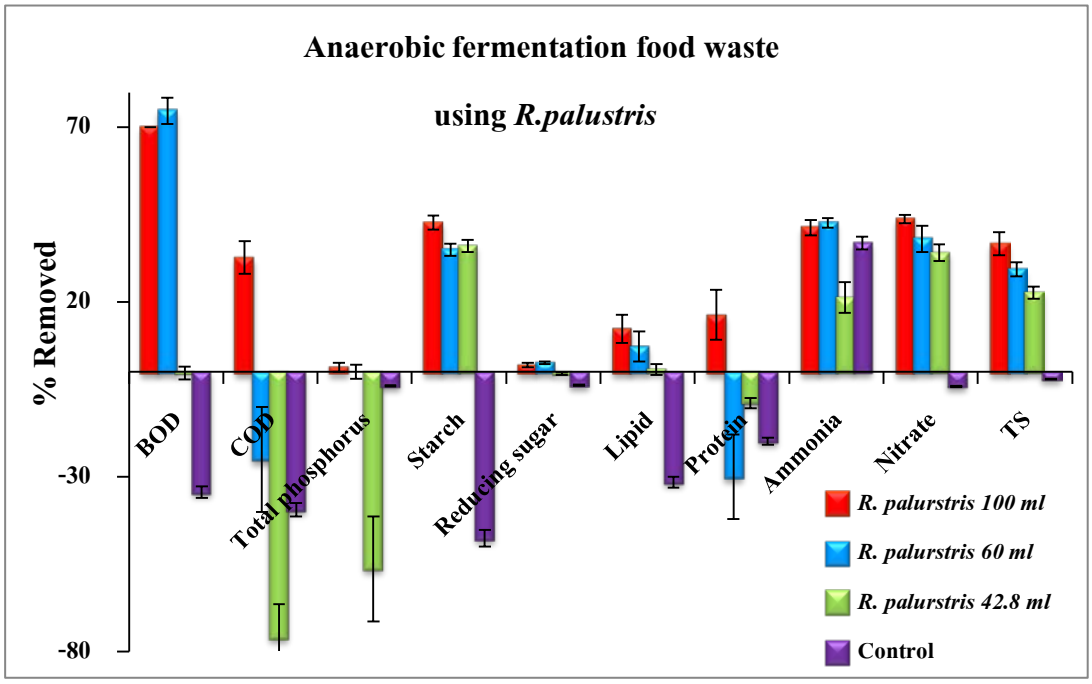


Fig. 4.22 Shown in optimizing volume of *R. palustris* for efficient digestion in 7 day.

Anaerobic fermentation was run on experimental medium of three different inoculation volumes of *R. palustris* with autoclaved food waste and a control (food waste only and no *Rhodopseudomonas* culture). Digestion of starch, reducing sugar, lipids, protein, ammonia, nitrate and reduction of BOD, COD, total phosphorus, and total solid are shown in Fig. 4.22 as percentage removal after 7 days incubation compared to the control at the beginning of the incubation. The waste volume was 300 gm in each treatment and the *Rhodopseudomonas* volumes were 100 ml, 60 ml and 42.85 ml of inoculum (BChl *a* = 9.7 µg/ml of inoculation culture). The *Rhodopseudomonas* cell suspension was centrifuged down at 5000 rpm for ten minutes and resuspended in tap water before being used as an inoculum. Thus the experiment with 100 ml of culture added would have been started with *Rhodopseudomonas* cells containing 97 µg of BChl *a*. All the experiments were incubated in the light on a magnetic stirrer including the control which was not inoculated with bacteria. % Removal of a material is defined as +ve

and apparent increase in the parameter after the 7 day incubation compared to the control at t = 0 is defined as –ve.

The experiment with the largest amount of inoculum contained 100 ml of *Rhodopseudomonas* cells and 300 ml of food waste: the BOD was reduced by about 70 % of BOD and the COD by about 33% (Fig 4.22), however, there was very little change in the reducing sugar (2%) or the total phosphorus (1%). The BOD experiment decreased more than COD as found by Pintucci, *et al*. (2013). *Rhodopseudomonas* digested 43% of the starch, 41% of the ammonia, *Rhodopseudomonas palustris* has the capacity to use inorganic nitrogen by breaking the triple bond of the dinitrogen (N_2), hydrogenating the N_2 to form ammonia (NH_3) but only fixes N_2 if no ammonia is available, 44% of the nitrate. The photosynthetic system of *Rhodopseudomonas* has only one photosystem (RC-2), which is very similar in structure to the PSII found in oxygen producing photosynthetic organisms (Larimer *et al*., 2004; Ritchie and Runcie, 2013). *Rhodopseudomonas* cannot use nitrate as a nitrogen source because it cannot convert nitrate into ammonia (Larimer *et al*., 2004; KEGG 2014), however they can use NO_3^- as an alternative electron acceptor for O_2 in oxidative phosphorylation and so when growing aerobically when they run out of O_2 they start using NO_3^- as an alternative electron acceptor before switching over to fully anaerobic metabolism. *Rhodopseudomonas* broke down 37% of the total solid, 16% of the protein and 12% of the lipid.

Rhodopseudomonads can digest some organic materials ignored by most organisms: for example *Rhodopseudomonas* bacteria (strain DK6) can metabolise latex rubber sheet wastewater (Kantachote *et al*., 2005). DK6 is most closely allied to *R. blastica* (Kantachote, *et al*., 2005) and removes organic carbon from the latex wastewater but needs added fixed nitrogen such as ammonia or amino acids. The *R. palustris* strain used in the present study (CGA009) is capable of fixing nitrogen but there are many strains of Rhodopseudomonads that have a limited capacity to fix-N_2. *Rhodopseudomonas* DK6 can reduce very high BOD and COD loads but requires fixed nitrogen. Moreover, purple non-sulfur bacteria isolated from shrimp pond sediments in Southern Thailand can remove heavy metals and sodium from contaminated shrimp ponds: two strains were isolated, NW16 and KMS24 (Panwichian *et al*., 2010).

It was found in the present study that *Rhodopseudomonas* readily broke down lipids but in certain circumstances lipid compounds can be inhibitory. Different types of organic

compounds can cause inhibition of other compounds, for example photo-metabolism of aromatic acids by *R. palustris* is sensitive to the presence of exogenous fatty acids. Long-chain fatty acids produced during disruption of the cell and associated with the chromatophore particles cause severe inhibition of benzoate and p-hydroxybenzoate photoassimilation when added to a suspension of intact cells. The main component of the isolated fatty acids was an octadecamonoenoic acid with minor amounts of stearic, palmitic, and palmitoleic acids (Dutton and Evans, 1970). Easily metabolized linear fatty acids are preferred over more difficult compounds such as aromatic and phenolic compounds.

The major limitations of methanogen bacteria for breaking down food wastes are that they cannot readily break down starches and cannot readily use lipids and fats and do not readily break down large molecular weight organic compounds. Rhodopseudomonads are a better choice because they readily break down starches, lipids and fats (Larimer *et al*., 2004; see KEGG, (2014) data in the Introduction). There are three other difficulties with methanogen bacteria: (a) many cannot be kept in pure culture and so if a culture fails in a fermentation vat a new starter culture has to be obtained from another sewage treatment plant, (b) they can be surprisingly sensitive organisms and starter cultures often die unexpectedly and (c) they are strict anaerobes and readily die if exposed to oxygen and so require specialist anaerobic facilities. Methanogens in sludge digesters are actually a community of many different interdependent organisms. Rhodopseudomonads have a much better profile of metabolisable organic material. Their major limitations are that they cannot break down pectins or cellulose (KEGG, 2014).

Food waste inoculated with 60 ml of *Rhodopseudomonas* gave very similar results to the experiment with the higher inoculum (Fig. 4.22). BOD was reduced about 70% but the COD *increased* by about 20% (-20%): this was probably due to mobilization of organic compounds by the *Rhodopseudomonas*. Digestion of starch (30%) and lipids (10%) were similar to the results of the higher inoculation and again there had been no substantial reduction in reducing sugars (3%) but proteins had been solubilised without digestion (-30%) compared to the control. About 40% of the ammonia, nitrate and total solids were removed. Soluble protein increased probably due to a physical breakdown of the autoclaved food waste. There was no significant reduction in phosphate.

Inoculation with 42.85 ml of *Rhodopseudomonas* achieved less digestion than the high inoculation volumes. 36% of the starch was removed, 34% of the ammonia and a lesser amount of nitrate (30%). The experiment digested only 1% of the lipid and had no significant effect on the amount of reducing sugar in the culture medium. The *Rhodopseudomonas* broke down some of the food waste without digesting it leading an apparent increase in BOD and COD compared to the control. The same effect resulted in an apparent increase in soluble protein (-9%) and an increase in soluble phosphorus (-56%).

The control experiment was not inoculated with *Rhodopseudomonas* but was incubated for 7 days under the same conditions as the *Rhodopseudomonas* incubations. The control experiment showed an increase in most of the parameters after 7 days incubation. This is probably mainly a mechanical effect of physically breaking down the autoclaved food waste after being stirred for 7 days and there might also have been some photolytic effects. BOD, COD, reducing sugar, lipid all increased about 20-30% compared to the contents of the autoclaved food waste at the beginning of the experiment. About 30% of the ammonia was apparently lost which is comparable to the loss of ammonia observed in the inoculated cultures. This suggests that some ammonia was lost by evaporation in all the experiments and not by assimilation by the cells. There was no reduction in nitrate indicating that the losses of nitrate observed in all the incubations containing *Rhodopseudomonas* was due to the activity of the cells.

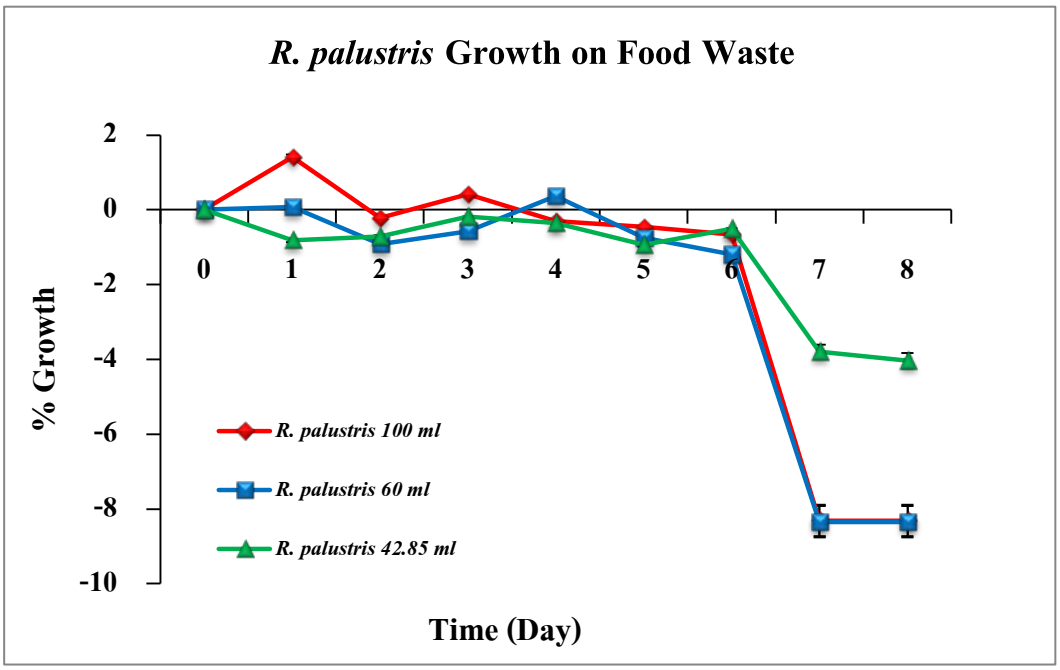


Fig. 4.23 Percent of growth of *Rhodopseudomonas palustris* for efficient digestion of food waste in 7 days.

Rhodopseudomonas palustris can digest a lot of organic compounds in the food waste in experiment but there was not a great growth of the *Rhodopseudomonas* cells. The amount of BChl *a* in the cultures was more or less constant over the first 6 days of the experiments with some loss of BChl *a* on days 7 & 8. The *Rhodopseudomonas* was digesting the food wastes (Fig. 4.22) but not growing very much (Fig 4.23). The bacteriochlorophyll *a* in experiment where 100 ml of *Rhodopseudomonas* cells were added was 1.42% growth, at day 2 and day 3 a slight decrease in growth (-0.22%) and on days 4–7 day they not grow (-0.30%, -0.45%, -0.65%, -8.31% growth, respectively). The experiments where 42.85 ml and 60 ml of *Rhodopseudomonas* cells were added showed little or no net growth from day 1 to day 6 but both then showed a significant decrease of up to -10% growth on days 7 and 8 of the experiment (Fig. 4.23).

4.3.3 Measurements of gas production by *Rhodopseudomonas* and setting suitable proportion between *Rhodopseudomonas palustris* inoculation and food waste.

Gas production experiments were run using suitable inoculations of *R. palustris* culture (100 ml) with food waste 300 g/FW (section 4.3.2). *Rhodopseudomonas* can use the organic compounds of food waste based on tap water and its major components such as protein, starch, oil, reducing sugar (Mekjinda and Ritchie, 2013a,b) etc. Experiments were set up with *Rhodopseudomonas* with autoclaved food waste (Experiment) and controls: the negative control was food waste not inoculated with *Rhodopseudomonas*. All experiments were incubated in the light and the gas collected in a pneumatic trough as previously described. The experiment produced large amounts of gas on the 2nd and third days of the incubation but production of new gas rapidly fell off to near zero after 7 days (Fig 4.24). Fig. 4.25 shows the cumulative totals.

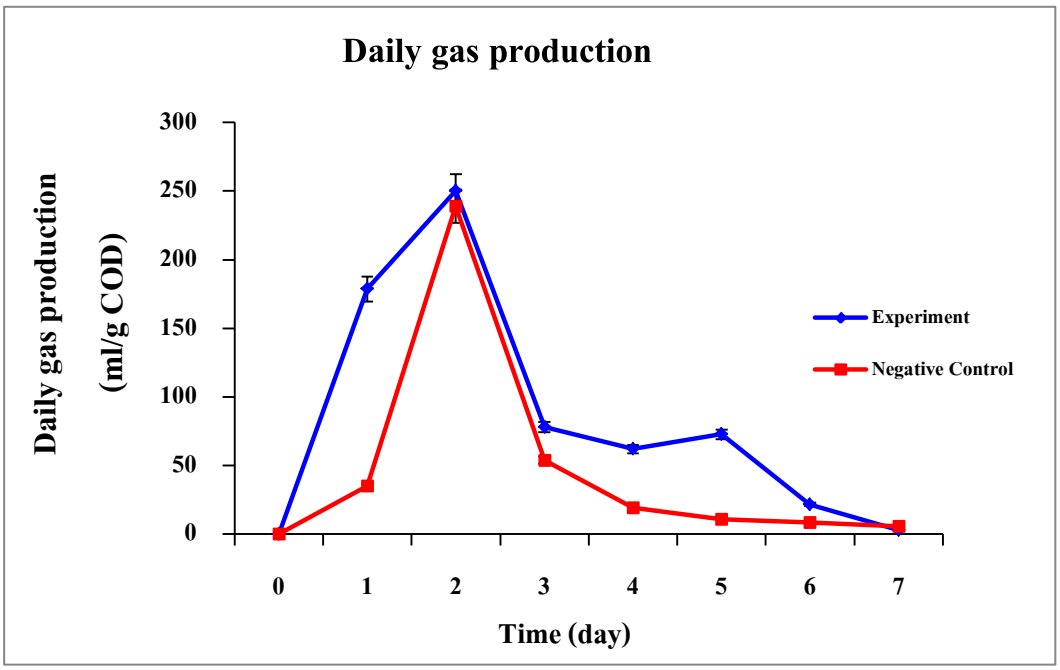


Fig. 4.24 Daily gas production from food waste using *Rhodopseudomonas*.

Fig. 4.24 shown the daily gas production from the experiment and negative control was food waste with no added culture. Gas production was very high on the 1st day (178 ml/g COD), 428 ml total gas/g COD on the 2nd day, 77 ml total gas/g COD on day 3, 61 ml total gas/g COD on day 4, 72 ml total gas/g COD on day 5, 21 ml total gas/g COD on day 6 and 2.7 ml total gas/g COD on day 7. The experiment produced the highest daily gas production on the 2nd day and then gas production rapidly fell off to negligible amounts at the end of the experiment. The total gas production of the negative control on the 1st day was 34 ml /g COD, 239 ml /g COD on the 2nd day, 53 ml /g COD on day 3, 18 ml /g COD on day 4, 10 ml/g COD on day 5, 8 ml /g COD on day 6 and 5 ml /g COD on 7 day. After day 2 the daily gas production was very low.

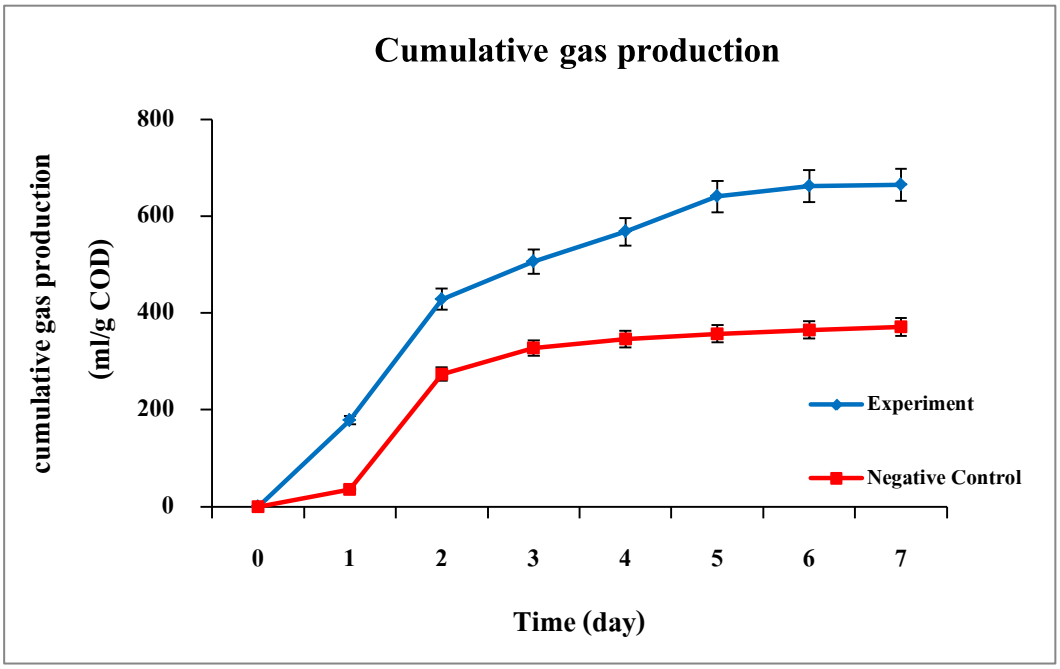


Fig. 4.25 Accumulation gas production from food waste using *R. palustris*.

Fig. 4.25 shows accumulation gas production from the experiment shown in Fig. 4.24. The negative control was sterile food waste with no added *R. palustris* culture. In the case of the inoculated experiment large accumulation of gas were produced: after 1 day, a total 178 ml of gas/g COD; 428 ml/g COD after 2 days; 506 ml/g COD after 3 days; 568 ml gas/g COD after 4 days; 640 ml/g COD after 5 days; 662 ml/g COD on 6 day and 664 ml of total gas/g COD on 7 day (One mole of H_2 would occupy 24.4 l at 25 ^{o}C). For food waste inoculated with *Rhodopseudomonas* the total gas volume increased every day from day 1 to day 7. However, after day 6 the rate of increase of total gas volume was very slow and had nearly stopped by day 7.

Almost no accumulation of gas occurred in the positive control where *Rhodopseudomonas* was present but no carbon source or electron source was provided. The unexpected result was for the negative control where sterilized food waste was incubated with no added *Rhodopseudomonas*. Substantial gas production was observed, particularly on day 2 of the experiment.

Figs 4.24 and 4.25 present the total gas production of the food waste /*Rhodopseudmonas* incubation experiments. The gas produced was analysed and the hydrogen gas was measured using a GC 8A series Shimadzu Gas Chromatograph. Figs 4.26 and 4.27 show daily production of H_2 and cumulative production of H_2 over the 7 days of the incubation experiment. Nitrogen fixation can increase the H_2 production yield from nitrogenase but H_2 production can occur without N_2 fixation but it is important to know that H_2 production is highly variable both between strains and under different growth conditions (Suwansaard *et al*., 2009). *Rhodopseudomonas palustris* has the peculiarity of encoding three different nitrogenases in its genome (Larimer *et al*., 2004; Oda *et al*., 2005). *Rhodopseudomonas* strains are also capable of hydrogen gas production both anaerobically in the dark and photosynthetically using organic compounds as a hydrogen source (Hillmer and Gest, 1977; Mangels *et al*., 1986; Suwansaard *et al*., 2009; Panwichian *et al,*. 2009; Suwansaard *et al*., 2010). *R. palustris* can produce hydrogen gas from palm oil milling effluent which has a very high oil and fatty acid content (VFAs-Volatile Fatty Acids) (Suwansaard *et al*., 2009). Furthermore, a strain of *R. spalustris*, TN1 isolated from Songkhla Lake in Southern Thailand was found to be an effective producer of hydrogen gas (Suwansaard *et al*., 2009; Suwansaard *et al*., 2010) from palm oil mill effluent.

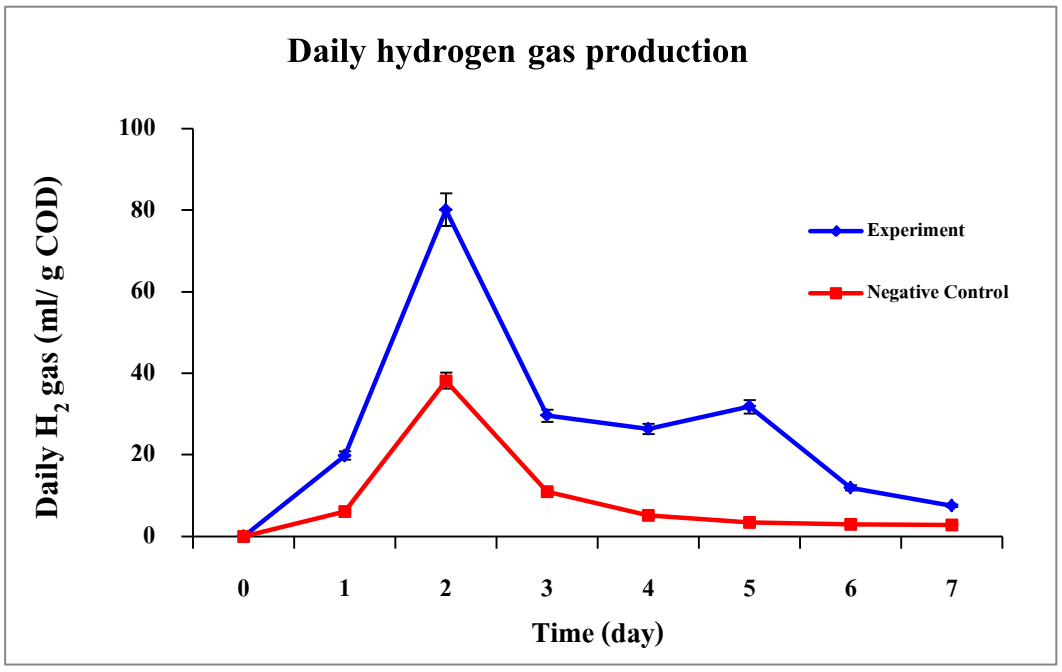


Fig. 4.26 Daily H_2 production from digestion of food waste in 168 hours using *R. palustris*.

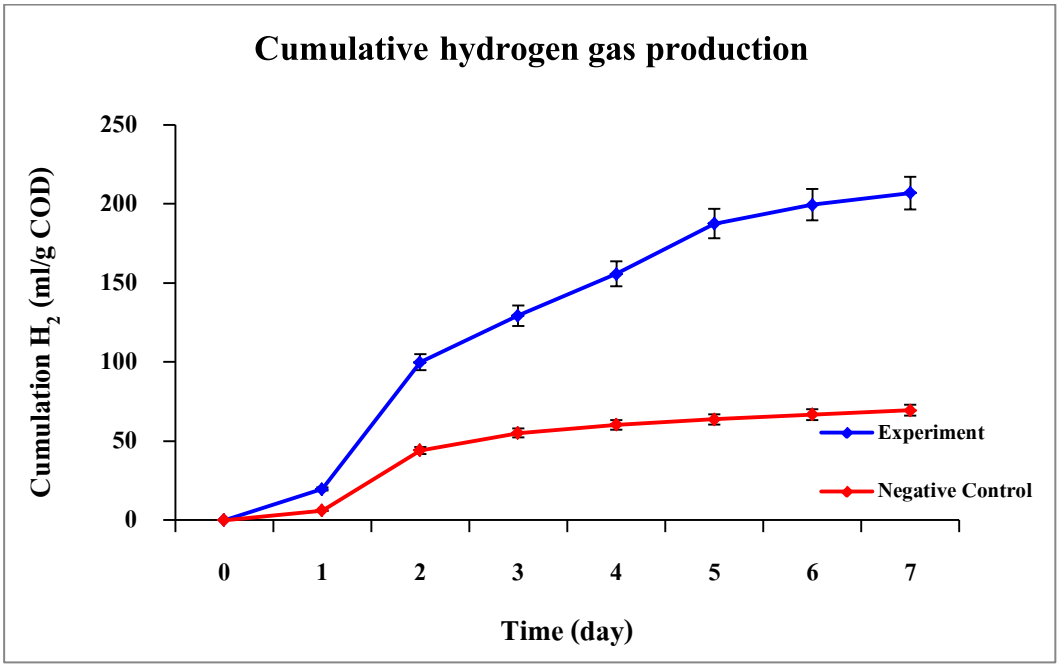


Fig. 4.27 Cumulative H_2 production from digestion of food waste in 7 days using *R. palustris*.

Rhodopseudomonas growing on food waste produced very high amounts of H_2 on the 2nd day of the incubation (80 ml H_2/g COD) but after the second day the rate of H_2 rapidly decreased on days 3, 4, 5, 6 & 7 (29, 26, 31,11, 7.5 ml H_2/g COD respectively) (Fig. 4.26). The negative control (sterile food waste only) produced about ½ of the H_2 gas as the *Rhodopseudomonas* inoculated food waste on day 2 (38 ml H_2/g COD) but production on subsequent days was very low.

The only other major gas found to be produced in the cultures was carbon dioxide (also analysed by gas chromatograph). A large amount of CO_2 was lost by the *Rhodopseudomonas*/food waste incubation and from the sterile food waste with no *Rhodopseudomonas* added at an almost constant rate of about 30 ml/day from day 1 to day 7 in both incubations. Photosynthetic bacterial digestion of restaurant food waste by photosynthetic bacteria could potentially produce useful amounts of hydrogen gas and would be capable of converting much of the organic matter of a waste effluent into CO_2 and hydrogen gas (Oh *et al.*,

2004; Merugu *et al*., 2011). To be commercially useful the CO_2 and H_2 would have to be separated.

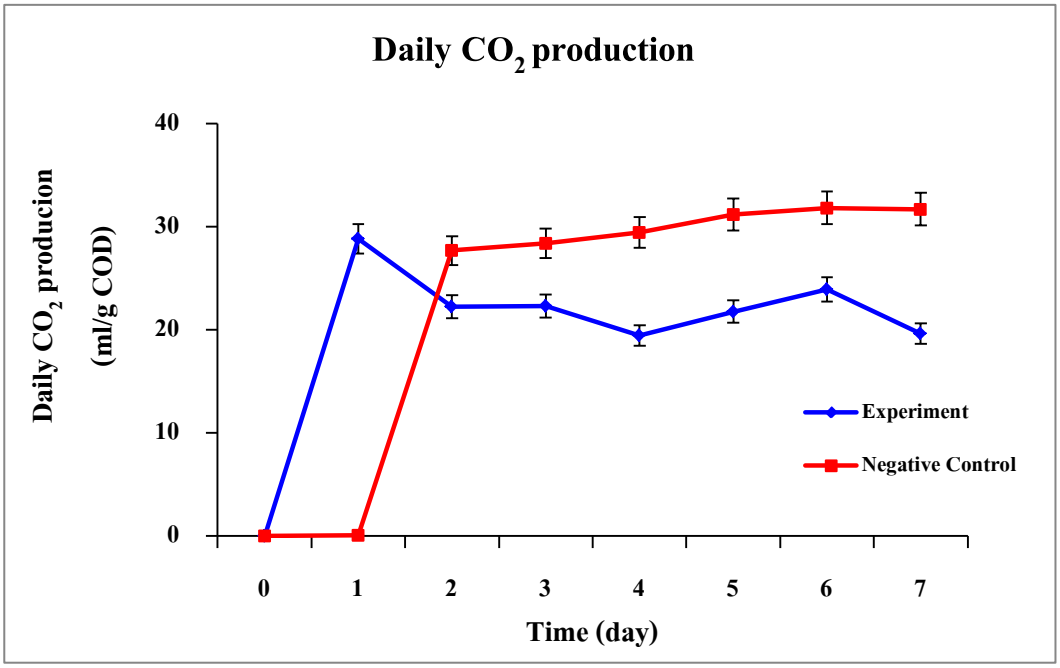


Fig. 4.28 Shown daily CO_2 production from digestion of food waste using *R. palustris*.

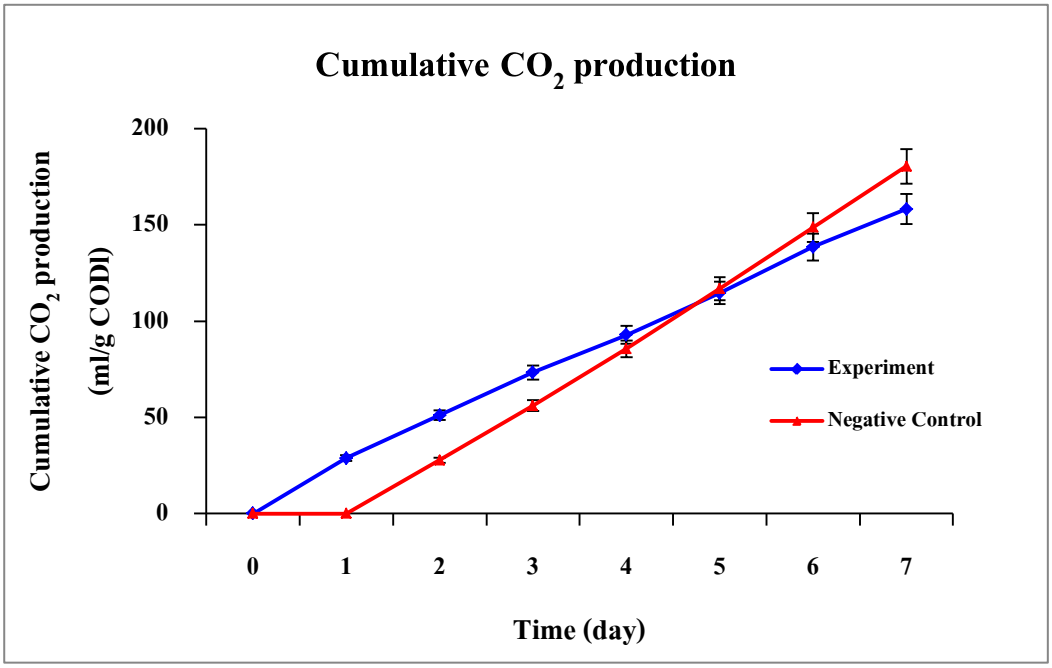


Fig. 4.29 Cumulative of CO_2 production from digestion of food waste using *R. palustris*.

Loss of CO_2 was very high after the first 24 hrs at an almost constant daily rate of about 30 ml/day in both the *Rhodopseudomonas*/food waste and food waste only incubations (Fig. 4.28 & 4.29). The incubations with the *autoclaved* food waste evolved large amounts of CO_2 but this does not appear to be of biological origin.

4.4. Digestion of food waste using yeast (*Saccharomyces cerevisiae*).

The value of a 2-stage fermentation process, first using yeast and then inoculating the culture with *Rhodopseudomonas* was investigated. The yeast incubation step was run in the dark, the *Rhodopseudomonas* incubation was run in the light. *Rhodopseudomonas* rapidly killed the yeast and took over the culture after inoculation and incubation in the light.

The first step was an anaerobic fermentation of 30 day using yeast based on the work of Suwannarat and Ritchie (2013a, b). Three lots of 125 g FW/l food waste were inoculated with 10 ml of yeast inoculums (about 4.6×10^6 of cells/ml). Yeast can remove as much as 77% DW of the original oils and 31% DW of the original starch (Fig. 4.30) but the yeast was only able to break down about 60% to 80% of the starch (Carneiro *et al*., 2007; Arsova, 2010), 74% DW of the original reducing sugar and COD went down by 59% but yeast did not remove very much total phosphate. Yeast incubation increased the Total P by mobilizing organic P in the food waste. Fig. 4.30 shows composition of food waste after anaerobic fermentation using yeast. Yeast grew well on food waste and can break down much of the organic matter (Mekjinda and Ritchie, 2013b), large amounts of the carbon content of the waste is converted into yeast protein (Fig. 4.30).

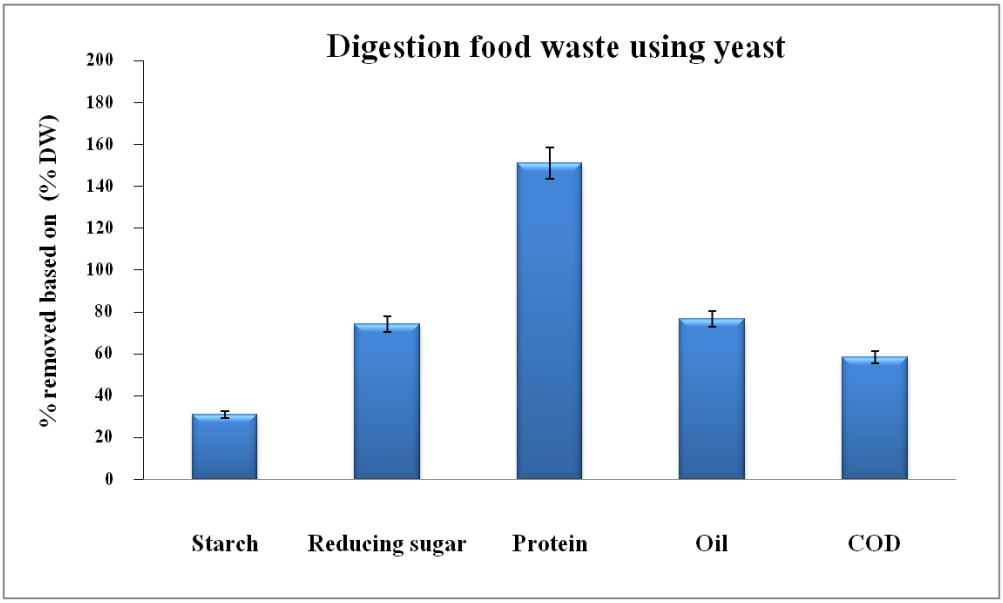


Fig. 4.30 Breakdown of food waste by anaerobic fermentation using *S. cerevisiae* (30 days) (redrawn after Mekjinda and Ritchie, 2013b).

The % value for the protein is greater than 100%. This value is expressed as removal from the original protein content. The cells grew well and made yeast protein using the carbon sources available and the ammonia and so reflects synthesis of protein by the yeast.

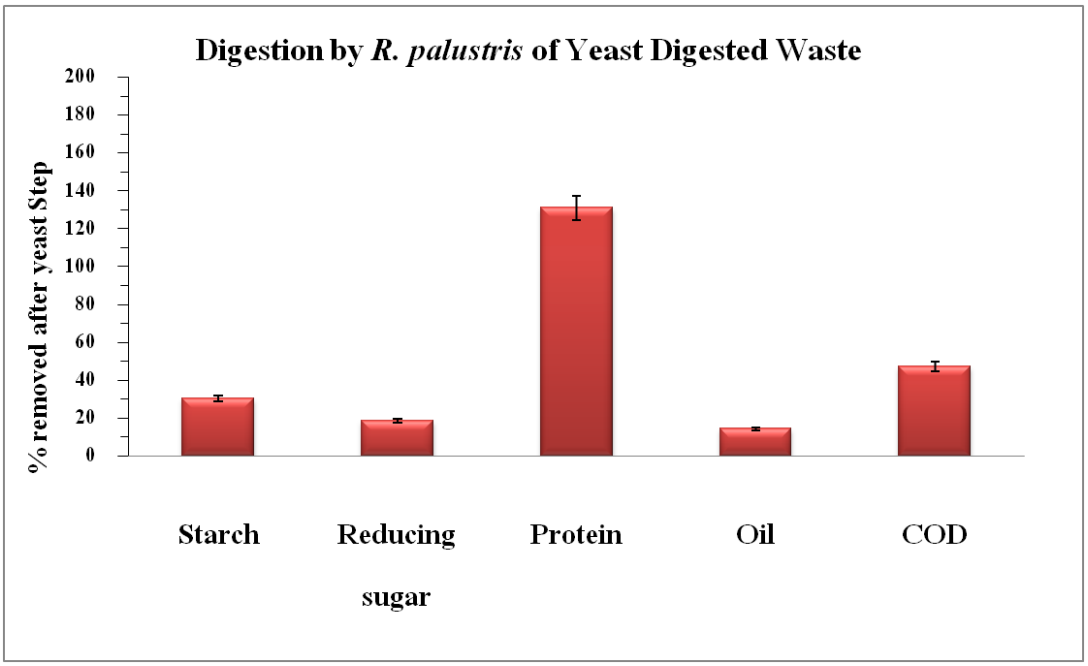


Fig. 4.31 Breakdown of food waste following yeast by anaerobic fermentation using *R. palustris* (7 day). (redrawn after Mekjinda and Ritchie 2013b).

The second stage of the incubation was started by adding 75 ml of *Rhodopseudomonas* which contained a total of about 12 µg of BChl *a* per ml of culture. The two stage process (Incubation with yeast (30 d) followed by *R. palustris* (7 d) (Fig. 4.30 & 4.31) removed 15% DW of the remaining oil, 30 % DW of the starch, reducing sugar content was further reduced by only 19% DW and COD went down by a further 47%. The protein synthesized by the yeast in the first stage of the incubation was utilized by the *Rhodopseudomonas* in the second stage incubation with *Rhodopseudomonas*. Nearly all the phosphorus was removed by the *Rhodopseudomonas* second stage digestion (98% DW total phosphorus). Fig. 4.32 shows a comparison of digestion of food waste by yeast and yeast digestion followed by *R. palustris* digestion. The two stage process can remove organic compounds better than the one stage yeast process because *R. palustris* can grow on a wider range of compounds than yeast. *Rhodopseudomonas* effectively removed all the soluble phosphorus and reduced COD and removed nearly all the remaining oil. The yeast/*R. palustris* two stage digestion process was

better at digesting food waste than using *R. palustris* as single step fermentation (Mekjinda and Ritchie, 2013b) and the *Rhodopseudomonas* incubations outlined above (Chapter 4.2 to 4.4).

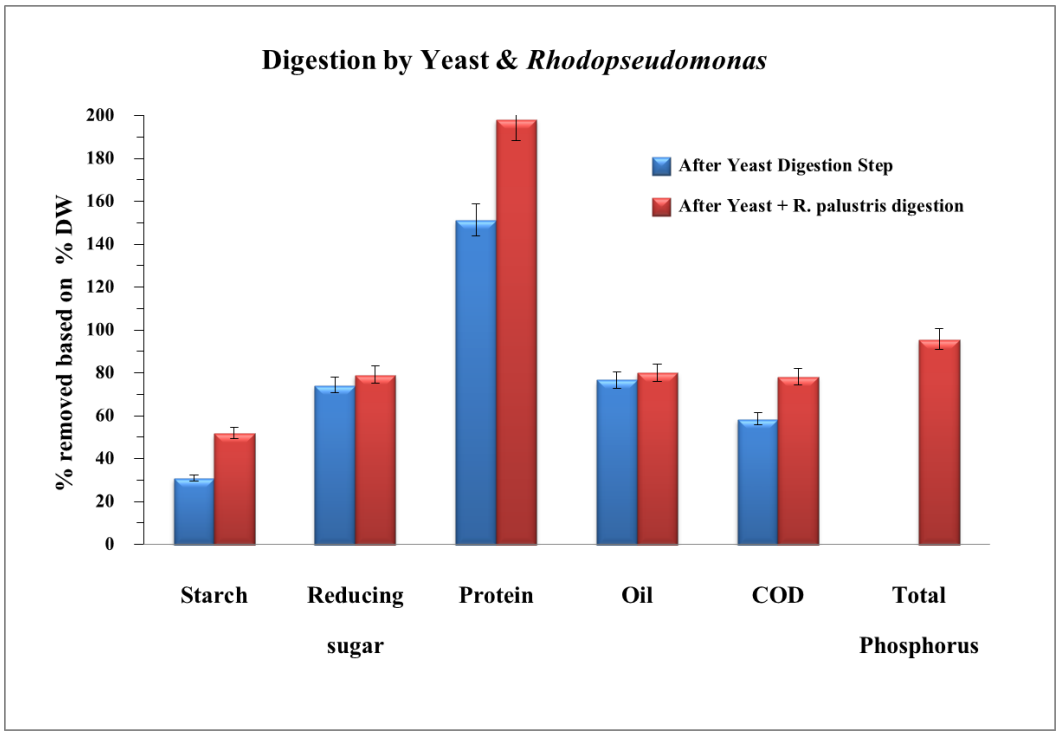


Fig. 4.32 Digestion of food waste using yeast and *Rhodopseudomonas palustris* two-stage digestion. After the *Rhodopseudomonas* digestion step soluble P was nearly zero.

Fig. 4.32 shows percentage of removal of starch, reducing sugar, protein, oil, COD and total phosphorus from food waste. Yeast in the first incubation step used most of the organic compounds present including protein, sugars, protein and oil but only part of the starch. The experiment using yeast only removed 31% of starch, 74% of reducing sugar, 151% of protein, 77% of oil, 59% of COD based on the original of food waste. Yeast did not remove any significant amount of total phosphorus. The *R.palustris* incubation step removed 52% of the starch, 79% of the reducing sugar, 198% of protein, 80% of the oil, 87% of the COD and 96% of total phosphorus based on the original food waste. Thus *Rhodopseudomonas* very effectively removed protein and total phosphorus. It is likely that *Rhodopseudomonas* stores large amounts

of polyphosphate in its cells–this is a common phenomenon in algae (Ritchie *et al*., 1997, Runcie *et al*., 2004, see KEGG, 2014 genomic information in Chapter 2).

4.4.1 Determine the effect of inoculation volume of *R. palustris* on food waste following a first-stage yeast incubation.

In the two-stage anaerobic fermentation of food waste with a first stage yeast fermentation followed by a *Rhodopseudomonas palustris* digestion 150 gFW of food waste was used in each experiment. The *Rhodopseudomonas* culture used for Experiment # 1 used 75 ml of cell culture, experiment # 2 used 70 ml of cell culture, and experiment # 3 used 65 ml of cell culture, experiment # 4 used 60 ml of cell culture, experiment # 5 used 55 ml of cell culture and experiment #6 50 ml of cell culture. The *Rhodopseudomonas* culture used to inoculate the 2nd stage contained 5 µg/ml BChl *a* /ml of culture. The *Rhodopseudomonas* treatments were incubated in the light. The control was not inoculated with *Rhodopseudomonas* but had been inoculated with yeast and so was incubated for a total of 32 days in yeast and was not incubated in the light.

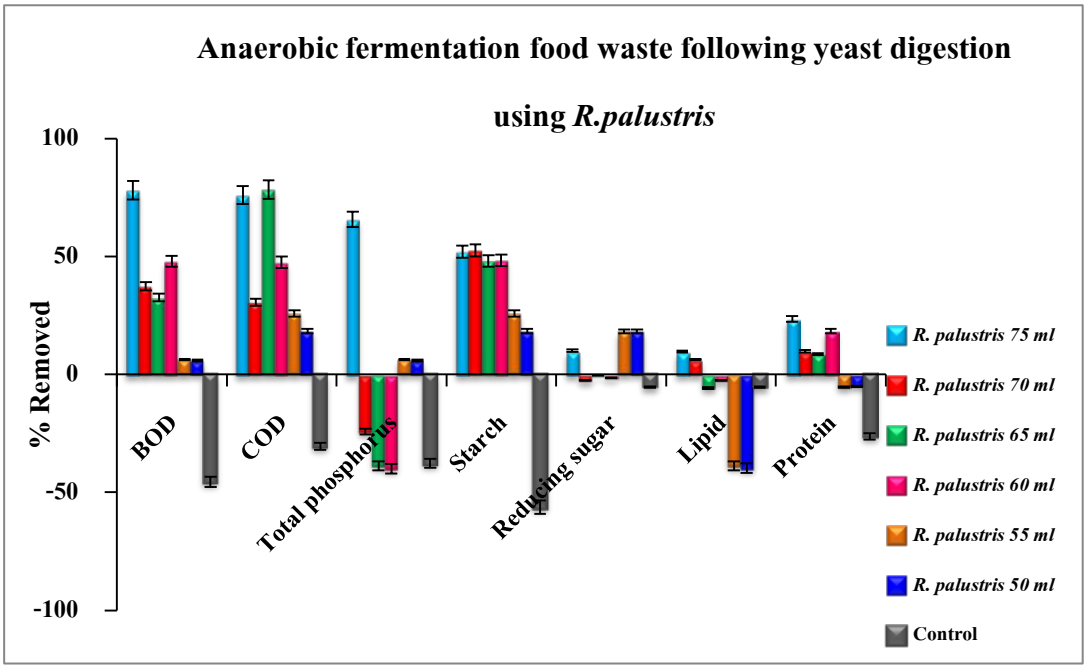


Fig. 4.33 Optimizing the volume of *R. palustris* for efficient digestion of food waste on food waste incubated for 25 days with yeast and then inoculated with *Rhodopseudomonas* for 7 days.

The experiment with 75 ml of *R. palustris* removed 78% of BOD, 76% of COD 66% of total phosphorus, 52% of starch, 24% of the protein and 10% of the reducing sugar and the oil. The experiment with 70 ml of *R. palustris* removed 37% of BOD, 31% of COD, 53% of the starch, 10% of the protein, 6% of the oil but they did not reduce the total phosphorus (-24%) and the reducing sugar (-3%). The experiment with 65 ml of *R. palustris* removed 33% of BOD 78% of COD, 48% of the starch, 9% of the protein, and did not remove total phosphorus (-39%) or the oil (-6%) and the reducing sugar (-1%). The experiment with 60 ml of *R. palustris* removed 48% of BOD, 48% of the COD, 48% of the starch, 18% of the protein and did not reduce the phosphorus (-40%), oil (-3%) or the reducing sugar (-1%). The experiment with 55 ml of *R. palustris* removed 6% of the BOD, 26% of the COD, 6% of the total phosphorus, 26% of the starch, 18% of the reducing sugar and did not remove the lipid (-39%) or the protein (-5%).

The experiment with 50 ml of *R. palustris* removed 6% of the BOD, 18% of the COD, 6% of the total phosphorus, 18% of the starch, 18% of the reducing sugar and then they cannot removed the lipid (-40%) and the protein (-5%). The experimental control had been incubated with yeast for a total of 32 days by the end of the experiment. Compared to measurements made after 25 days, there was no removal of BOD, COD, phosphorus or starch, in fact they increased but protein did decrease: BOD (-46%), COD (-31%), the total phosphorus (-38%), starch (-38%), reducing sugar (-5%), lipid (-5%) and the protein (+26%).

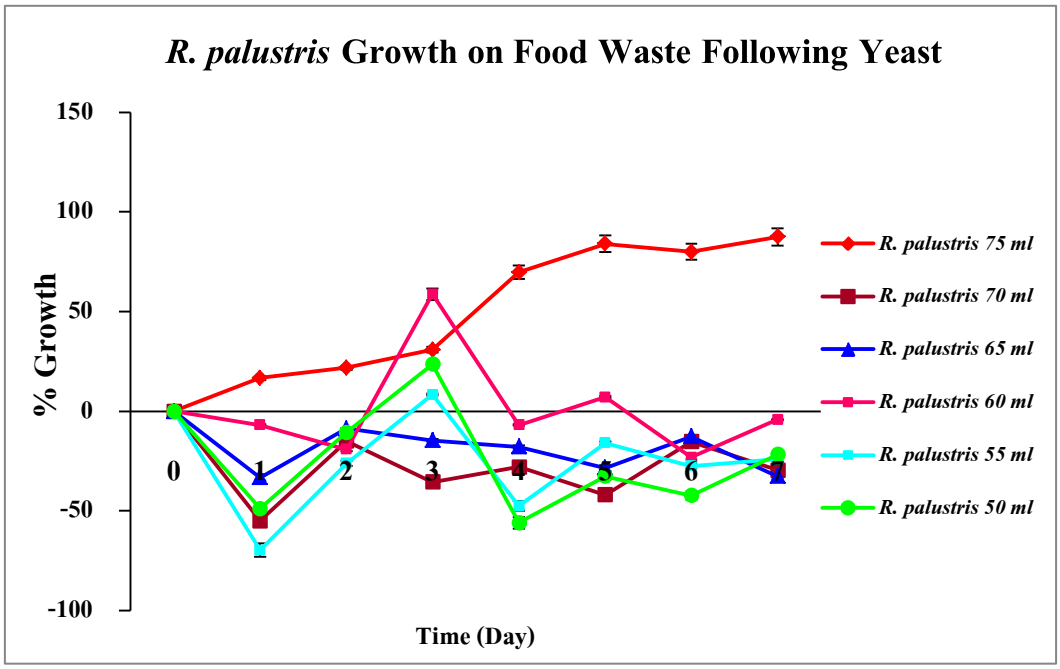


Fig. 4.34 Percent of growth of *R. palustris* for efficient digestion of food waste following a first-stage yeast incubation for 7 day. *Rhodopseudomonas* took over the culture from the yeast in all cases but in all except the heaviest inoculation there was a lag-phase.

Fig. 4.34 shows the growth of *Rhodopseudomonas* over the course of the experiment (7 days). The *Rhodopseudomonas* in most of the experiments died off during the first few days of the experiment and then gradually recovered to near the initial inoculation level by the end of the experiment. In the case of the food waste given the heaviest inoculation of *Rhodopseudomonas* 50 % (75 ml of cells 5 µg/ml BChl *a*) the cells did not die off but grew continuously over the course of the experiment. This result suggests that a heavy inoculation with *R. palustris* is needed for *R. palustris* to establish it self and overwhelm the yeast present.

4.4.2 Measurement of gas production by *R. palustris* from food waste following a first-stage yeast digestion

An experiment was set up with 150 gFW of food waste incubated with yeast for 25 days in a first-stage incubation followed by a *Rhodopseudomonas* digestion using 75 mol of inoculum (5 µg/ml BChl *a*) for 7 days. The experiments were set up in 500 ml serum bottles. The negative control experiment was food waste following yeast digestion but no addition of *Rhodopseudomonas*. Gas production was analyzed using a GC 8A series Shimadzu Gas Chromatograph.

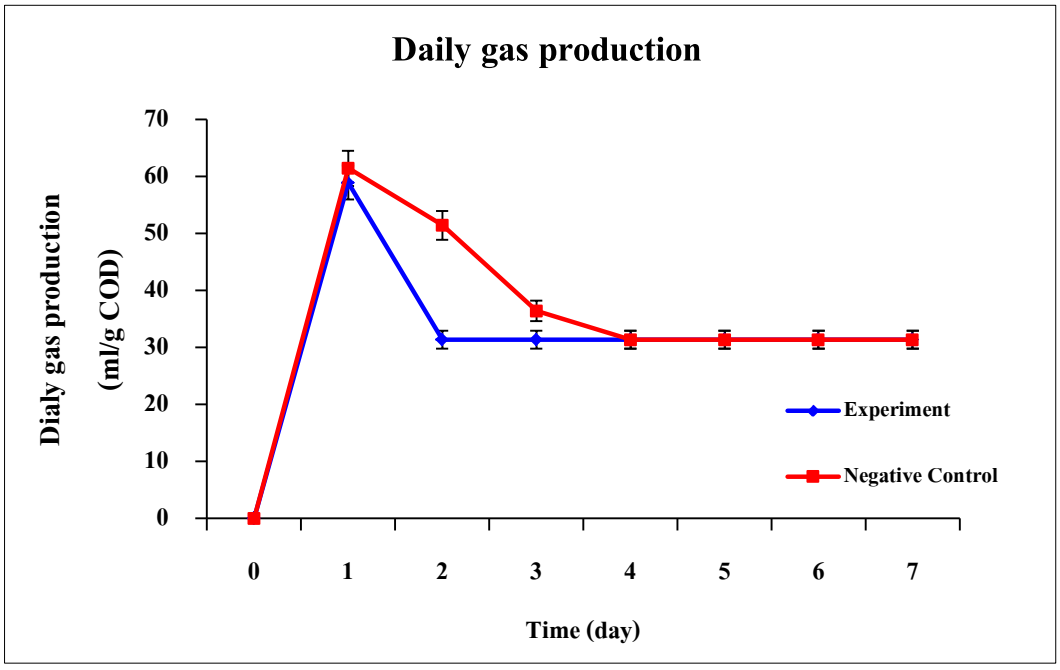


Fig. 4.35 Daily gas production from food waste following yeast using *R. palustris*

Fig. 4.35 shows the daily gas production over 7 days. On day 1 the *Rhodopseudomonas* culture produced a large amount of gas (58 ml/g COD) but after day 1 to day 7 the *Rhodopseudomonas* inoculated culture experiment stopped producing any more gas than the two controls (31.42 ml of gas/g COD). The negative control (no addition of *Rhodopseudomonas*) initially produced high amounts of gas, the first day it produced 61.42 ml /g COD, 51.42 ml /g

COD on day 2 and 36.42 ml /g COD on day 3 but after day 3 they produced a very low (31.42 ml/g COD) amounts of gas each day.

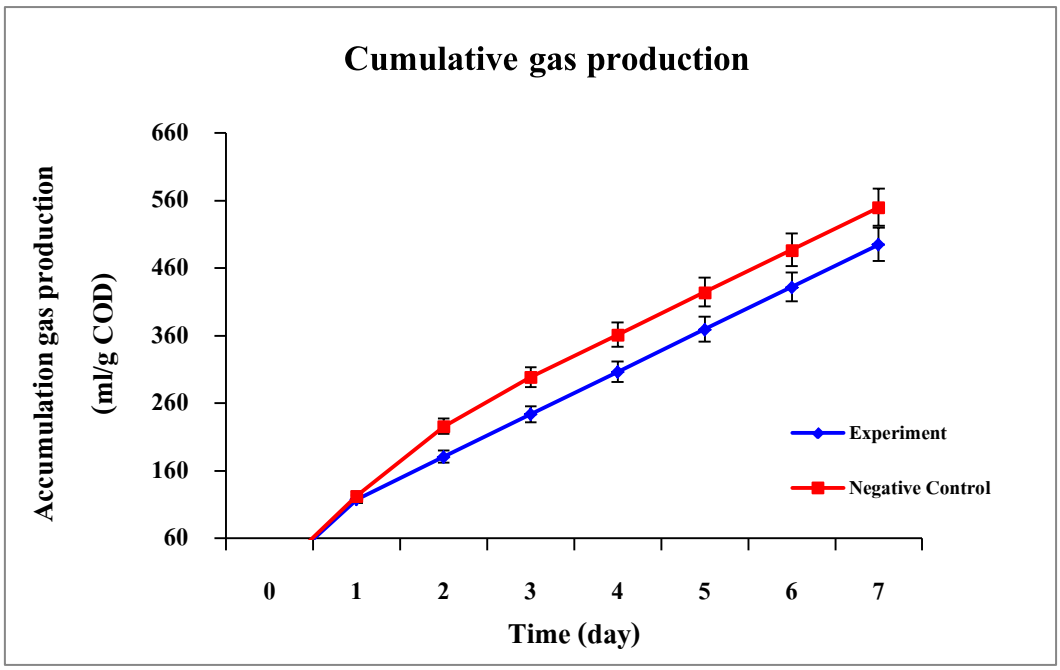


Fig. 4.36 Accumulation gas production from food waste followed yeast using *R. palustris*

Fig. 4.36 shows the cumulative gas production using the data shown in Fig. 4.35 All three experimental treatments produced a steady rate of gas production of about 58.9 ml/g COD per day over about 3 days. Because of the more sustained production of gas the negative control actually produced more gas over the 7 day incubation than the food waste inoculated with *Rhodopseudomonas* after the initial 25 day incubation with yeast.

Large amounts of gas were produced by all three treatments. The food waste incubated with yeast then inoculated with *Rhodopseudomonas* produced a total of 247.5 ml /g COD in 7 days. The negative control produced similar amounts of gas over the 7 days even though the food waste was not inoculated with *Rhodopseudomonas* but the original yeast inoculum (25 days old) was allowed to continue to grow for a further 7 days (275 ml /g COD of the total gas on day 7).

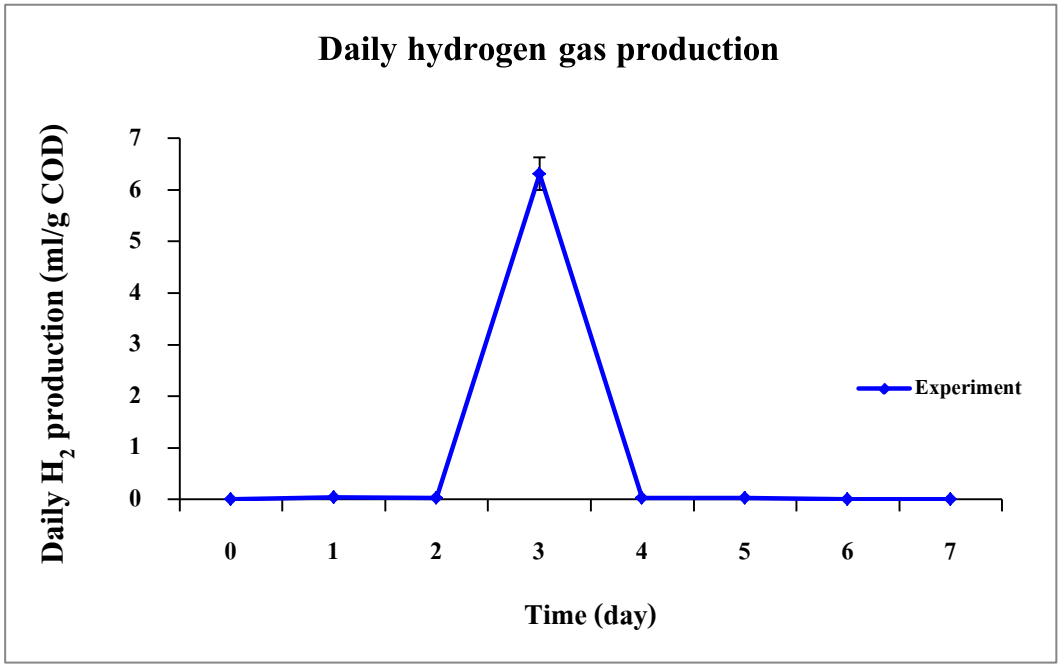


Fig. 4.37 Daily H_2 production from digestion of food waste following in 7 day using *R. palustris*.

Hydrogen in the evolved gas was measured using the Gas chromatograph. No hydrogen was found in the negative control which had been incubated with yeast for 25 days and the incubation was allowed to continue. Hydrogen gas was produced by the treatments inoculated with *Rhodopseudomonas* but the control produced CO_2 but no hydrogen. The cultures which were first incubated with yeast for 25 days and then incubated with *Rhodopseudomonas* at first produced very little hydrogen on the 1st day produced only 0.0354 ml /g COD of hydrogen gas. On day 3 the culture produced a larger amount of hydrogen gas (6.3163 ml of H_2/g COD), but after day 3 the culture produced very little H_2. Hydrogen production was a short-lived spike.

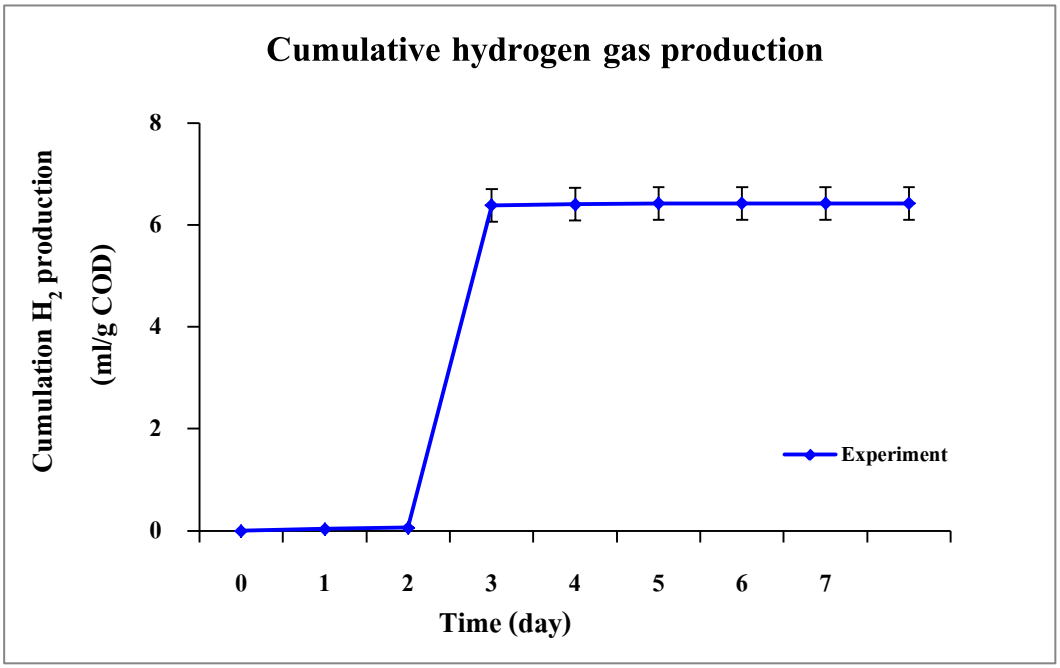


Fig. 4.38 Cumulation H_2 production from digestion of food waste following in 7 days using *Rhodopseudomonas palustris*. The negative control produced no hydrogen gas.

Fig. 4.38 shows the cumulative production of H_2 shown in Fig. 4.37. The accumulation of hydrogen gas from food waste following yeast digestion and then inoculation with *Rhodopseudomonas*. The Yeast digested food waste inoculated with *Rhodopseudomonas* only produced about 6.4 ml of H_2/g COD in 7 days, nearly all of it on the 3rd day of the incubation.

The amounts of hydrogen produced in these 2-fermentation stage experiments were very low compared to the 7 day incubation experiments shown in Figs 4.26 & 4.27 where fresh food waste was inoculated with *Rhodopseudomonas* without first-step yeast fermentation. Thus yeast had used most of the carbon sources useable by *Rhodopseudomonas* to produce H_2 before the second fermentation step. A two stage fermentation is not successful in producing significant amounts of hydrogen from food waste.

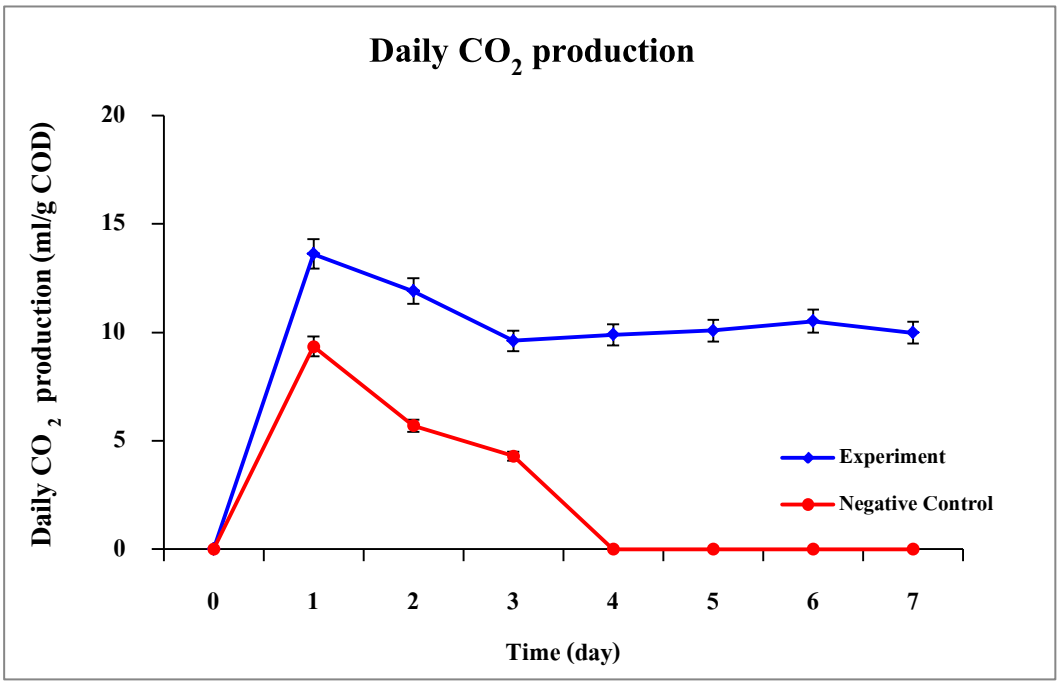


Fig. 4.39 Daily CO_2 production from digestion of food waste following a 25 day yeast incubation followed by a 7 day incubation using *Rhodopseudomonas palustris.* Only a small proportion of the gas produced by *Rhodopseudomonas* is H_2, most of it is CO_2.

The CO_2 production from food waste following yeast using *Rhodopseudomonas* was measured. The food waste pre-incubated with yeast and then inoculated with *Rhodopseudomonas* produced very high volumes of CO_2 (13.6 ml CO_2/g COD) but then slowed down to about 11.9 ml CO_2/g COD per day for the remainder of the experiment. The volume of CO_2 gas on the first day was 13.6 ml CO_2/g COD, 11.9 ml CO_2/g COD on day 2, 9.6 ml CO_2/g COD on day 3, 9.8 ml CO_2/g COD on day 4, 10 ml CO_2/g COD on day 5, 10.5 ml CO_2/g COD on day 6, and 9.9 ml CO_2/g COD on day 7. The negative control produced very high CO_2 on the first day (9.3 ml CO_2/g COD), 5.7 ml CO_2/g COD on day 2, 4.2 ml CO_2/g COD on day 3 and after day 3 they stopped producing CO_2.

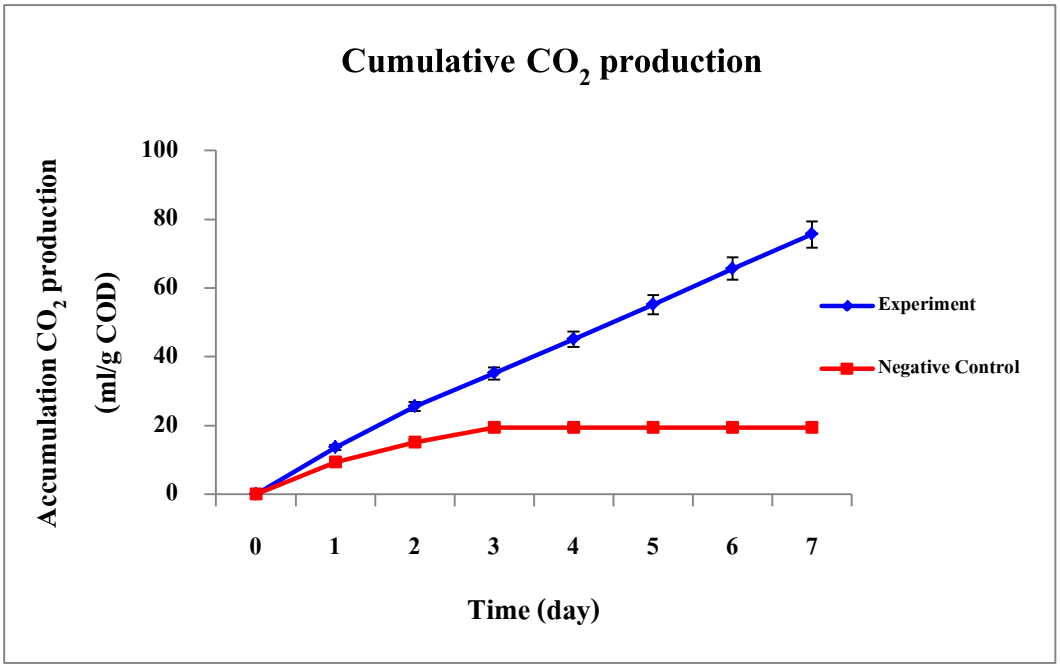


Fig. 4.40 Cumulation CO_2 production from digestion of food waste following yeast digestion for 25 days followed by a 7-day incubation using *Rhodopseudomonas palustris.* Only a small proportion of the gas produced by *Rhodopseudomonas* was H_2, most of it was CO_2.

Fig. 4.40 shows the cumulative gas production of CO_2 using the data from Fig. 4.39. The cumulativeCO_2 produced by the food waste pre-incubated with yeast for 25 days and then inoculated with *Rhodopseudomonas* was linear with time and by 7 days had produced 150 ml of CO_2 or about 20 ml/day. The negative control produced 19 ml of CO_2 on the first day and 30 ml after 2 days but by day 3 had stopped producing extra CO_2. The negative control with food waste and incubated with yeast for 25 days and then allowed to continue incubating with yeast but with no inoculation with *Rhodopseudomonas* produced some CO_2 in the first few days of the experiment but then stopped producing new CO_2. As pointed out above most of the gas produced in the two-step incubation experiment was CO_2 and not H_2 (Figs 4.35, 4.36, 4.37, 4.38, 4.39, 4.40).

CHAPTER 5

Conclusions

5.1 Conclusion

This study was on the possible use of the photosynthetic bacterium, *Rhodopseudomonas palustris* to break down bulk food waste because *R. palustris* can produce hydrogen gas under anaerobic conditions and they can digest vegetable, starch, sugarcane juice and whey to produce hydrogen gas.

It was shown that *R. palustris* can use the organic compounds found in food waste such as glutamate (amino acids), flour starch, protein (BSA), glucose, and vegetable oil. *R. palustris* was grown routinely in PM medium with acetate and benzoate as carbon sources and thiosulphate as an electron source but grew successfully on food waste diluted with tap water. Thus food waste provides a source of organic carbon, useable electron sources, vitamins and minerals for growth. This makes growing *Rhodopseudomonas* on food waste in a simple bioreactor a viable proposition.

Incubations of *Rhodopseudomonas* on food waste were successful in breaking down the food waste and produced large amounts of H_2 gas and require very little light to be able to grow photoheterotrophically. *Rhodopseudomonas* (970 μg BChl *a*) in 100 ml of water and 300 gFW of food waste was able to remove 70 % of the BOD and reduced the COD by about 33% however, there was very little change in the reducing sugar (2%) or the total phosphorus (1%). The *Rhodopseudomonas* used 43% of the starch, 41% of the ammonia, 44% of the nitrate (as an alternative to O_2 as an electron acceptor in oxidative phosphorylation respiration), 37% of the total solid, 16% of the protein and 12% of the lipid. The hydrogen production from food waste was very high (maximum 80 ml of H_2/g COD /day) but only occurred during the first few days of the incubations and rapidly fell to zero by day 7 of the incubations. *R. palustris* can digest a lot of

organic compounds in the food waste but there was not a great growth of the *Rhodopseudomonas* cells.

A two-stage anaerobic digestion using yeast as the first stage, followed by a *R. palustris* digestion was tested because *R. palustris* is able to use a very wide range of organic compounds (Larimer et al. 2004) and it was thought that *R. palustris* might be able to produce useable amounts of H_2 from food waste that had already been partially digested using yeast. A two-stage fermentation experiment was set up where food waste was first digested using yeast for 25 days and then inoculated with *Rhodopseudomonas* and incubated for 7 days. One control was where a 25-day yeast digestion was allowed to incubate for a further 7 days without addition of *Rhodopseudomonas* and a second control was set up with *R.palustris* inoculated into PM medium with no carbon or electron source and incubated for 7 days. The yeast digestion stage lowered the COD, removed much of the reducing sugar and proteins but digestion of oil and starch very low. Yeast removed little phosphorus and increased the total P by mobilizing organic P in the food waste. In the 2^{nd} stage yeast digested food waste was inoculated with 75 ml of *R. palustris* (375 μg BChl *a*). *R. palustris* removed 78% of BOD, 76% of COD, 66% of total phosphorus, 52% of starch, 24% of the protein and 10% of the reducing sugar and the oil. The second-stage incubation did improve the breakdown of carbon compounds over incubation using a single stage yeast incubation.

H_2 production from the second-stage *R. palustris* digestion was very low (<13 ml of H_2/g COD). *R. palustris* grows well on food waste after partial digestion by yeast and breaks down cooking oils and starches that yeast cannot digest quickly. The two stage process is highly successful in improving the digestion of food waste but not in producing H_2. Apparently most of the organic material useable by *R. palustris* to produce H_2 gas is also useable by yeast to produce ethanol by alcohol fermentation and so after the yeast digestion step there is little organic carbon left which is useable by *R. palustris* to produce H_2. The idea of generating alcohol from a yeast digestion and then producing H_2 from a second stage fermentation using photosynthetic bacteria does not appear to be a viable proposition.

5.2 Suggestions for Future Work

1. Despite its photosynthetic mechanism being an anaerobic process *Rhodopseudomonas* is able to grow well in simple pondages to deal with piggery waste.

2. Digested food waste was not expected to contain high levels of heavy metals. This needs to be confirmed experimentally.

3. *R. palustris* as a second stage digestion agent has some value to break down carbon compounds but significant hydrogen production should not be expected

4. Using *R. palustris* as a second digestion stage in a simple pond setup might be a viable method of removing toxic organic compounds from sludges of fermentors.

5. *R. palustris* is very good at removing phosphate. This is environmentally important in preventing algal blooms.

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Appendices

Appendices I

Standard Method mainly based on APHA 1 998

1. Chemical Oxygen Demand (COD) - Closed Reflux Method analysis

Apparatus

- Heating block
- Digestion vessel size16 x 100 mm screw Type

Reagents

- 294.185 g/mole of Potassium dichromate ($K_2Cr_2O_7$). Needs to be dried in a hot air oven 103 ^{O}C for 2 hours then cooled in a desiccator.
- Conc. H_2SO_4
- Ag_2SO_4, technical grade
- $HgSO_4$
- Ferrous Ammonium Sulphate, FAS
- 0.0167 M $K_2Cr_2O_7$: 0.4913 g of $K_2Cr_2O_7$ was dissolved in 50 ml of distilled water, 16.7 ml of concentrated sulfuric acid was added slowly and diluted to 100 ml with careful mixing and cooling.
- Ferroin Indicator: 1.485 g of 1, 10-phenanthroline monohydrate and 695 mg $FeSO_4.7H_2O$ were dissolved in distilled water, and then diluted to 100 ml.

Procedure

Wash culture tubes and caps with 20% H_2SO_4 before first use to prevent contamination. Refer to Table 3 for proper sample and reagent volumes. Make volumetric measurements as accurate as practical; use Class A volumetric ware. The most critical volumes are of the samples and digestions solution. Use a microburette for titrations. Measure H_2SO_4 to ± 0.1 ml. The use of hand-held pipettors with non-wetting (polyethylene) pipet tips were practical and adequate. Place sample in culture tube to ampoule and add digestion solution. Carefully run sulfuric reagent down inside of vessel so an acid layer is formed under the sample-digestion solution layer. Tightly cap tubes or seal ampoules, and invert each several times to mix completely.

Place tubes or ampoules in block digester preheated to 150 ^{o}C and reflux for 2 h behind a protective shield. Cool to room temperature and place vessels in test tube rack. Some

mercuric sulfate may precipitate out but this will not affect the analysis. Add 0.05 to 0.1 ml (1 or 2 drops) ferroin indicator and stir rapidly on magnetic stirrer while titrating with standardized 0.1 M FAS. The end of point is sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of sample.

Table A1.1-Sample and reagent quantities for various digestion vessels.

Digestion Vessel	Sample (ml)	Digestion Solution (ml)	Sulfuric Acid Reagent (ml)	Total Final Volume (ml)
Culture tubes:				
16 x 100 mm	2.5	1.5	3.5	7.5
20 x 150 mm	5.0	3.0	7.0	15.0
25 x 150 mm	10.0	6.0	14.0	30.0

Calculation COD

$$\text{COD as mg } O_2/L = \frac{(A - B) \times M \times 8000}{\text{ml sample}}$$

Where: A = ml FAS used for blank

B = ml FAS used for sample

M = morality of FAS

8000 = mill equivalent weight of oxygen x 1000 ml/L

2. BOD analysis (Azide method)

Apparatus

- Incubation bottles size 300 ml
- Incubator $20 \pm 3\ ^{O}C$
- Burette
- Volumetric Cylinder size 100 ml

Reagents

- Diluted H_2SO_4 2.8 ml of concentrated H_2SO_4 added slowly to distilled water and adjusted to a volume of 100 ml with distilled water.
- Standard Starch solution: soluble starch laboratory grade 20 g dissolved in 1000 ml of hot distilled water.
- Manganese sulphate solution: 480 g of Manganese sulphate tetrahydrate ($MnSO_4.4H_2O$) or 400 g Manganese sulphate dihydrate ($MnSO_4.2H_2O$) or 364 g Manganese sulphate monohydrate ($MnSO_4.H_2O$) dissolved in distilled water made up to a volume of 1000 ml. Manganese sulphate solution was checked that it showed no reaction when added to the potassium iodide solution.
- Alkali – Iodide – Azide solution: 500 g Sodium hydroxide (NaOH) or 700 g Potassium hydroxide (KOH), 135 g Sodium iodide (NaI) or 150 g Potassium iodide (KI) are first dissolved in about 800 ml distilled water and then made up to 1000 ml. 10 g of Sodium azide (NaN_3) was the first dissolved in 40 ml of distilled water and then added to the Alkali–Iodide solution and the total volume adjusted to 1000 ml with distilled water.
- 0.025 N of Standard sodium thiosulphate titrant: 6.205 g of Sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) and 0.4 g of Sodium hydroxide (NaOH) was dissolved in distilled water and adjusted to 1000 ml.
- 0.025 N Standard potassium bi-iodate solution: 0.8124 g of Potassium bi-iodate [$KH(IO_3)_2$] dissolved in distilled water and adjusted to 1000 ml.
- Magnesium sulphate solution: 22.5 g of Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) dissolved in distilled water and adjusted to 1000 ml.
- Calcium chloride solution: 27.5 g of Calcium chloride ($CaCl_2$) dissolved in distilled water and adjusted to 1000 ml.
- Ferric chloride solution: 0.25 g of Ferric chloride hexahydrate ($FeCl_3.6H_2O$) dissolved in 1000 ml of distilled water total volume.
- Phosphate buffer solution: 8.5 g of Potassium dihydrogen phosphate (KH_2PO_4) 21.75 g of Dipotassium hydrogen phosphate (K_2HPO_4), 33.4 g Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$), and 1.7 g of Ammonium chloride (NH_4Cl) dissolved in 500 ml of distilled water and adjusted to 1000 ml.

- 1 N of Sodium hydroxide (NaOH): 40 g of NaOH dissolved in distilled water and adjusted to 1000 ml.

- 1 N of Sulphuric acid: 28 ml of Conc. H_2SO_4 (18 M, 36N) added slowly to 600 ml of distilled water and adjusted to 1000 ml kept in brown bottle and room temperature.

Procedure

1) 1 ml of Magnesium sulphate solution, Calcium chloride solution, Ferric chloride solution, and Phosphate buffer solution 1ml per 1 liter of water

2) Equilibrate the solution to the atmosphere by aeration for about 1 – 2 hrs.

3) The water is then poured into a BOD bottles and capped using the special BOD bottle gas plugs. Oxygen was measured after preparation for the Day (0) DO_0 measurements and the other bottles incubated at 20±3 ^{O}C for 5 days. Dissolved oxygen was determined on the maturity date (DO_5: Dissolved oxygen after 5 day standard incubation) and compared to the dissolved O_2 recorded for the DO_0 bottles.

Determination of Dissolved Oxygen (DO)

1) 1 ml of Manganese sulphate Solution and 1 ml of Alkali - Iodide-Azide solution were added to the BOD bottles, taking care not to add bubbles of air to the water in the BOD bottle. The BOD bottle cap is then carefully replaced and the contents mixed carefully 15 times.

2) The brown/white precipitate was allowed to settle.

3) 1 ml H_2SO_4 was then added avoiding any loss of the oxidised floc. The cap is replaced and the bottle shaken back and forth until the floc redissolves. I_2 is released from the floc in the presence of H_2SO_4 in equimolar amounts for the amount of oxygen dissolved in the water.

4) Pour 99 ml of water from the BOD bottle.

5) The Iodine present is titrated with 0.025 N sodium thiosulphate standard solutions until the solution is light yellow. Starch solution is then added and the titration is continued until the dark color disappears. The volume of sodium thiosulphate titrant is recorded. One mole of thiosulphate titrates one mole of I_2. Hence, for a standard thiosulphate of 0.025 N then 1 ml of titrant is equivalent to 25 µmoles of oxygen (O_2) or 0.4 mg of O_2.

Calculation of BOD value

$$BOD\ (mg/L) = DO_0 - DO_5$$

DO_0 = The DO of the sample titration in the early days

DO_5 = Average DO of the sample titration at after incubator 5 days

3. Total Solid analysis

Apparatus

- Evaporating dishes
- Water bath or Steam bath
- Desiccators
- Analytical Balance
- Hot air oven
- No specific reagents required.

Procedure

Note: Solid samples usually required 0.5 to 2 grams, slurry samples required 2-5 grams, and liquor samples required 10 ml, per duplicate. Liquor samples should be filtered through a 0.2 μm pore size filter prior to analysis. Convection oven method was used for liquor samples.

Convection oven method

1) Ceramic weighing dishes were dried by placing them in a 105 ± 3 ºC drying oven for a minimum of four hours. The dishes were cooled in a desiccator. Using gloves or tweezers to handle the dishes, pre-dried dish was weighed to the nearest 0.1 mg. The weight was recorded.

2) The sample was thoroughly mixed and then an appropriate amount was weighed out to the nearest 0.1 mg, into the weighing dish. Liquor samples were passed through a 0.2 μm filter prior to analysis (Millipore Corp, Bedford, MA, USA). The weight of the sample plus weighing dish was recorded. Three replicates were used routinely.

3) The samples were placed into a convection oven at 105 ± 3 ^{o}C for a minimum of four hours. The sample was removed from the oven and allowed to cool to room temperature in a desiccator. The dish containing the oven-dried sample was weighed to the nearest 0.1mg and the weight recorded. Overnight drying was used routinely.

Calculations

The percent total solids, or percent dissolved solids for a liquor sample was calculated as follows:

$$\% \text{ Total Solid} = \frac{(\text{Weight}_{\text{dry dish plus dry sample}} - \text{Weight}_{\text{dry sample}}) \times 100}{\text{Weight}_{\text{sample}}}$$

4. pH

Apparatus

- pH meter (Lutron WA-20117SD)

Procedure

1) pH electrode was calibrated using standard buffers (pH 4 and pH 7.2).

5. Total phosphate analysis

Apparatus

- Tubes
- Rack
- Autoclave
- Dropper
- Stirring rod
- Beaker

Reagent

- Sulfuric acid, H_2SO_4, 5N: 70 ml conc H_2SO_4 diluted to 500 ml with distilled water.
- Potassium antimonyl tartrate solution: 1.3715g $K(SbO)C_4H_4O_6 \cdot \frac{1}{2}H_2O$ was dissolved in 400 ml distilled water in a 500 ml volumetric flask and diluted to volume. Stored in a glass-stopper bottle.
- Ammonium molybdate solution: 20 g $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ dissolved in 500 ml distilled water. Stored in a glass-stoppered bottle.

- Ascorbic acid, 0.1 M: 1.76 g ascorbic acid dissolved in 100 ml distilled water. The solution is stable for about 1 week at 4 ^{o}C.

- Combined reagent: The above reagents were mixed in the following proportions for 100 ml of the combined reagent: 50 ml *5N* H_2SO_4, 5 ml potassium antimonyl tartrate solution, 15 ml ammonium molybdate solution, and 30 ml ascorbic acid solution. The mixture was mixed after addition of each reagent in the order set out above. If turbidity formed in the combined reagent the solution was shaken and allowed to stand few minutes until turbidity disappeared before proceeding. The reagent is stable for 4 h.

- Potassium persulfate ($K_2S_2O_8$), 10 g in 200 ml distilled water. This solution was prepared in daily.

Procedure

1) 50 ml or an appropriate amount of sample was diluted to 50 ml with distilled water.

2) 1 drop phenolphthalein indicator was added. If a red color developed, sulfuric acid solution was added until color just disappeared.

3) 1 ml of sulfuric acid solution was added and 10 ml of standard ammonium persulphate was added).

4) The sample was autoclaved gently for 30 minutes at 120 ^{o}C.

5) After cooling, 1 drop of phenolphthalein was added and the solution was neutralized to a faint pink color with 1 N sodium hydroxide.

6) The solution was diluted with distilled water to 100 ml. 25 ml samples were taken for assay.

7) 4 ml of combined reagent was added to the 25 ml sample.

8) At least 10 minutes (but not more than 30 minutes) was allowed for color development.

9) Absorbance was measured at 880 nm using a Shimadzu UV-1601 spectrophotometer (*Shimadzu*, Kyoto, Japan) and sample phosphate calculated from a standard curve as mg P (Fig. A2.1).

6. Ammonia analysis

Apparatus

- Tubes with Caps
- Rack
- Stirring rod
- Tube and Crap
- Rack
- Stirring rod
- Water bath

Reagent

- Distilled water
- Phenol solution : 5 g of phenol dissolved into 50 ml ethyl alcohol 95% (V/V)
- Sodium nitroprusside solution (Na_2Fe $(CN)_5NO.2H_2O$) : 0.5 g of Sodium nitroprusside solution ($Na_2Fe(CN)_5NO.2H_2O$) was dissolved in 100 ml of distilled water kept in a brown bottle.
- Oxidizing solution: 1 g of sodium dichloroisocyanurate, $C_3Cl_2N_3NaO_3$ or potassium dichloroisocyanurate, $C_3Cl_2N_3KO_3$ was dissolved in 5 g of NaOH, 50 g of tri-sodium citrate dehydrate ($Na_3C_6H_5O_7.2H_2O$) and dissolved in distilled water adjusted to a total volume of 250 ml.
- Ammonia standard solution: 0.165 g of ammonia sulphate ($(NH_4)_2$ SO_4) (analytical reagent grade) dissolved in distilled water adjusted to volume of 1000 ml. The stock standard solution was kept in a brown bottle.

Procedure

Preparation of standard curve

1) 5 ml of stock ammonia standard solution was diluted to 500 ml with distilled water.

2) 0, 5, 10, 20 and 40 ml from stock solution were placed into 50 ml volumetric flask and adjusted to 50 ml with distilled water. Standards were: 0, 0.035, 0.070, 0.140 and 0.280 mg-N/L respectively. The zero was used as the blank.

3) 2.5 ml of phenol solution, 5 ml of oxidizing solution and 2.5 ml of Sodium nitroprusside were added to each 50 ml standard. After a 1 hr incubation absorbance were read at 640 nm and a standard curve of A_{640} vs. Ammonia (mg-N) prepared.

Analysis of food waste

1) Samples were assayed routinely using 10ml rather than 50 ml volumes. 10 ml of sample was placed in a screw cap tube to avoid loss of ammonia as gas.

2) 0.5 ml of phenol solution, 1 ml of oxidizing solution, and 0.5 ml of Sodium nitroprusside were added to a 10 ml sample volume and mixed. After incubation for 1 hr the absorbance was read at 640 nm and the standard curve used to calculate ammonia as mg N (Fig. A2.2).

7. Nitrate analysis

Apparatus

- Tubes with Caps
- Rack
- Stirring rod
- Tube and Crap
- Rack
- Stirring rod
- Water bath

Reagent

- Distilled water free of nitrite and nitrate must be used in preparation of all reagents and standards.

- Sodium chloride solution (30%): 300 g NaCl dissolved in distilled water and dilute to 1 L.

- Sulfuric acid solution: 500 ml conc. H_2SO_4 was carefully added to 125 ml distilled water with mixing. The solution was cooled and keep tightly stoppered to prevent absorption of atmospheric moisture.

- Brucine-sulfanilic acid reagent: 1 g brucine sulfate $[(C_{23}H_{26}N_2O_4)_2 \cdot H_2SO_4 \cdot 7H_2O]$ and 0.1 g sulfanilic acid ($NH_2C_6H_4SO_3H \cdot H_2O$) dissolved in 70 ml hot distilled water. 3 ml conc.

HCl was then added, cooled, mixed and diluted to 100 ml with distilled water. Stored in a dark bottle at 5 °C. This solution is stable for several months; the pink color that develops slowly does not effec its usefulness. The bottle was marked with a warning: CAUTION: Brucine Sulfate is toxic; take care to avoid ingestion.

- Potassium nitrate stock solution: 1.0 ml = 0.1 mg NO_3-N. 0.7218 g anhydrous potassium nitrate (KNO_3) dissolved in distilled water and diluted to 1 liter in a volumetric flask. The solution is stable for at least 6 months.

- Potassium nitrate standard solution: 1.0 ml = 0.001 mg NO_3-N. 10.0 ml of the stock KNO_3 solution was diluted to 1 liter in a volumetric flask. This standard solution should be prepared fresh weekly.

- Acetic acid (1 + 3): 1 volume glacial acetic acid (CH_3COOH) diluted with 3 volumes of distilled water.

- Sodium hydroxide (l N): 40 g of NaOH dissolved in distilled water. After cooling it was diluted to 1 L.

Procedure

1) Adjust the pH of the samples to approximately 7 with acetic acid or sodium hydroxide. If necessary, filtration was used to remove turbidity.

2) The required numbers of sample tubes were set up in a test tube rack to handle reagent blank, standards and samples. Space tubes were spread evenly throughout the rack to allow for even flow water of incubation bath water between the tubes. This assists in achieving uniform heating of all tubes.

3) If it was necessary to correct for color or dissolved organic matter which will cause color on heating, a set of duplicate samples were run to which all reagents except the brucine-sulfanilic acid had been added.

4) 10.0 ml of standards were pipetted and samples or an aliquot of the samples were diluted to 10.0 ml - into the sample tubes. Contents of tubes were mixed by swirling and place rack in cold water bath (0 - 10°C).

5) The samples in this study were not saline and so a correction for salinity using blanks with added NaCl were not necessary.

6) 10.0 ml of sulfuric acid solution was pipetted into each tube and mixed by swirling. Tubes were allowed to come to thermal equilibrium in the cold bath. Care needed to be taken that temperatures had equilibrated in all tubes before continuing.

7) 0.5 ml brucine-sulfanilic acid reagent was added to each tube (except the interference control tubes, if they were needed) and carefully mixed by swirling, then the rack of tubes was placed in the 100°C water bath for 25 minutes.

CAUTION: It is critical that all tubes are incubated at the same temperature and so the hot water bath needs to have a circulating pump. After the hot water incubation the rack of tubes is removed from the hot water bath and immersed in a cold water bath to allow them to reach thermal equilibrium (20-25°C).

8) Absorbances were read against the reagent blank at 410 nm and the standard curve used to calculate nitrate as mg N (Fig. A2.3).

8. Starch analysis

Apparatus

- Beaker
- Stirring rod
- Rack
- Stirrer or magnetic mixer

Reagent

- Ethyl alcohol : C_2H_5OH 95%
- Sodium hydroxide : NaOH
- Glacial acetic acid : CH_3COOH
- Potassium iodide : KI
- Iodine : I_2
- Standard Starch solution: soluble starch laboratory grade 20 g dissolved in 1000 ml of hot distilled water.

Procedure

Mechanism of Action of Iodine test for starch. The use of Lugol's iodine reagent (IKI) is useful to distinguish starch and glycogen from other polysaccharides. Lugol's iodine yields a blue-black

color in the presence of starch. Glycogen (animal starch) reacts with Lugol's reagent to give a brown-blue color. Other polysaccharides and monosaccharides yield no color change; the test solution remains the characteristic brown-yellow of the reagent. Starch and glycogen form helical coils. Iodine atoms can then fit into the helices to form a starch-iodine or glycogen-iodine complex. Plant starches in the form of amylose and amylopectin have less branches than glycogen. This means that the helices of starch are longer than glycogen, therefore binding more iodine atoms. The result is that the color produced by a starch-iodine complex is more intense than that obtained with a glycogen-iodine complex. Most of the starch present in food waste would have been plant starch but there would have been some glycogen present from meat in the food waste. Standard plant starch was used in this study as the standard and no differentiation could be made between plant and animal starch.

Preparation of Reagent Solutions and Standard Curve

1) Sodium hydroxide 2 N: 80 g of sodium hydroxide was dissolved in 800 ml of distilled water and adjusted to 1000 ml after cooling.

2) Glacial acetic acid 1 N: 60 ml of glacial acetic acid was dissolved in 800 ml of distilled water then adjusted to 1000 ml.

3) Iodine solution: 0.200 g of Iodine and 2 g of potassium iodide were dissolved in 80 ml of distilled water and adjusted to 100. This solution is not stable and needs to be replaced periodically.

4) 1 ml of Ethyl alcohol and 9 ml of Sodium hydroxide was added to 0.04 g of amylose (soluble starch) and the volume made up to 100 ml with distilled water and stirred for 10 min.

5) Prepare 5 x 100 ml of volume flasks, adding 70 ml of distilled water to each bottle.

6) To the first bottle 0.4 ml of glacial acetic acid was added , second bottle added 0.8 ml of glacial acetic acid, third bottle added 1.2 ml of glacial acetic acid, fourth bottle added 1.6 ml of glacial acetic acid, fifth bottle 2 ml of glacial acetic acid were added. 2 ml of Iodine was added to each of the standards.

7) 1, 2, 3, 4, 5 ml of the standard starch solution was then pipette into the five respective volumetric flasks and each adjusted to 100 ml.

8) A blank was prepared by adding add 2 ml of glacial acetic acid, 2 ml of Iodine and adjusting to 100 ml using distilled water.

9) Absorbance was measured at 620 nm using the prepared blank and a standard curve of A_{620} vs. starch (mg) was prepared. An example standard curve is shown in (Fig A2.4).

Analysis of food waste

1) 1 ml of Ethyl alcohol and 9 ml of Sodium hydroxide was added to 5 g of food waste and the volume made up to 100 ml with distilled water and stirred for 10 min.

2) 5 ml of digested food waste was taken and added to 70 ml of distilled water, 2 ml of glacial acetic acid, 2 ml of Iodine and made up to 100 ml.

3) Absorbance was measured at 620 nm with a blank as zero. A standard curve was used to calculate starch content (Fig A2.4).

9. Reducing sugar analysis

Apparatus

- Beaker
- Stirring rod
- Rack
- Stirrer or magnetic mixer

Reagent

- Standard glucose 10 mg/ml: 1 g of glucose dissolved in distilled water to 100 ml volume.
- 1.5 M of sulphuric acid: 20 ml of concentrated sulphuric acid (18.75 M) was pipetted slowly with mixing into about 200 ml of distilled water then adjust to 250 ml.
- 10% of NaOH (2.5 M): 25 g of NaOH dissolved in distilled water adjusted to 250 ml.
- DNS reagent: 10 g of DNS was first dissolved in 200 ml of 2 M NaOH. Sodium potassium tartrate 300 g was dissolved in water made up to 500 ml and the two solutions were then mixed together and adjusted to 1 L. The DNS reagent was kept in a brown bottle.

Procedure

Preparation of glucose standards for a standard curve

1) A working solution of glucose concentration 2 mg/ml was prepared.

2) A series of standard solutions of glucose were prepared from the working solution using 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 275 µl volumes in tubes and the total volume brought up to 500 µl.

3) Each test tube was incubated in a water bath at 37 ^{o}C for 30 min and then cooled down to room temperature.

4) 0.5 ml of DNS reagent was added and each test tube was incubated in a water bath at 100 ^{o}C for 15 min

5) 4 ml distilled water was added and each tube was cooled in an ice bath.

6) Absorbance of each known glucose solution was measured at 540 nm and used to make a standard curve (A_{540} vs. Glucose (µg)) using the zero as a blank (Fig. A2.5).

Analysis of reducing sugar of food waste

1) 5 g of food waste was added to 50 ml of distilled water heated to 50 ^{o}C for 10 min to ensure dissolution of the reducing sugars present.

2) The extract was then filtered with Whatman No.1 filters and the volume adjusted with distilled water to 100 ml.

3) Dilution step. 10 ml of the extracted sample was then placed in a flask and the volume made up to 100 ml with distilled water.

4) 1 ml of diluted sample was added to 1 ml of DNS reagent and 2 ml of distilled water and then mixed.

5) The test tube was then incubated in a hot water bath at 100 ^{o}C 5 min and then cooled (as for the standards above).

6) Absorbance was measured at 540 nm and the standard curve used to calculate glucose equivalents in mg (Fig. A2.5).

10. Lipid analysis (APHA 1998)

Apparatus

- Beaker
- Stirring rod
- Rack
- Stirrer or magnetic mixer
-

Reagent

- Petroleum ether

Procedure

Preparation of vegetable oil standards for a standard curve

1) Total lipid analysis using standard graph comparison of standard solution of lipid dissolved in various solvents.

2) Prepared stock standard: 10 mg of standard cooking oil was dissolved in petroleum ether made up to 50 ml, equal to 0.2 mg / ml or 200 ppm. Commercial Palm Cooking Oil was used as standard (Oleen Cooking Oil).

3) Process of preparing a standard curve: test tubes were set up as shown in Table 4 and absorbance measured at 210 nm. A calibration curve was prepared of absorbance vs. concentration of standard solution of lipid (in ppm).

Table A1.2-Preparation of lipid stock standard at different concentrations.

Tube no.	Stock standard (ml)	Solvent (ml)	Concentration of oil (ppm)
1	0	10	0
2	1	9	20
3	2	8	40
4	3	7	60
5	4	6	80
6	5	5	100
7	6	4	120

Analysis of food waste

1) 5 g of food waste was used for analysis

2) 50 ml of petroleum ether was added and shaken vigorously to dissolve the lipid in the organic solvent.

3) Particulates were allowed to settle and the absorbance of the supernatant measured at 210 nm and the standard curve used to calculate lipid in micrograms/ml (ppm) (Fig. A2.6).

11. Protein analysis (Lowry et al. 1951)

Apparatus

- Beaker
- Stirring rod
- Rack
- Stirrer or magnetic mixer

Reagent

- Reagent C:

Solution A: 1 g of $NaCO_3$ dissolved into 50 ml of 0.4% NaOH (NaOH 0.2 g in 50 ml of distilled water)

Solution B_1: 1% $CuSO_4 \cdot 5H_2O$ (0.02 g of $CuSO_4 \cdot 5H_2O$ dissolved in 2 ml of distilled water)

Solution B_2: 2% $Na_3C_6H_5O_7 \cdot 2H_2O$ (0.04 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ dissolved in 2 ml of distilled water)

Reagent C = solution A: solution B_1: solution B_2 in ratios of 50: 0.5: 0.5. Reagent C must be made up and used new every time an assay is run.

- Folin–Ciocalteus phenol reagent: yellow stock solution 2 N: needs to be diluted 1:1 before use. 1 N concentration (1:1) kept in brown bottle at room temperature. It must be used fresh every time.

Procedure

Preparation of BSA standards for a standard curve

1) Working solutions were prepared of bovine serum albumin (BSA) standard. The stock concentration used was 1 mg/ml, prepared as 0.1 g of bovine serum albumin in 100 ml of distilled water. These can be kept as frozen aliquots of a few ml each.

2) Preparation of bovine serum albumin (BSA) Standard curve. 0, 0.03, 0.06, 0.09, 0.12, 0.15, 0.18, 0.21, 0.24, 0.27, and 0.30 ml volumes of BSA standard (1 mg/ml) were pipetted into test tubes, adding water to adjust volume to 0.30 ml in each tube.

3) 3 ml of reagent C was added to each tube at room temperature for 20 min after mixing.

4) 0.3 ml of Folin–Ciocalteus phenol reagent was added and mixed immediately then kept at room temperature for 30 min.

5) Absorbance was measured at 750 nm using the zero as the blank.

Analysis of food waste

1) 5 g of food waste was placed in 100 ml of distilled water and a 500 µl was taken for protein analysis in a test tube.

2) 3 ml of reagent C was added to the test tube, mixed and left for 20 min.

3) 0.3 ml of Folin–Ciocalteu phenol reagent was added and mixed immediately then left for 30 min.

4) Absorbance was measured at 750 nm (A_{750}) using distilled water as blank.

5) The standard curve of A_{750} vs. BSA (mg) was used to calculate the protein content of the unknown sample using a curve fitted by linear regression (Fig. A2.7).

Appendices II

Standard Curves

1. Total phosphorus

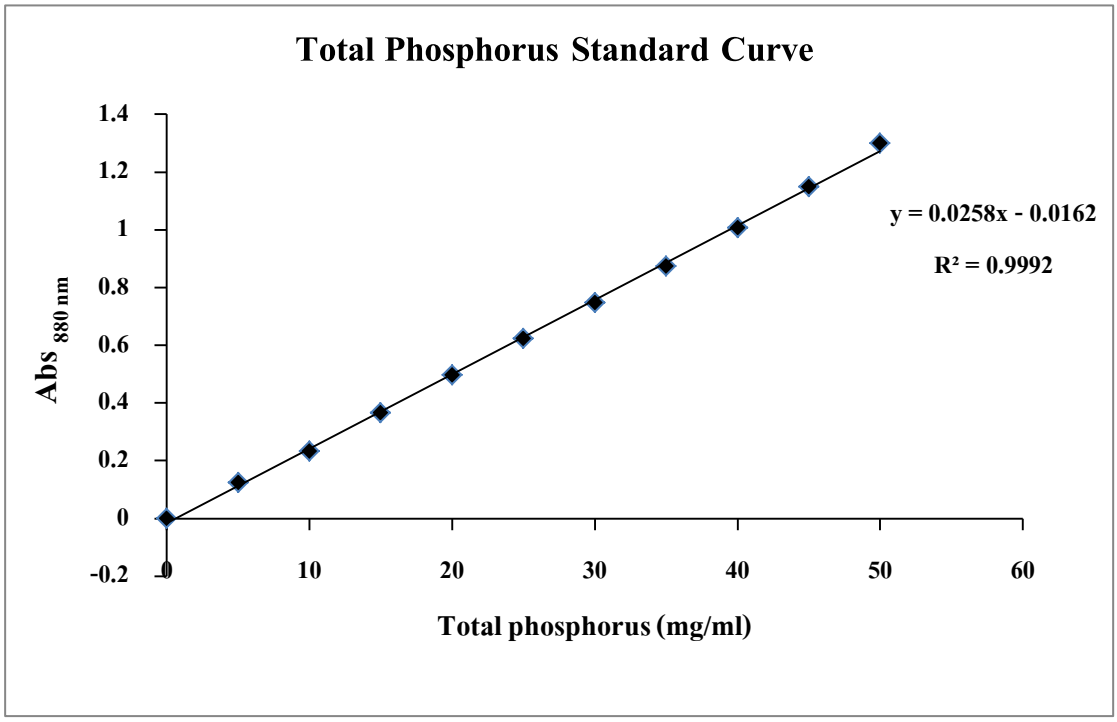

Fig. A2.1-Total phosphorus standard curve

2. Ammonia standard curve

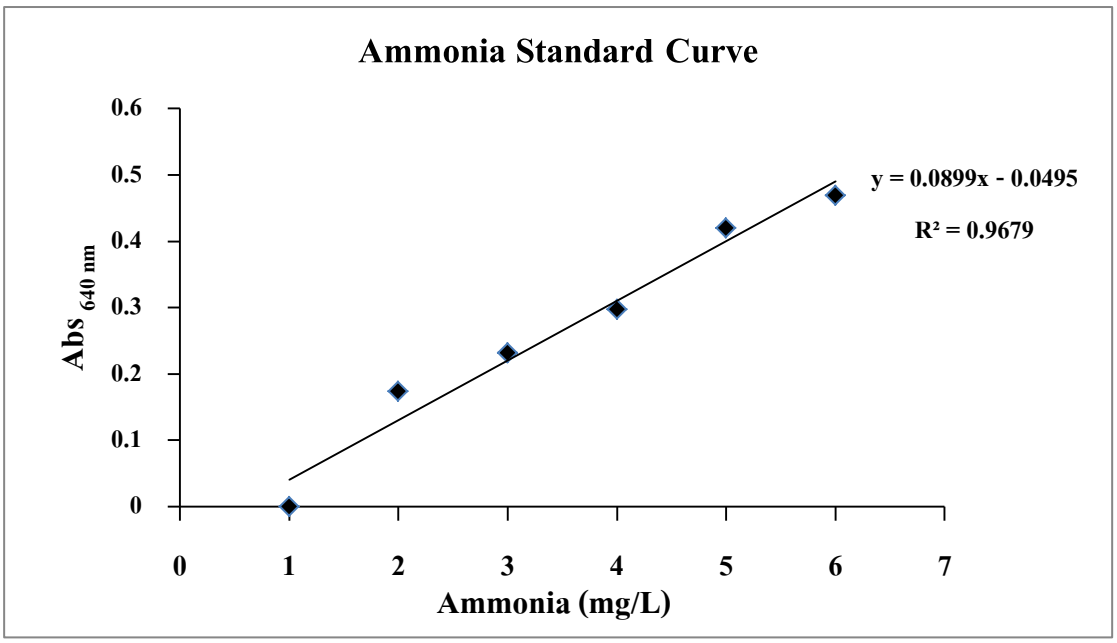

Fig. A2.2-Ammonia standard curve

3. Nitrate – N standard curve

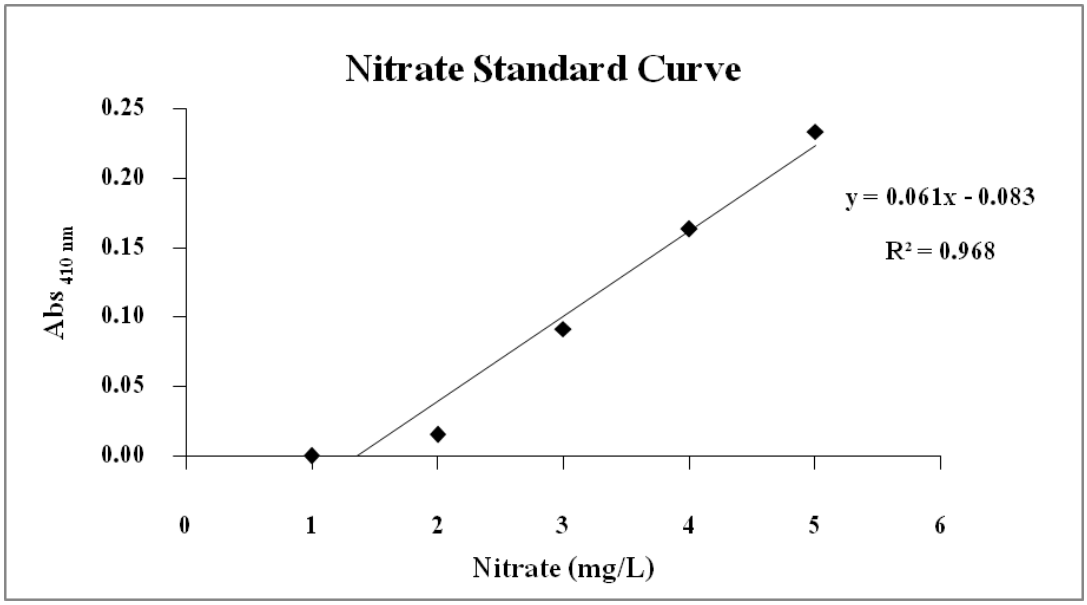

Fig. A2.3-Nitrate-N standard curve

4. Starch standard curve

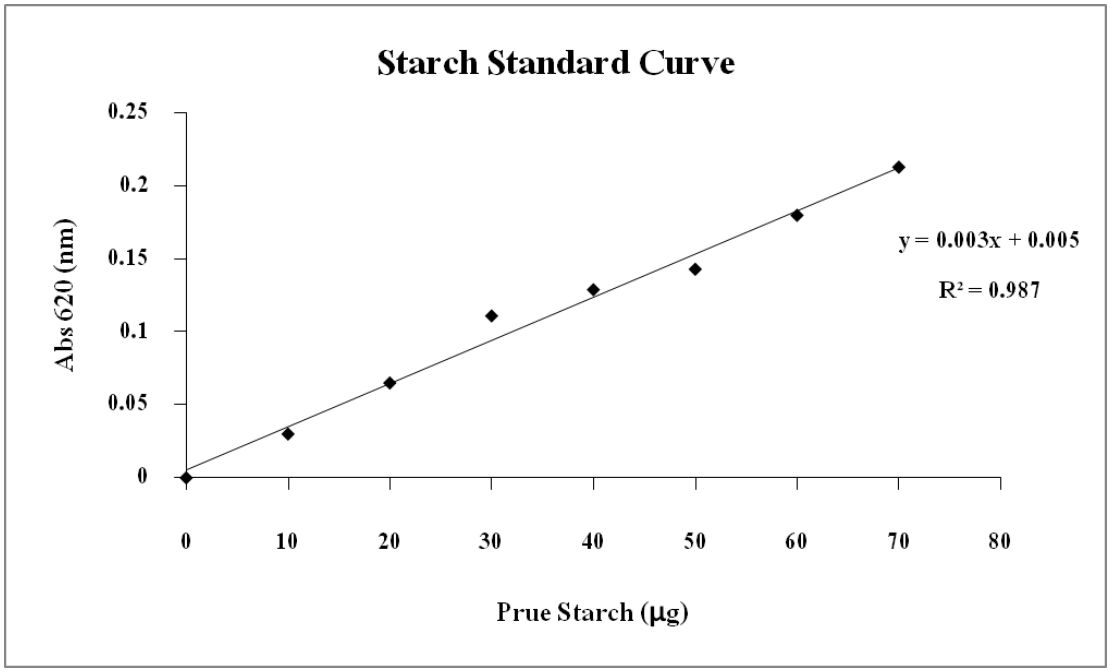

Fig. A2.4-Starch standard curve.

5. Glucose standard curve

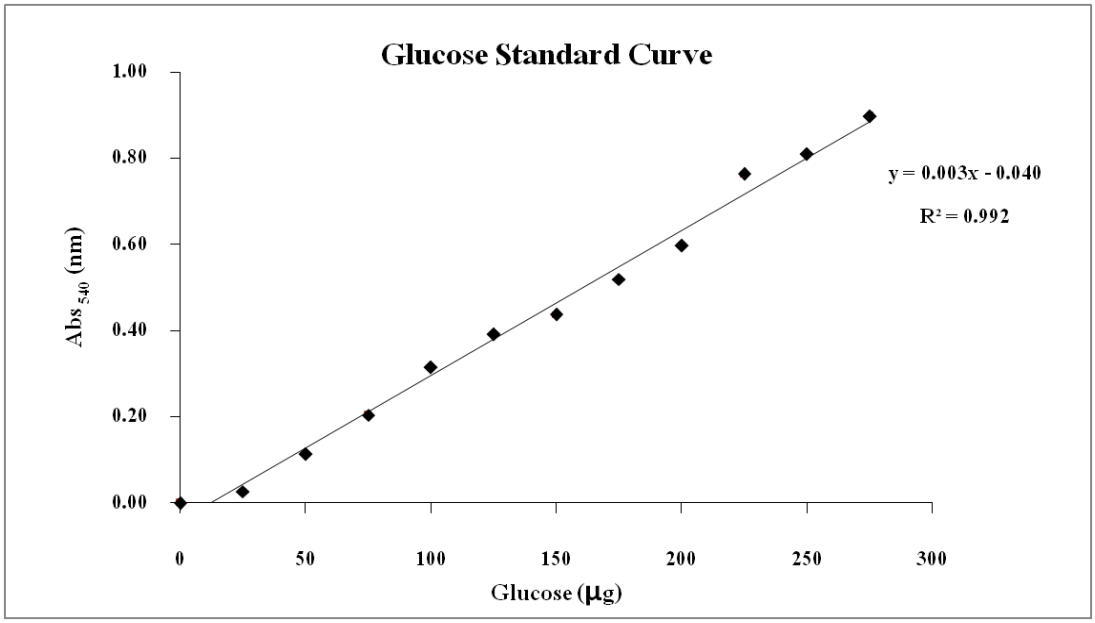

Fig. A2.5-Glucose standard curve

6. Oil standard curve

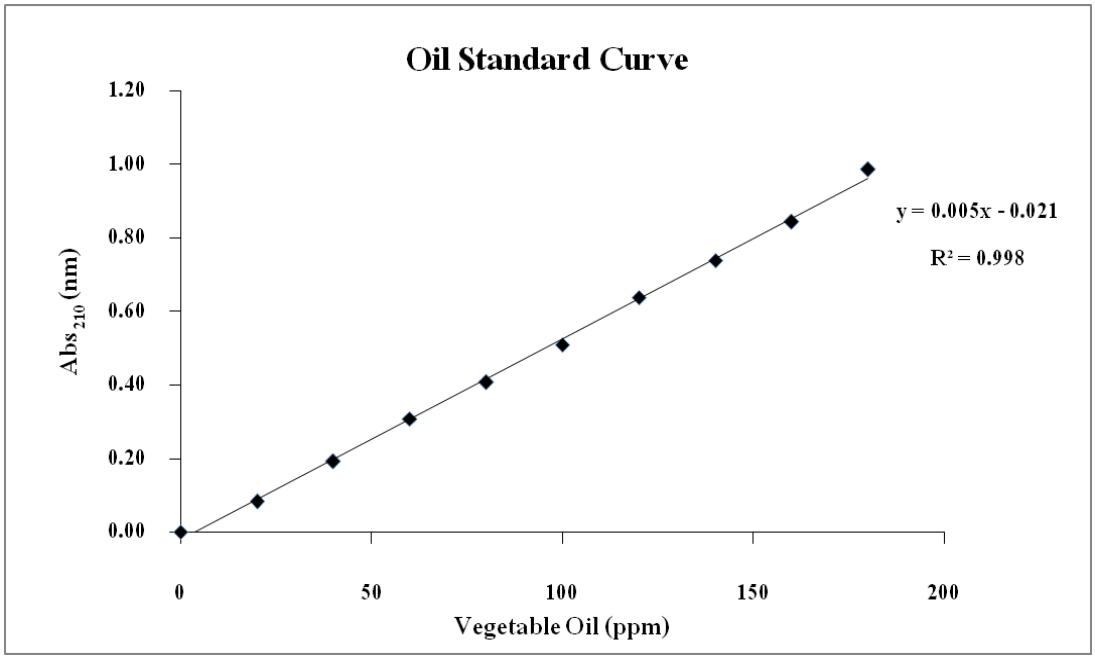

Fig. A2.6-Cooking Oil standard curve

7. Protein standard curve

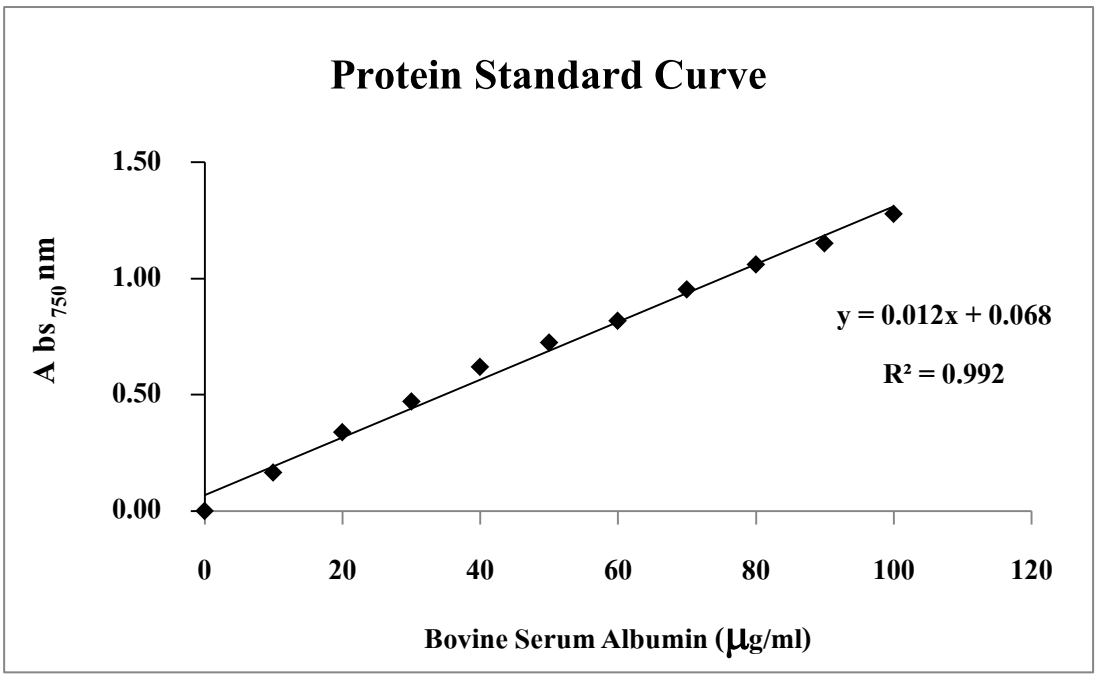

Fig. A2.7-Protein standard curve

8. Bottle of volume standard curve

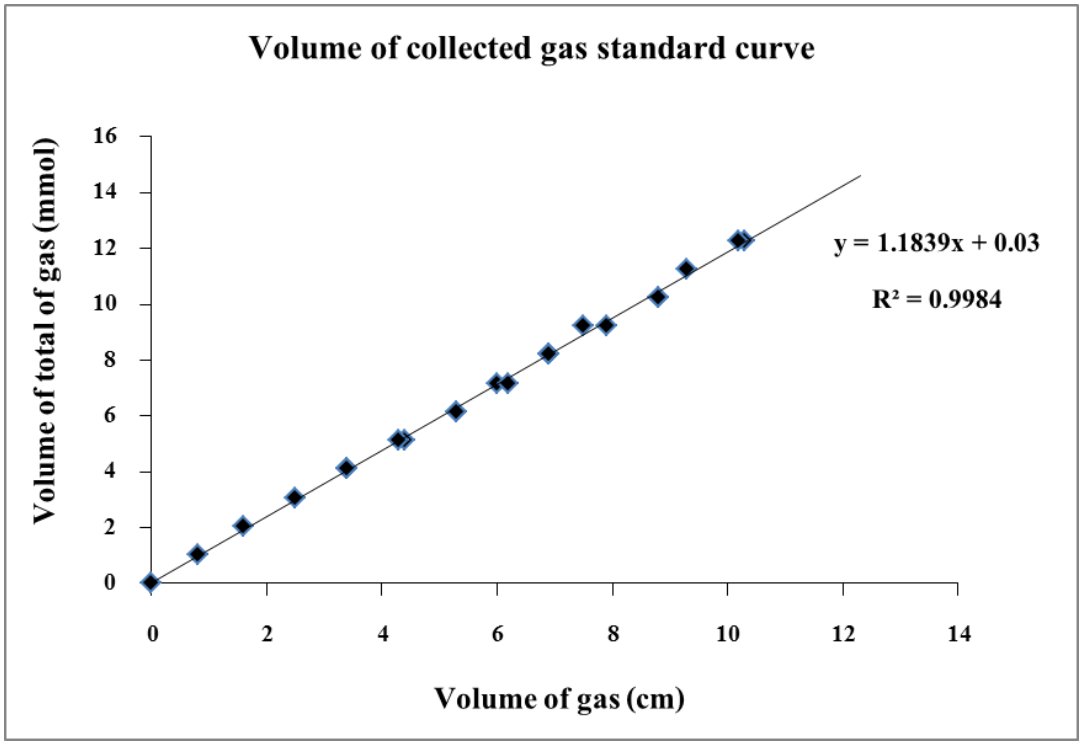

Fig. A2.8-Bottle of volume of collected gas

Appendices III

Culture Media for *Rhodopseudomonas*

Media for *Rhodopseudomonas*

PM media (Kim and Harwood 1991**)**

Table A3.1-PM medium

PM component	For 1000 ml PM (ml)	For 500 ml PM (ml)
Double distilled water	800	400
0.5M Na_2HPO_4	25	12.5
0.5M KH_2PO_4	25	12.5
10% $(NH_4)_2SO_4$	10	5
Concentrated base	1	0.5
0.1M $Na_2S_2O_3 \cdot 5H_2O$	1	0.5
2 mg/ml *p*-aminobenzoic acid (PABA)	1	0.5
	Bring volume to 1000 ml	Bring volume to 500 ml

pH should be around 6.8. 45 min autoclave.

- Pour PM into round flask, flush with argon gas for 30 min, close with rubber stopper, and place medium and 16 ml hungate tubes (including rids and rubber stopper) into anaerobic glove box

- Pour 10 ml into hungate tubes, and close with rubber stopper and rid

- Take tubes from glove box, and autoclave

- Add carbon sources from stock solutions (e.g., acetate and succinate).

PM/Acetate/Yeast Extract (PMAcY) Agar

Table A3.2-PM/Acetate/Yeast Extract (PMAcY) Agar

PMAcY component	For 1000 ml PMAcY	For 500 ml PMAcY
(1) 2x PM	500 ml	250 ml
(2) Yeast extract (Final 0.1%)	1 g	0.5 g
Agar	15 g	7.5 g
Double distilled water	500 ml	250 ml
Autoclave (1) and (2) separately, mix, and add		
2M Sodium acetate (final 20 mM)	10 ml	5 ml

Nitrogen-fixing medium (NF)

Table A3.3-Nitrogen-fixing medium (NF)

NF component	For 1000 ml NF (ml)	For 500 ml NF (ml)
Double distilled water	800	400
0.5M Na_2HPO_4	25	12.5
0.5M KH_2PO_4	25	12.5
Concentrated base	1	0.5
0.1M $Na_2S_2O_3 \cdot 5H_2O$	1	0.5
2 mg/ml *p*-aminobenzoic acid (PABA)	1	0.5
	Bring volume to 1000 ml	Bring volume to 500 ml

pH should be around 6.8. 45 min autoclave.

- Pour NF into round flask, flush with argon gas for 30 min, close with rubber stopper, and place medium and 27 ml anaerobic tubes (including rubber stopper and aluminum seal) into anaerobic glove box

- Pour 10 ml into anaerobic tubes, and close with rubber stopper and seal with aluminum seal

- Take tubes from glove box, and autoclave

- Add carbon sources from stock solutions (e.g., acetate and succinate).

Component for *Rhodopseudomonas* media

Concentrated base

Table A3.4-Concentrated base

Concentrated base component	For 1000 ml concentrated base
Nitrilotriacetic acid (NTA-free acid)	20 g
$MgSO_4$ anhydrous	28.9 g
$CaCl_2 \cdot 2H_2O$	6.67 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0185 g
$FeSO_4 \cdot 7H_2O$	0.198 g
Metal 44	100 ml

EDTA (ethylenediaminetetraacetic acid) can be used as a replacement for NTA. Dissolve NTA separately in 600 ml water and neutralize with KOH (14.6 g KOH), add other components, and dissolve in order given. Adjust to pH 6.8 before making to final volume of 1000 ml. A precipitate forms when adjusting the pH from the acid side of 6.8 with KOH (need about 100 ml of 1M KOH), but eventually will redissolve with stirring. When the pH is near 6.8, the color of the solution changes from a deep yellow to straw color. Then, filter sterilize and store in glass bottle wrapped with aluminum foil. Store at 4ºC for at lease one year.

Metal 44

Table A3.5-Metal 44

Metal 44 component	For 1000 ml metal 44
EDTA (free acid, not sodium salt)	2.5 g
$ZnSO_4 \cdot 7H_2O$	10.95 g
$FeSO_4 \cdot 7H_2O$	5 g
$MnSO_4 \cdot H_2O$	1.54 g
$CuSO_4 \cdot 5H_2O$	0.392 g
$Co(NO_3)_2 \cdot 6H_2O$	0.25 g
$Na_2B_4O_7 \cdot 10H_2O$	0.177 g

Add EDTA to 800 ml distilled water with stirring and adjust pH about 5.0 with 10M NaOH to get EDTA dissolved. Add the other metals in order given (Do not add components until the previous one has completely dissolved), and then make to final a volume of 1000 ml (final pH is around 2.4, a clear and lime green solution). Then, filter sterilize and store in glass bottle wrapped with aluminum foil. Store at 4ºC indefinitely.

Phosphate solutions

Table A3.6-Phosphate solutions

To make	For 1000 ml	For 500 ml
0.5M Na_2HPO_4 (FW 141.96)	70.98 g	35.49 g
0.5M KH_2PO_4 (FW 136.09)	68.045 g	34.0225 g

Filter sterilize.

Others

Table A3.7-Others reagent

To make	For 500 ml	For 250 ml
10% $(NH_4)_2SO_4$	50 g	25 g
0.1M $Na_2S_2O_3 \cdot 5H_2O$ (FW 248.2)	12.41 g	6.205 g
2 mg/ml *p*-aminobenzoic acid (PABA)	1 g	0.5 g

Filter sterilize (PABA bottle should be wrapped with aluminum foil).

Carbon solutions

Table A3.8-Carbon source solutions

To make	For 200 ml	For 100 ml
2M Sodium acetate, trihydrate (FW 136.08)	54.432 g	27.216 g
1M Sodium succinate (FW 270.15)	54.03 g	27.015 g

Filter sterilize 2 times. For anaerobic stocks, filtrate solution (once filter sterilized) into autoclaved bottle and flush with N_2 gas for 30 min.