DISSERTATION- II
REPORT

**BIOHYDROGEN PRODUCTION AND ANALYSIS FROM FRUIT
AND DAIRY WASTES**

SUBMITTED TO
DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIOTECHNOLOGY AND BIOSCIENCES
LOVELY PROFESSIONAL UNIVERSITY
PHAGWARA, PUNJAB

SUBMITTED BY
SALMAN AHMAD KHAN
REG NO: 11307406

UNDER GUIDANCE OF
MS. SOUMYA SRIVASTAVA
ASSISTANT PROFESSOR
DEPARTMENT OF MICROBIOLOGY AND
BIOPROCESSENGINEERING,
LOVELY PROFESSIONAL UNIVERSITY,
PUNJAB, INDIA

ACKNOWLEDGEMENT

Apart from the efforts by me, the success of dissertation- II project depends largely on the encouragement and the guidance of others. I take this opportunity to extend my esteemed regards to those people who have been instrumented in the successful completion of my pre dissertation project.

A formal statement of acknowledgment is hardly sufficient to express my regards to personalities who have rendered their valuable support, help and guidance to meet this end. To begin with I offer my thanks to the almighty and my beloved parents who have always encouraged and supported me. First of all a special thanks to my university Lovely Professional University, Phagwara, Punjab for integrated studies in Biotechnology for giving me the opportunity to carry out this project as an integral part of my M.Tech Biotechnology.

I am entirely indebted to Ms. Soumya Srivastava (Mentor) for her valuable and constant assistance, cooperation, guidance, listening to my queries and correcting the various documents with attention and care during the entire period. It is due to her immense knowledge the expertise that has encouraged me to start with a challenging and prestigious project.

I feel privileged to offer my sincere thanks to Dr. Neeta Raj Sharma (HOS) and Dr. Himanshu Singh (COS) for providing a wonderful opportunity that has brought a revolutionary change in my life and I would also like to thanks SSS-NIRE, Kapoorthala for helping me in carrying out analysis part of project.

With all these people it would have possible for us to successfully complete our project.

SALMAN AHMAD KHAN

(11307604)

ABSTRACT

The worldwide energy consumption is rapidly increasing leading to reduction in fossil fuel reserves as well as increase in erratic prices, global warming, ecosystem imbalance, health hazards. Therefore there is need for alternative fuel which is renewable and environment friendly. Biohydrogen serves as green technology for current energy demands. Hydrogen has a high energy yield of 122 kJ/g, which is 2.75 times greater than those available hydrocarbon fuels. The biological method of hydrogen production results to a win-win solution and opened a new avenue for utilization of renewable and inexhaustible energy sources. In this present study the fermentation of fruit and dairy waste was carried out by facultative anaerobe. *Enterobacter aerogenes* (MTCC 111) was used as micro-organism for production of gas and fatty acids. The waste containing carbohydrates were used as substrate. These substrate were categorized by Reducing sugar test, BOD test, COD test, TDS test and TSS test. The slurry was pretreated by heating. The process was run for 10 days with limited air inside serum glass bottles containing 800ml slurry, sucrose 20g/l and nutrients solution 12.34 g/l. The amount of gas produced was estimated by COD reduction of wastes. The yield of hydrogen from fruit waste was 79.87 ml H_2/ mg COD reduced and dairy waste was 50.68 ml H_2/ mg COD reduced. The byproducts were analyzed by FTIR and HPLC analysis confirming the production of acetic acid, lactic acid, propionic acid and ethanol.

KEYWORDS: *Green technology, Enterobacter aerogenes, fatty acids, BOD, COD, fermentation.*

CERTIFICATE

This is to certify that **SALMAN AHMAD KHAN** bearing registration no. **11307406** have completed Dissertation-II project report (BTY 731), titled **"BIOHYDROGEN PRODUCTION AND ANALYSIS FROM FRUIT AND DAIRY WASTES"** under my guidance and supervision. No part of the report has ever been submitted for any other degree at any university.

The report is fit for submission and the partial fulfillment of the conditions for the award of **M.Tech. Biotechnology**.

Mentor: Ms. Soumya Srivastava
UID: 15898
Date:
Lovely Professional University
Phagwara, Punjab

DECLARATION

I, hereby declare that this project report entitled "**BIOHYDROGEN PRODUCTION AND ANALYSIS FROM FRUIT AND DAIRY WASTES**" is carried out by **SALMAN AHMAD KHAN** under the supervision of "Ms. Soumya Srivastava" for the partial fulfillment of Degree of M. Tech. Biotechnology, Lovely Professional University, Phagwara (Punjab).

STUDENT'S NAME:

Salman Ahmad khan

11307406

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CHAPTER 1
INTRODUCTION

Fossil fuels are the major sources of energy, and they account for about 80% of global energy demand. But they are characterized with a lot of problems, which include non-renewability, erratic prices, global warming, ecosystem imbalance, health hazards, and other environmental/agricultural effects like pollution which contributes more on greenhouse and release of toxic gases like gases like CO_2, SO_2, NOx and other pollutants leading to global warming and food shortage. Therefore, there is need for a renewable, healthier, more environment friendly, abundant/secure, and sustainable alternatives. Biofuels potentially provide these advantages and are increasing in global demand and the resources would be exhausted within decades (Sarma *et al*., 2012).

The energy crisis is the concern that the world's demands on the limited natural resources that are used to power industrial society are diminishing as the demand rises. These natural resources are in limited supply. While they do occur naturally, it can take hundreds of thousands of years to replenish the stores. Our present oil reserves will last 40 years at most and will decline significantly well before then, so it's time to investigate new sources of energy. Globally, experts are working hard to find out how renewable sources of energy can be used to better fulfill our energy needs. This process is difficult and time consuming but significant changes are already occurring (Winter *et al*., 2005).

1.1 *World dependence on energy*

The developing countries such as China and India with the rising energy demand effected global economy and are likely to consume ever more energy. Countries like United Arab Emirates, and Egypt which are the major exporters of crude oil would fail to meet the demands by 2042. India's dependence on imported fossil fuels rose to 38% in 2012, despite the country having significant domestic fossil fuel resources.India ranked as the fourth-largest energy consumer in the world in 2011, following China, the United States and Russia (Kazim *et al*., 2001).

The country's energy demand continues to climb as a result of its dynamic economic growth and modernization. According to World Bank data, India is the third-largest economy on a purchasing power parity basis and has the world's second-largest population (U.S. Energy Information Administration, International Energy Statistics).
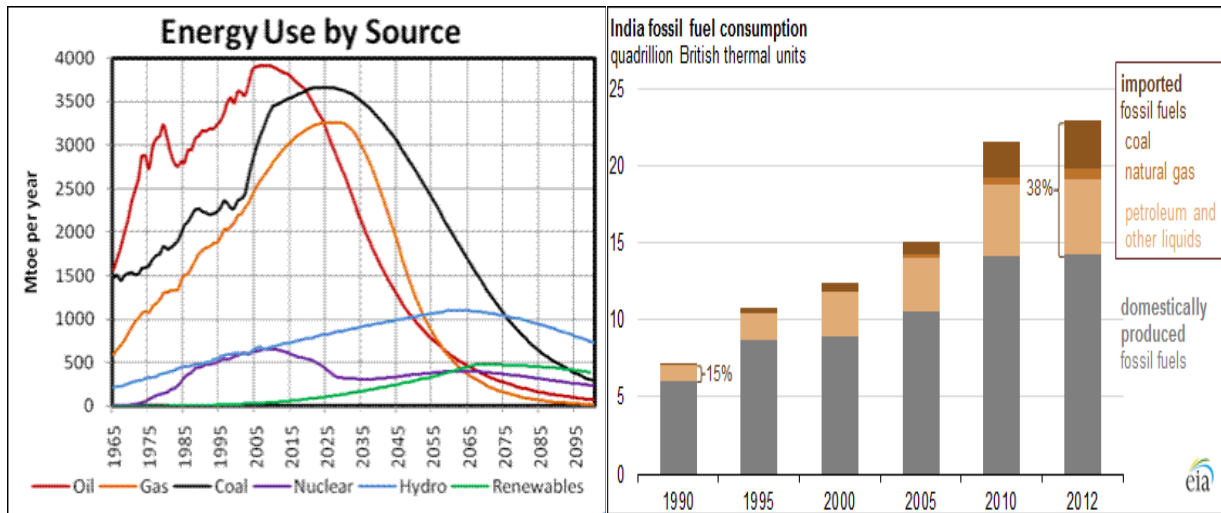
Figure 1: Energy dependence of world and fuel consumption of India. (U.S. Energy Information Administration, International Energy Statistics)

In the realm of growing energy demands, depleting fossil fuel reserves, and increasing pollution loads manifested due to fossil fuel usage .The biofuels signifies a sustainable and futuristic alternative to fossil fuels. There has been increase in development of biofuels plants were operating in Europe (91%), Germany had 35% of all plants, followed by Denmark (17%) Sweden, Switzerland and Austria (8%), Asia (7%) and India (4%) (Bolzonella *et al*., 2003).
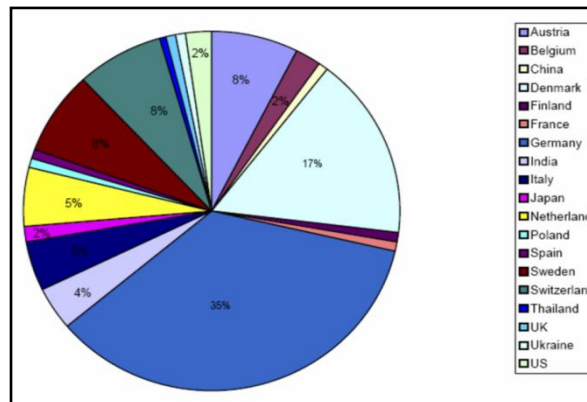
Figure 2: Biofuels plants usage across the world (Bolzonella *et al*., 2003).

1.2 *Bio hydrogen as a green technology:*

In this context, hydrogen has gained significant attention as a "green technology" and future energy carrier due to its clean and sustainable nature. Considerable interest has been generated regarding H_2 production by biological routes employing photo-biological, dark fermentation, thermochemical, enzymatic etc (Zang *et al.*, 2000).

1.3 *Need for bio-hydrogen production:*

Hydrogen gas is also used widely as feedstock for the production of chemicals, hydrogenation of fats and oils in food industry, production of electronic devices, processing steel and also for desulfurization and re-formulation of gasoline in refineries. Hydrogen can be produced domestically from several sources, reducing our dependence on petroleum imports. Hydrogen produces no air pollutants or greenhouse gases when used in fuel cells; it produces only nitrogen oxides (NOx) when burned in ICEs.Based on the National Hydrogen program of the United States, the contribution of hydrogen to total energy market will be 8.10% by 2025 (fuel economy, 2009).

1.4 *Comparison of biohydrogen from other fuels:*

Hydrogen has three times higher energy content than petrol and methane but because of its low density (0.0000899 kg/l at 20°C) it has very low energy content per unit volume. The properties and a comparison of hydrogen with other fuels are presented in table 1 (Midilli *et al*., 2005).

Fuel	Energy content mass / HHV (MJ/kg)	Energy content volume / HHV (MJ/l)
Petrol	47.4	34.8
LPG* (liquid)	48.8	24.4
LNG** (liquid)	50	23.0
Hydrogen (liquid)	141.9	11.9
Hydrogen (gas)	141.9	0.012
Methane (gas)	50.2	0.039
*Liquefied petroleum gas. **Liquefied natural gas.		

Table 1: Hydrogen vs other fuels (Midilli, *et al*., 2005)

1.5 *Biohydrogen production methodologies:*

1.5.1 *Conventional production method*

Conventional hydrogen gas production methods are steam reforming of methane (SRM), and other hydrocarbons (SRH), non-catalytic partial oxidation of fossil fuels (POX) and auto thermal reforming which combines SRM and POX. Those methods are all energy intensive processes requiring high temperatures (>850°C) (Armor, 1999).

Biomass and water can be used as renewable resources for hydrogen gas production. Despite the low cost of waste materials used, high temperature requirement (T = 1200°C) is still the major limitation for this. Electrolysis of water may be the cleanest technology for hydrogen gas production. However, electrolysis should be used in areas where electricity is inexpensive since electricity costs account for 80% of the operating cost of hydrogen production process (Kim *et al.*, 2003).

1.5.2 *Microbial role in production:*

Bio-hydrogen production can be realized by both anaerobic and photosynthetic micro-organisms using carbohydrate rich and non-toxic raw material. Under anaerobic condition, hydrogen is produced as a byproduct during conversion of organic waste into organic acids which are then used for methane generation. Acidogenic phase of anaerobic digestion of waste can be manipulated to improve hydrogen production. Photo synthetically processes include algae which use CO_2 & H_2O for hydrogen gas production. Some photo heterotrophic bacteria utilize organic acids such as lactic, acetic and butyric acid to produce H_2 and CO_2 (Levinin *et al.*, 2004).

1.5.3 *Bio-chemicals methods*

The production of bio-hydrogen is a microbial conversion process carried out bacteria capable of synthesizing hydrogen producing enzymes such as hydrogenase and nitrogenase in dark and photo-fermentation respectively. Biological hydrogen production is classified into four categories:

(1) Bio-photolysis of water using solar energy and algae /cyanobacteria

(2) Photo-decomposition of organic compounds using light energy and photosynthetic bacteria,

(3) Fermentative hydrogen evolution to breakdown carbohydrate-rich substrate to hydrogen and other products such as acid and alcohols using anaerobic bacteria and

(4) Hybrid system combining dark and photo-fermentation either directly or in a series -type (Kothari *et al*., 2012).

1.6 *Hydrogen Economy:*

Hydrogen has been put forward as a new energy carrier in a system called "*Hydrogen Economy*", which would be used as a fuel and to transport and store energy in the way that electricity is used. Hydrogen as an energy carrier has many advantages due to its non-toxicity, high energy content, yielding only water in combustion, and its flexibility to be used both in fuel cells as well as internal combustion engines (Gregory 1973).

1.7 Advantages of biohydrogen:

Hydrogen has significant advantages as an energy resource:

- The combustion of hydrogen only produces water vapor, which is a non-greenhouse gas and also do not cause environmental and atmosphere pollution.
- The combustion of hydrogen in automobiles is 50% more efficient than gasoline.
- Hydrogen battery is deemed as future supply for automobiles.
- Hydrogen gas has a three times higher energy content than petrol and methane and also from hydrocarbon fuels on mass level.
- Hydrogen shows double conversion efficiency when used in fuel cell instead of gas turbine.
- Petroleum and chemical industries like fossil fuels processing and ammonia manufacturing.
- Hydrogenation agent to increase the level of saturated fats and oil.
- Metal production and fabrication.
- Shielding gas in welding methods such as atomic hydrogen welding.
- Filling gas in balloons and airships and also as fuel for rocket propulsion.
- Energy storage technology and electronic industry.
- Production and processing of silicon.
- Power generation with fuel cells.

(Reith 2003; Armor 2005; Dong 2007; Scragg, 2009; Mohammed 2011).
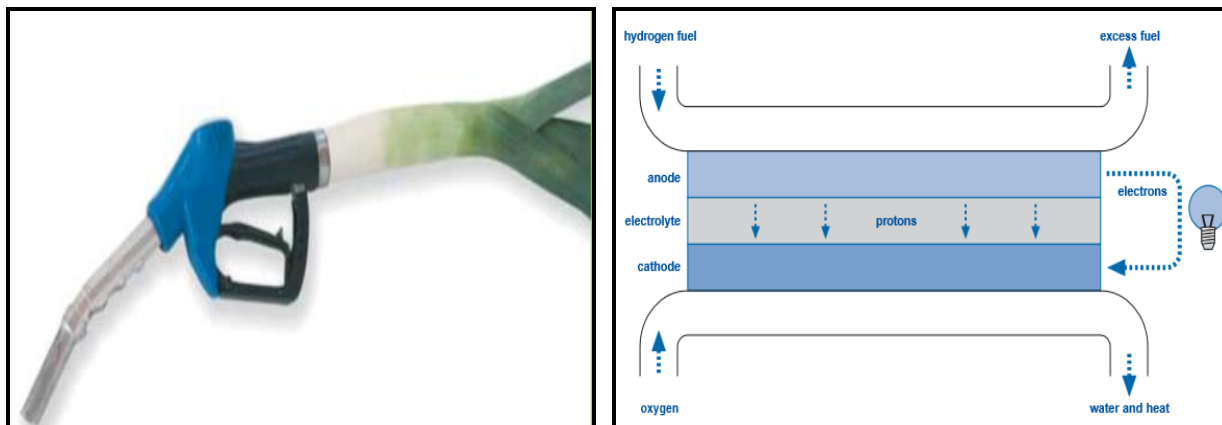
Figure 3: Applications of biohydrogen (Reith *et al*., 2003)

1.8 Challenges in production:

Hydrogen as a gas, in ambient conditions, occupies a large volume (11 m3/kg) for storage. Therefore, the main challenge in hydrogen storage is to reduce the volume of gas in equilibrium with the environment. Hydrogen can be stored as pressurized gas, liquefied hydrogen, in metal hydrides, in nanostructured/ porous material, in hydrogen-rich chemical or on the surface of adsorption compounds (Krishna *et al*., 2012).

Currently, issues in early developmental stages include, the weight of the absorbing material-mass of a tank would be about 600 kg compared to the 80 kg of a comparable compressed H_2 gas tank, lower desorption kinetics, recharge time and pressure, heat management, life cycle cost, container compatibility and optimization (Riis *et al*., 2006)

Hydrogen distribuition over long-distance distance done as a liquid in super- insulated, cryogenic, over-the-load-tankers, railcars and barges. The transported liquid hydrogen is then vaporized for end uses. The blending hydrogen into natural gas pipeline possess another issues concerning that includes, benefits of blending, extent of natural gas pipeline network, end-use systems impact , safety, downstream extraction and also leakage (Melaina, *et al.,* 2013).

CHAPTER 2
TERMINOLOGY

FOSSIL FUELS:

Fossil fuels are fuels formed naturally such as anaerobic decomposition of buried dead organisms. These fuels contain high percentages of carbon and include coal, petroleum, and natural gas. Other more commonly used derivatives of fossil fuels include kerosene and propane.

SRM:

Steam Reforming Methane is a method for producing hydrogen, carbon monoxide, methane and other useful products from hydrocarbon fuels such as natural gas. This is accomplished by processing device called a reformer which causes reaction with steam at high temperature with the fossil fuel.

FACULTATIVE ANAEROBE:

A facultative anaerobe is an organism that makes atp by aerobic respiration if oxygen is present, but is capable of switching to fermentation or anaerobic respiration if oxygen is absent. *Staphylococcus spp*., *Streptococcus spp*., *Escherichia coli*, *Listeria spp*., and *Shewanella oneiensis* are example of facultative anaerobe.

HYDROGENASE:

Bacterial cells can get benefit from uptake activity of hydrogenase through formation of reducing equivalents required for cell's metabolism. A hydrogenase is an enzyme responsible for catalysis of reversible oxidation of molecular hydrogen. It also consume and produce H_2 depending upon conditions.

BATCH REACTOR:

The batch reactor is the generic term for a type of vessel widely used in the process industries. These type of reactors are used for a variety of process operations such as solids dissolution, product mixing, chemical reactions, batch distillation, crystallization, liquid/liquid extraction and polymerization.

CHAPTER 3
SCOPE OF STUDY

Today's energy requirements are heavily dependent on fossils fuels such as oil, coal and natural gas. Due to extensive use of fossils fuels their environmental consequences have already begun to surface. The primary cause of global warming and acid rain is the excessive use of fossil fuels only. These started to affect the earth's climate, weather, vegetation and aquatic ecosystem. The global warming and natural energy considerations, a non-polluting and renewable energy source needs to be developed.

The scope of present study is to develop an alternative fuel to combat current energy problems. Thus, producing biohydrogen from waste by action of bacteria serves as a clean energy source obtained from wastes having potential energy substitute for fossils fuel. In near future it will be widely used in fuel cells.

CHAPTER 4
AIMS AND OBJECTIVES

AIM: Production and analysis of bio-hydrogen from fruit and dairy waste.

OBJECTIVES:

- To characterize substrates under different categories.
- To produce hydrogen gas and fatty acids from wastes.

CHAPTER 5
REVIEW OF LITERATURE

The biological hydrogen production is a promising and sustainable process where renewable organic waste can be used as energy generating source. Availability of huge quantities of wastewater coupled with anaerobic treatment can be considered to be a useful methodology to reduce pollution load along with hydrogen generation. For economical production of hydrogen, carbohydrates and proteins are the main components. The fermentative evolution is more advantageous than photochemical evolution for mass production of hydrogen by microorganisms, where various wastewaters can be used as substrates (Kothari *et al.,* 2006).

In photo-fermentation organic materials or biomasses are converted into hydrogen and carbon dioxide by photosynthetic bacteria under the simultaneous use of solar energy. It takes place under anoxic or anaerobic conditions by using photosynthetic microorganism and sunlight as energy. The optimal temperature is 30–35°C and pH 7.0. One of the groups of micro-organisms capable of photo-fermentation are purple non-sulphur (PNS) bacteria, which under anaerobic conditions utilizes simple organic acids. Species of such bacteria include *Rhodobacter sphaeroides, Rhodopseudomonas palustris, Rhodobacter capsulatus and Rhodospirillum rubrum.* (Azwar *et al.,* 2014)

Dark fermentation is the fermentative conversion of organic substrate and biomass materials to produce bio-hydrogen which takes place in anaerobic conditions at 30 to 80°C without the presence of light. Unlike bio-photolysis process that produces only H_2, the products of dark-fermentation are mostly H_2 and CO_2 combined with other gases, namely CH_4 and H_2S, depending on the reaction process and the used substrate. Additionally, compared to other biological methods dark-fermentation has several advantages, such as its ability to produce hydrogen continuously without the presence of light, higher hydrogen production rate, process simplicity, lower net energy input and utilization of low-value waste raw materials (Ni *et al*., 2006)

According to chemicals reactions occurring biohydrogen production, the complete oxidation of glucose to hydrogen and carbon dioxide yields a maximum of 12 mole hydrogen per mole of glucose (Eq. 1). However, there are no metabolic energy is obtained in this case. Currently this high yield reaction is not reported in fermentative systems (Reith *et al.*, 2003).

$$C_6H_{12}O_6 + 6H_2O \text{-----------} > 12H_2 + 6CO_2 \quad \Delta G_0 = +3.2kJ \quad \text{(Eq. 1)}$$
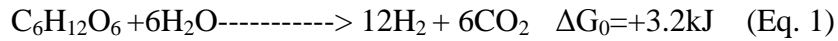
Similarly, the available hydrogen production from glucose is determined by the butyrate/acetate ratio. When acetic acid is the end-product, a theoretical maximum of 4 moles hydrogen per mole glucose is obtained (Eq. 2) and when butyrate is the end-product, a theoretical maximum of 2 moles hydrogen per mole glucose is produced (Eq.4) (Reith *et al.*, 2003).

$C_6H_{12}O_6 + 2H_2O \text{---------} > 4H_2 + 2CH_3COOH \text{ (acetate)} + 2CO_2 \qquad \Delta G_0 = -206kJ \text{ (Eq.2)}$

$C_6H_{12}O_6 + 2H_2O \text{---------} > 2H_2 + 2CH_3CH_2COOH \text{ (propionate)} + 2CO_2 \quad \Delta G_0 = -231kJ \text{ (Eq. 3)}$

$C_6H_{12}O_6 + 2H_2O \text{----------} > 2H_2 + CH_3CH_2CH_2COOH \text{ (butyrate)} + 2CO_2 \quad \Delta G_0 = -254kJ \quad \text{(Eq. 4)}$
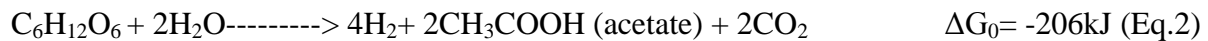
The highest theoretical yields of hydrogen are associated with acetate as the fermentation end-product. In practice, high hydrogen yields are associated with a mixture of acetate and butyrate fermentation products, and low H_2 yields are associated with propionate and reduced end-products such as alcohols and lactic acid (Levin *et al.*, 2004).
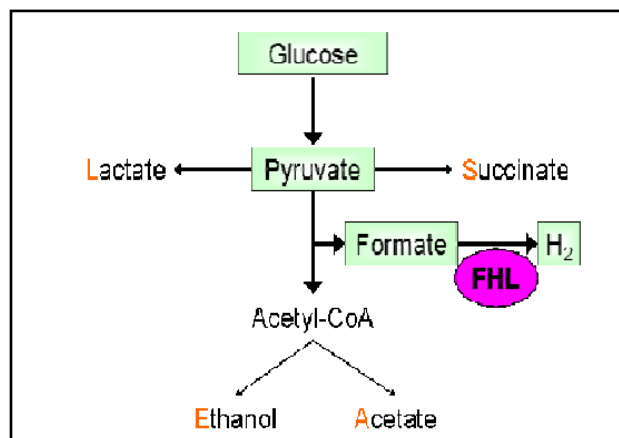
Figure 4: The biological pathway of hydrogen production (Datar *et al.*, 2004).

Reactors	Bacterial strains	Substrate	HRT (h)	Operating conditions pH	Operating conditions Temp (°C)	Maximum H_2 yield	References
Batch	POME sludge	Food waste	-	7	55	593 ml H_2/ g carbohydrate	(Nazlina, et al., 2009)
Fed-batch	*Clostridium sp.*	Swine manure	16	5	35	18.7×10^{-3} g H_2/ g TVS	(Zhu, et al., 2009)
AnSBR	Seed sludge	Food waste	36	5.3 ± 1	35 ± 1	0.5 moles H_2/ mole hexose added	(Kim, et al., 2010)
Batch	*Clostridium sp.* R1	Carbohydrate	-	6	30	3.5 moles H_2/ mole cellobiose	(Ho, et al., 2010)
Batch	*Bacillus coagulans* IIT-BT S1	Sludge as substrate	12	6	37	37.16 ml H_2/ g COD consumed	(Kotay & Das, 2010)
AnSBR	Seed sludge from a dairy manure	Liquid swine manure	16	5.0	37 ± 1	1.50 moles H_2/ mole glucose	(Wu, et al., 2010)
1. Batch 2. CSTR	*Clostridium acetobutylicum* and *Citrobacter freundii*	Xylose	-	6.8	45	1. 0.71 moles H_2/ mole xylose 2. 1.97 moles H_2/ mole xylose	(Mäkinen, et al., 2012)
Batch	Bacterial hydrolysis	Grass silage	-	7	37	37.8 ± 5.8 ml H_2/ g silage	(Li, et al., 2012)
Batch	*Clostridium pasteurianum*	Dry Grass	-	7	35	72.21 ml H_2/ g-dry grass	(Cui & Shen, 2012)
Batch	Sewage sludge	Food waste		6.0 ± 1	35 ± 1	2.26 moles-H_2/ mole hexose	(Im, et al., 2012)
Batch	Anaerobic digested sludge	Distillery wastewater		5.5	37	1 l H_2/ l medium	(Wicher, et al., 2013)
Semi-continuous	Seed anaerobic sludge	Glucose		6–8	35	7 mmol H_2/ g_{dwt}-h	(Kan, 2013)

*TVS means total volatile solids *g_{dwt} means gram dry weight

Figure 5: Studies on hydrogen production by dark-fermentation (Azwar, *et al*., 2014)

The biohydrogen production potential of carbohydrates is approximately 20 times higher than that of lipids. One attractive way to produce hydrogen is biological production, and it can be done through either by dark fermentation of low cost substrate from waste or by photo processes through photolysis by splitting water. However, production of H_2 by dark fermentative processes is technically much simple than photo processes (Bartacek *et al.,* 2007).

In methanogenic pathway, acidogenic processes produce hydrogen and Volatile Fatty Acids (VFA) and are thereby considered an effective and promising means to produce clean energy hydrogen. Fermentative hydrogen production can be achieved by anaerobic acid-forming bacteria such as clostridium sp. or facultative anaerobes such as *Enterobacter* sp.(Levin *et al.,*2004).

Many anaerobic organism can produce hydrogen from carbohydrate containing wastes. The organisms belonging to genus *Clostridium* and *C. buytricum*, *C. Thermolacticum*, *C. Pasterurianum, C. Paraputrificum* M-21 and *C. bifermentants* are obligate anaerobes and spores forming organisms. *Clostridia* species produce hydrogen gas during the exponential growth phase. In the batch growth of Clostridia the metabolism shifts from a hydrogen /acid production phase to stationary growth phase. Investigations on microbial diversity of a mesophilic hydrogen producing sludge indicated the presence of clostridia species as 64.6% (Fang *et al*., 2002).

Clostridium thermolacticum can produce hydrogen from lactose at 58^0C. One more hydrogen producing bacterial strain *Klebisalle oxytoca* HP1 was isolated from hot springs with maximal hydrogen production rate at 35^0C. *T. thermosaccharolyticum* and *Desulfotomaculum geothermicum* strains producing hydrogen gas in thermophilic acidogenic culutures.. A hyperthermophilic archeon, *Thermococcus kodakaraesis* with 85^0C optimum growth temperature was isolated from geothermal spring in japan and identified as a hydrogen producing bacteria. Ueno investigated that the hydrogen gas production capacity of some thermophilic organisms belonging to the genus *Thermoanaerobacterium* has been investigated (Shin *et al*., 2004).

E. aerogenes characteristic are smaller, rod-shaped cells that are motile and encapsulated compared to others in the same family of *Enterobacteriaceae*. The complete genomic information (88% is coded) has not been entirely sequenced as of yet, however, there is some research that shows studies on mutations as well as show evidence of replication through plasmids. *Enterobacter* species contain a subpopulation of organisms that produce a beta lactamase at low-levels (Fraser *et al* , 2006).

Enterobacter aerogenes has been plated on several different medias and have been observed under several types of testing. The results are as follows- *E. aerogenes* tested negative when treated with/for: Indol, Methyl red, Hydrogen sulfide (by way of TSI), Urease, Arginine dihydrolase, Phenylalanine deaminase, and Dulcitol. *E. aerogenes* tested positive when treated with/for: Voges-Proskauer, Simmons' citrate, KCN, Motility, Lysine decarboxylase, Ornithine decarboxylase, Gas from glucose, Lactose, Sucrose, Manntiol, Salicin, Adonitol, Inositol,

Sorbitol, Arabinose, Raffinose, and Rhamnose. Delayed positive results were obtained from: Gelatin (22°C) and Malonate (Fraser *et al.,* 2006).

Enterobacter aerogenes grows under both aerobic and anaerobic conditions, it assimilated glucose at a rate as high as 17 mmol glucose/(g-dry cell h) and consequently evolved hydrogen at near-equal speed. The yields of hydrogen, though, were small, at 1.0 mol from 1 mol glucose and 2.5 mol from 1 mol sucrose. The optimum temperature for bacterium growth was about 40 °C and the optimum pH for hydrogen evolution was roughly 5.7, though 7.0 was the optimum pH for growth. This bacterium immediately restored the respiratory function to its aerobic condition when cultivation was changed from anaerobic to aerobic conditions (Tanisho *et al.,* 1983)

The ability of *Enterobacter aerogenes* to produce hydrogen through the fermentation of a variety of sugars, including glucose, galactose, fructose, mannose, mannitol, sucrose, maltose, and lactose, has led scientists to investigate the use of this bacteria's metabolism as a means of acquiring clean energy. Many bacteria can produce hydrogen through fermentation at a neutral pH, and *E. aerogenes* is no exception. Its optimal pH for hydrogen production is between 6 and 7. However, maintaining that high of a pH is difficult during fermentation because the process yields acidic products such as acetic acid, succinic acid, and lactic acid, and the accumulation of those products causes a decrease in pH (Kumar *et al*., 2001).

Microorganism	Temperature (^{0}C)	Substrate	Reference
Clostridium sp. no 2	36	glucose, xylose	(Taguchi *et al*., 1994; Taguchi *et al*., 1995; Taguchi *et al.,* 1996)
C. paraputrificum M-21	37	GlcNAc1	(Evvyernie *et al*., 2001; Evvyernie *et al*., 2000)

C. butyricum *LMG1213tl*	36	glucose	(Heyndrickx *et al*., 1986)
Pyrococcus *furiosu 98*	98	glucose	(Nakashimada *et al*., 1999)
C. butyricum *LMG1213tl*	36	glucose	(Heyndrickx *et al*., 1986)
Thermotoga *neapolitana*	55	Glucose	(Sakai et al., 2005) Pyrococcus furiosu 98 glucose (Nakashimada *et al*., 1999)
Thermotoga *neapolitana*	55	glucose	(Sakai *et al*., 2005)
Thermotoga elfii	65	glucose	(de Vrije *et al*., 2002; Ravot *et al*., 1995; van Niel *et al*., 2002)
Clostridium *thermocellum*	70	glucose, sucrose	(Yokoyama *et al.,* 2007b)
Caldanaerobacte *r subterraneus*	70	glucose, sucrose	(Yokoyama *et al*., 2007b)
Enterobacter	35	Glucose	(Lu *et al*., 2007; Nath *et al.,* 2006; Sen and Das 2005; Shin *et al*., 2007; Thompson *et al*., 2008)

E. aerogenes	38	glucose	(Tanisho and Ishiwata 1994; Tanisho *et al.*, 1987; Tanisho *et al.,* 1983)
E. cloacae IIT-BT 08 wt	36	glucose, sucrose	(Kumar and Das 2000; Kumar and Das 2001; Kumar *et al.*, 2001; Tanisho *et al.*, 1987)
E. cloacae IIT-BT 08 m DM11	36	glucose	(Kumar and Das 2001; Kumar *et al.*, 2001)
E.aerogenes E.82005	38	molasses	(Tanisho and Ishiwata 1994)
E. aerogenes HU-101 wt	37	glucose	(Rachman *et al.*, 1998)
C. butyricum IFO13949 +	37	Starch	(Yokoi *et al.*, 2002; Yokoi *et al.*, 1998; Yokoi *et al.,* 2001)

Table 2: Hydrogen producing species bacteria (Reith *et al.,* 2003).

The hydrogen producing aerobic cultures such as *Aeromonos spp.*, Pseudomonas spp., and vibrio spp. were identified. Anaerobic cultures like *Actinomyces spp., Porphyromonos spp.*, Beside to clostridium spp. have been detected in anaerobic granular sludge. The hydrogen yield varied between 1 and 1.2 mmol/mol glucose when culture were cultivated under anaerobic conditions. Hydrogen production by thermotogales species and Bacillus sp. were detected in mesophilic acidogenic cultures (Shin *et al.*, 2004).

Organism	Substrate	Mode of operation	pH/ temperature	Volumetric H_2 production (L/L med)	Yield (mol H_2/mol substrate)
Anaerobic bacteria					
C. butyricum EB6	POME	Batch	5.5/37 °C	3.2	–
C. butyricum ATCC19398	Glucose (3 g/L)	Batch	7.2/35 °C	0.94	1.8
C. acetobutyricum M121	Glucose (3 g/L)	Batch	7.2/35 °C	0.88	2.29
C. tyrobutyricum FYa102	Glucose (3 g/L)	Batch	7.2/35 °C	0.63	1.47
C. beijerinckii L9	Glucose (3 g/L)	Batch	7.2/35 °C	1.19	2.81
C. thermolacticum	Lactose (10 g/L)	Continuous	7/58 °C	–	3.0
C. thermocellum 27405	Delignified wood fiber	Batch	6.3/60 °C	–	1.6
C. tyrobutyricum	Glucose (5 g/L)	Immobilized	HRT 2 h	7.2 L H_2/L d	223 ml/g hexose
Facultative anaerobic bacteria					
E. aerogenes ATCC29007	Glucose (118.06 mM)	Batch	6.13/38 °C		425.8 ml H_2/g DCW h
Klebsiella oxytoca HP1	Glucose (10 g/L)	Batch	7.0/65 °C	87.5 ml H_2/L h	1.0
Citrobacter sp. Y19	Glucose (10 g/L)	Batch	7.0/36 °C	32.2 mmol H_2/g cell h	2.49
E. asburiae SNU-1	Glucose (25 g/L)	Batch	7.0/30 °C	398 ml H_2/L h	–
Thermophilic bacteria					
T. thermosaccharolyticum PSU-2	Sucrose (10 g/L)	Batch	6.2560 °C	12.12 mmol H_2/L d	2.53
T. saccharolyticum JW/SL-YS485	Xylose (4 g/L)	Batch	6.2/55 °C	–	0.88
T. maritima DSM3109	Glucose (7.5 g/L)	Batch	6.5/65 °C	0.275	1.67
T. neapolitana DSM4359	Glucose (10 g/L)	Batch	7.0/65 °C	0.29	1.84
Caldicellulosiruptor saccharolyticus DSM8903	Sucrose (10 g/L)	Batch	7/70 °C	8.4 mmol H_2/L	5.9

Figure 6: Yield of hydrogen gas from different microorganisms (Mei-ling *et al*., 2009).

Food waste and food processing wastes are potential feedstocks for bio-hydrogen production. However, the effects of variation of carbohydrate, fat, protein, cellulose and hemicellulose contents in the mixture of food waste are not clearly understood by bio chemical means. For example the highest bio-hydrogen yield of 2.79 mmol H_2 L^{-1} d^{-1} was obtained by fang from rice slurry which is rich in carbohydrate content while highest bio-hydrogen achieved the yield of 18.4 mmol H_2 L^{-1} d^{-1} from vegetable kitchen waste which is rich in cellulose content (Lee *et al*.,2008)

Carbohydrate has been reported to be the most suitable feedstock for bio-hydrogen production although other components in food waste such as fat, protein, cellulose can also be used as substrate (Lay *et al*., 2003). Theoretically, hydrogen is difficult to be produced from lipid and protein degradation. Carbohydrate rich substrate such as rice slurry, food waste rich in carbohydrate content wheat is concluded to be suitable substrates for bio hydrogen production. (Kim *et al*., 2011).

During carbohydrate hydrolysis, hydrolytic bacteria produce simple sugars such as sucrose, glucose, xylose or hexose. Glucose is an easily biodegradable carbon source, present in most of the industrial effluents and can be obtained abundantly from agricultural wastes. Theoretically Glucose is an easily biodegradable carbon source, present in most of the industrial effluents and can be obtained abundantly from agricultural wastes. According to reaction stoichiometry, bioconversion of 1mol of glucose is formed when butyrate is the end product (Kapdan *et al.,* 2006).

The highest hydrogen yield from glucose is around 2.0-2.4mol/mol. Production of butyrate rather than acetate may be one of the reasons for deviations from the theoretical yield. The partial biodegradation of glucose could be less than 95% glucose was degraded, the yield could be less than 1.7mol H_2 /mol glucose. Therefore, the utilization of substrate as an energy source for bacterial growth is the main reason for obtaining the yields lower than theoretical estimations (Ueno *et al.,* 2001).

A yield of 4.52 molH_2/mol sucrose in CSTR with 8h hydraulic residence time and this yield are higher than the other reported studies such as 3.47mol H_2/ mol sucrose in CSTR and 1.5mol H_2 /mol sucrose in USAB at same time HRT. However, the yield from glucose was only 0.91 mol H_2/ mol sucrose under the same operating conditions in CSTR. The optimization of C/N ratio at 47 provided efficient conversion of sucrose to hydrogen gas with a yield of 4.8mol H_2/ mol sucrose (Chen *et al*., 2003).

Similarly, cumulative hydrogen production from sucrose was 300ml while it was only 140 ml from starch, *Enterobacter cloacae* ITT-BY 08 produces 6mol H_2 /mol sucrose which is the highest yield among the other tested carbon sources (Kumar *et al.,* 2004).

The maximum hydrogen yield 3mol H_2/ mol lactose although theoretical yield is 8 mol H_2/ mol lactose. The results of these studies indicated that the higher hydrogen yields could be obtained from sucrose compared to other simple sugar. However, the yield per mole hexose remains almost the same for all types disaccharides (Collet *et al*., 2003).

The starch containing materials are abundant in nature and have great potential to use as a carbohydrate source for hydrogen production. According to stoichiometry, a maximum of 553 ml hydrogen gas is produced from 1gm of starch with acetate as a byproduct (. However, the yield may be lower than the theoretical value because of utilization of substrate for cell synthesis. Liu showed the maximum specific hydrogen production rate was 237 ml H_2 per gm VSSd when 24g/l edible corn was used as a substrate by *C.pasteurianum* (Zhang *et al*., 2003).

The dry sweet potato starch residue for hydrogen production by mixed culture of *C.butyricum* and *E. Aerogenes*. The hydrogen obtained in long term repeated batch operations was 2.4mol H_2 /mol glucose from 2% starch residue containing waste water (Zhang *et al*., 2003).

The oils and dairy product are the source of lipids in food waste and food processing waste. In anaerobic hydrolysis by microorganisms, bacteria secrete lipase for lipid hydrolysis. The hydrolysis of triacylglycerides resulted in free fatty acid and chains and glycerol. During the beta oxidation, glycerol and free fatty acids could be further hydrolyzed to acetyl-CoA and acetate resulting in hydrogen evolution from NADH oxidation and the oxidation state is different depending on the number of double bonds of the side chains of fatty acids (Cirne *et al*., 2007).

The proteins are polypeptides formed by joining covalently linked amino acids. Food waste and food processing waste from cheese, whey, casein, fish meat, chicken and eggs contain significant amount of protein. During anaerobic degradation of protein, hydrogen-producing bacteria such as *Clostridium sp*. hydrolyze protein into polypeptides and amino acids by secreting protease enzyme. Then the amino acids are further broken down into volatile fatty acids, carbon dioxide, hydrogen, ammonia, and reduced sulfur (Lay *et al*., 2003).

The cellulose used as a hydrolysate for fermentation by *Clostridium spp*. During an 81 h period of stationary culture, the organism consumed 0.92mmol glucose per hr and produce 4.10mmol H_2 per hr. The same culture was also used for hydrogen production from pure xylose or glucose and enzymatic hydro-lysate of Avicel cellulose or xylan. The hydrogen yield from the hydro-lysate was higher than that of carbohydrates as 19.6 and 18.6 mmol/gm of substrate consumed (Taguchi *et al*., 1995).

The production potential from micro crystalline cellulose at mesophilic condition with heat digested sludge was investigated by. Increasing cellulose concentration resulted in lower yields with the maximum value of 2.18 mol H_2 /mol cellulose with 12.5 g/lt cellulose concentration. However, 25g/l cellulose concentration provided the highest specific hydrogen production of 11.16 mmol/gm VSSd. The maximum hydrogen obtained in study was 102ml/g cellulose, which is only 18% of theoretical yield low yield was explained as partial hydrolysis of cellulose (Lay *et al.*, 2001).

Peels of fruits such as bananas, apples, oranges, guavas, cantaloupes, pears, papaya, pineapples, seeds of mangoes, apricots, peach and pectin is extracted from citrus peel and apple pomace are used as substrate in hydrogen production for many years as well as the main solid waste produced by the dairy industry is the sludge resulting from wastewater purification. Wastewater results from tank, truck and storage tank washing, pipe line washing and sanitizing. It contains whole milk products, cheese/whey/curd, milk solids, and milk wastes (Nazlina *et al.*, 2013).

The dairy industry in India on an average has been reported to generate 6-10 liters of waste water per liter of the milk processed. Depending upon the process employed, product manufactured and housekeeping exercised. The waste water of diary contain large quantities of milk constituents such as casein, lactose, fat, inorganic salt, besides detergents & sanitizers used for washing (Kolhe *et al.*, 2009).

Biohydrogen production from food waste and food processing waste containing large amount of cellulose such as jackfruit peels, apple waste, pineapple waste and food waste containing kimchi and vegetable kitchen waste results in interesting and varied. In order to avoid this problem, some studies applied physical treatments such as steam explosion or chemical treatments such as acid or alkaline treatment to disrupt the rigid structure of cellulosic and lignocellulosic materials and to saccharify the sugars for the production of bio-hydrogen by cellulosic bacteria in anaerobic conditions (Lay *et al.*, 1999).

From confectioners, apple, potato processor industrial effluents and also from domestic waste water. The highest production yield was obtained as 0.21t H_2 /gm COD from potato processing

waste water. Molasses is another carbohydrate substrate and it is a good source of sucrose. The maximum and available rate of hydrogen production continuous operation with *E.aerogenes* strain E.820005 was 36 and 20 mmol H_2 /lt hr , respectively. Whereas researcher Tanisho investigated the available yield was1.5mol H_2 sugar expressed interm of sucrose.Immobilization of cultures of polyurethane foam increase the yield to 2.2mol H_2 / mol sugar (Ginkel *et al*., 2005).

The hydrogen production through anaerobic fermentation using wastewater as substrate has been attracting considerable attention. One such feasible source is dairy wastewater, which contains complex organics, such as polysaccharides, proteins and lipids, which on hydrolysis form sugars, amino acids, and fatty acids. Anaerobic digestion of organic substrates to produce methane and carbon dioxide has been a well-developed biological treatment for wastewater and solid waste (Atif *et al*., 2005).

Among the fruits, global top four fruit commodities i.e. melon, banana, apple, and grape were selected as feedstock for the experimental investigation. Fruit waste containing organic polymers e.g. carbohydrate is subjected to dark fermentation process in which the polymers are hydrolyzed to sugars (Khamdan *et al*., 2009).

According to report submitted by Food and Agriculture Organization (FAO), UN in year 2009 that the dark fermentation of the selected fruits waste was conducted successfully to produce H_2, without any methane gas being detected. Among the four, banana yielded higher H_2 with potential global production more than 309 million cubic metric based on the 10% of harvested banana in 2009 being wasted. Simulation calculation of H2 production from the four fruits was also presented. Total H2 production can reach 726 million m^3 (FAO, 2009).

No	Fruits	Quantity (tons)[a]	Percent Wasted	H_2 Yield (mmol/g VS)	Potential H_2 Production (STP m^3)
1	Melon	101 000 000	10	5,96	185 808 197
2	Banana	95 000 000	10	8,61	309 093 490
3	Apple	71 700 000	10	7,30	153 941 162
4	Grape	66 900 000	10	7,28	77 457 569
	Total				726 300 418

Figure 7: Hydrogen yield from different fruits (Volume of harvested fruit in 2009, FAO UN)

There were more than 400 million tons of fresh fruits being harvested in 2009 to meet global demand. Throughout the cultivation until consumption, some portion of the fruits turn into waste. According to Global Food Losses, at least 10% of fresh fruits turn into waste during cultivation in agriculture sectors (Global food losses, 2011).
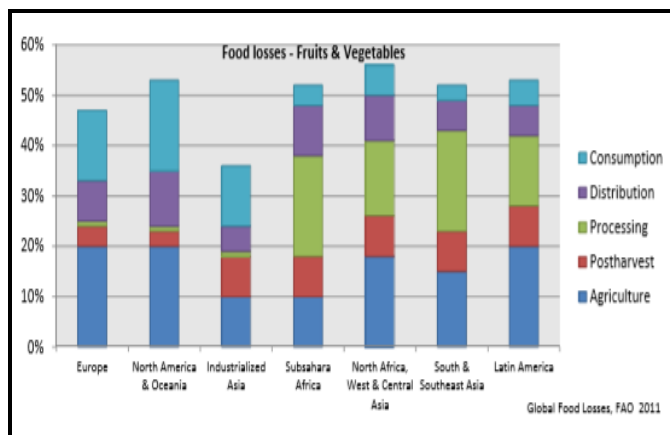

Figure 8: Global food losses FAO, 2011

Biochemical oxygen demand value gives the amount of oxygen required by microorganisms. It is necessary to provide standard conditions of nutrient, supply, pH. Absences of microbial because of the low solubility of oxygen in water strong wastes are always diluted to ensure that the demand does not increase in available oxygen. Low value of BOD is comparatively in winter months may be due to lesser quantity of total solids, suspended solids in water as well as to the quantitative number of microbial population (Avasan *et al*., 2001).

Waste water of food and dairy industry contains large quantities of milk constituents such as casein, lactose, Fat, inorganic salts. Besides detergents and sanitizers used for washing. All these components contribute largely towards their high biochemical oxygen demand of effluents of textile industry. From the different unit BOD value of mixed effluent ranged between 320 mg/l to 720 mg/l and final effluent 80 mg/l to 640 mg/l (Trivedi *et al*., 1986).

COD test is useful in pinpointing toxic condition and presence of biological resistant substances. Importance of organic matter in the ecology of bloom. The value of COD of untreated effluent was 1230 mg/l and that of treated effluent was 594 mg/l. It is also observed COD value of industry waste water ranges from 300 mg/l to 2400 mg/l. (Trivedi *et al*., 1986).

The maximum concentration of total dissolved solids in summer, which increased in rainy seasons. While, the minimum value was found in winter probably because of stagnation. In summer most vegetation is decaying, so rise in the amount of dissolved solids was neutral as the products of decaying matter which were settled in the water. In the rainy season less concentration of total dissolved solids are obtained, due to the concentration of the dissolved solids are obtained due to the dilution of waste effluents with rain water. It is reported that total dissolved solids in range 488 mg/l in the waste water from Jayanthi Nalla (Hosetti *et al*., 1994).

Effluent from the different food and dairy industries may have the different amount of solid particulate matter. When the effluent flows through the open drainage system particulate matter is expected to show greater degree of variance. If the effluent is highly acidic then the solid may dissolved in it, therefore it is necessary to evaluate effluent for the particulate matter. It is observed that the T.S.S. & sugar mill effluents is 220 to 790 mg/l (Avsan *et al.,* 2001).

PARAMETERS	High	Medium	Low
pH	8.0	7.2	7.0
BOD at 20 ^{o}C in mg/L	350	200	50
COD in mg/L	1000	500	250
Total Solids in mg/L	1300	700	200
Dissolved in mg/L	1000	500	250
Suspended in mg/L	350	220	100

Table 3: General characteristics of sewage wastewater (Ramprasad *et al*., 2012)

The pH level has an effect on enzyme activity in microorganisms, since each enzyme is active only in a specific pH range and has maximum activity at its optimal pH. It has been accepted in hydrogen research that pH is one of the key factors affecting the hydrogen production. Hydrogen fermentation pathways are sensitive to pH and are subject to end-products (Craven *et al*., 1998).

The results indicate that the control of pH was crucial to hydrogen production. It has been reported that under unoptimal pH, the hydrogen fermentation process shifted to solvent production or prolonged the lag phase (Temudo *et al*., 2007).

The lactate production was always observed together with sudden change of environment parameters, such as pH, HRT, and temperature, which indicated the culture was not adapted to the new environment (Demirel *et al.,* 2004).

The pH optimum around 5.5 was also reported by most of the other researchers for hydrogen production using HSW as substrate. Moreover, at unoptimum pH, a fermetnation pathway changeing from acetate pathway to butyrate pathways was detected and thus decreased the hydrogen production. Yasin in 2009, explained hydrogen been most produced at initially at pH 7.0. The hydrogen producing bacteria might be suppressed if too low pH was applied initially and could effect substrate utilization (Alzate *et al*., 2007).

The production at initial pH 7.0 resulted in higher bio-hydrogen yield when compared to bio-hydrogen production at controlled pH 5.5 range. Low ph at the initial stage of bio-hydrogen production resulted in suppression of hydrogen-producing bacteria for further degradation of the substrate. The addition of acid and alkali during hydrolysis process resulted in excess chemical addition and unstable bacterial studies which suggested the suitable ph of bio-hydrogen production to be controlled at pH 5.0-6.0. Therefore, neutral initial pH resulted in high bio-hydrogen yield due to bacterial adaptation to hydrolysis stages in the anaerobic degradation pathway (Yasin *et al.,* 2011).

According to very higher pH leads to cell maintenance disruption in which energy will be used to maintain neutrality instead of producing bio-hydrogen. Due to lack of ATP function, the activity of enzymes will be inhibited in hydrogen producing bacteria. Lay reported that initial pH 5.0 slowed down hydrogen-producing bacteria germination and lowered hydrogenase enzyme production. Very alkaline pH resulted in a long lag phase of bio-hydrogen production due to bacterial adaptation time to the alkaline cultures broth to produce organics acids as well as bio-hydrogen (Zong *et al*., 2009).

On addition of buffer and alkali such as a sodium hydroxide, potassium hydroxide and calcium carbonate are required to improve bio-hydrogen production. The livestock manure can provide

high buffering capacity and wide variety of nutrient of nutrient and nitrogen necessary for optimal bacterial growth (Pan *et al*., 2008).

The temperature affects the hydrogen producing bacteria activities and hydrogen production rate. Dark hydrogen fermentation reactions can be operated at different temperatures: mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C) or hyperthermophilic (>80°C) (Nath *et al*., 2006).

In most of dark fermentation experiments are conducted at $35-55C^0$. The extreme thermophilic process provides a number of advantages compared with the mesophilic and thermophilic. Firstly, the hydrogen production is much higher at extreme-thermophilic conditions than at mesophilic and thermophilic conditions (Levin *et al.,* 2004).

The extreme-thermophilic anaerobic hydrogen fermentation can achieve more hydrogen production and higher hydrogen production rates than mesophilic hydrogen fermentation. It has been reported that at exteme-thermophilic condition (70°C), hydrogen yield reached the theoretical maximum of 4 mole hydrogen per mole glucose, where the ones at mesophilic and thermophilic conditions were normally less than 2 mole hydrogen per mole glucose (Van Groenestijn *et al.,*2002).

At high fermentation temperature it was thermodynamically favorable for a hydrogen-producing reaction as the high temperature resulted in the increase in the entropy term, and made dark hydrogen fermentation more energetic while the hydrogen utilization processes were negatively affected with temperature (Hallenbeck *et al.,* 2005).The yield of bio-hydrogen from cheese whey was 0.78 and 1.03mol H_2 mol^{-1} glucose at mesophilic temperature (35^0C) and thermophilic temperature (55^0C) respectively (Kargi *et al.,* 2009).

The poor performance of bio-hydrogen from food waste obtained in mesophilic temperature at 35^0C was due to lactic acid production that inhibited the growth of hydrogen producing bacteria. They proved that bio-hydrogen could be effectively produced at thermophilic temperature from

food waste naturally even though no pre-treated inoculum was used as a seed culture due to the suppression of lactic acid bacteria (Kim *et al.,* 2011).

The experiments on bio-hydrogen production at 55^0C while 35^0C was effective for methane production. Heat -treatment or the use of thermophilic temperature during bio-hydrogen production resulted in generation of hydrogen producing bacteria and the suppression of acid producing bacteria. Thus, biological production from food waste and food processing waste was suggested to be effective at thermophilic temperature between 50 and 60^0C (Chu *et al.,* 2008).

During anaerobic degradation, intermediate by-products such as a actcetic acid, buytric acid and lactic acid and propionic acid are produced. The production of hydrogen is accompanied by production of acetic acid and butyric acid. In contrast, no hydrogen could be produced if the by-products are lactic acid and propionic acid (Ren *et al.,* 2006).

The study on pre-treatment such as heat, chemical or pH shock to food waste or seed culture are therefore applied to inhibit the effects of lactic acid bacteria during fermentation and to inhibit the effects of lactic acid bacteria during fermentation bacteria. In harsh environment, for example during pre-treatment germinate while hydrogen consuming bacteria could not survive and thus can be suppressed (Kim *et al.,* 2009).

The sequential dark and photo fermentation is rather a new approach in biological hydrogen gas production. There is limited number of studies carried out on sequential hydrogen gas production system.it has several advantages over single stage dark or photo-fermentation processes. The effluents of dark fermentation in hydrogen production provides sufficient amount of organic acids for the photo-fermentation. Therefore, the limitation by the organic acid availability would be eliminated. Bio-hydrogen production can be obtained when two system are combine. Further utilization of organic acids by photo-fermentative bacteria could provide better effluent quality in term of COD. The system should be well controlled to provide optimum media composition and environmental condition for the two microbial components of the process (Yokoi *et al.,* 1995).

CHAPTER 6

MATERIALS AND EQUIPMENTS

6.1 MATERIALS

Medias and chemicals:

S.No.	Materials	Company
1.	Autoclave	NSW Pvt. Ltd.
2.	Face Mask	Smart Care
3.	Glass wares	Borosil Glass
4.	Hot air oven	NSW Pvt. Ltd.
5.	Incubator	Yorco Incubator Bacteriological
6.	Laminar air flow	Rescholar Equipment
7.	Microwave	INALSA
8.	Microscope	Magnus
9.	Micropipette	P'Fact A
10.	Microtips	TARSONS
11.	Orbital shaker	REMI
12.	pH meter	Hanna
13.	Plastic wares	Poly lab
14.	Refrigerator	LG
15.	Weighing balance	Adventurer
16.	Centrifuge	REMI
17.	Spectrometer	Equip-tronics

6.2 EQUIPMENTS

Equipments and instruments:

S.No.	Chemicals/Medias	Company
1.	EMB agar	Himedia LaboratoriesPvtLtd.
2.	EMB broth	Himedia LaboratoriesPvtLtd.
3.	Ethanol	ChangshuYangyuan Chemical
4.	Nutrient broth	Himedia LaboratoriesPvtLtd.
5.	Nutrient agar	Himedia Laboratories Pvt Ltd.
6.	Dinitrosalicylic acid	Titan Biotech Ltd.
7.	NaOH	Central Drrug HousePvtLtd.
8.	Sodium potassium tartrate	Central Drug HousePvtLtd.
9.	Phenol	Central Drug HousePvtLtd.
10.	Sodium sulphite	Titan Biotech Ltd.
11.	Peptone	Himedia LaboratoriesPvtLtd.
12.	Yeast extract	Himedia Laboratories Pvt Ltd.
13.	Glucose	Himedia Laboratories Pvt Ltd.
14.	Potassium dichromate	Titan Biotech Ltd.
15.	Sodium thiosulfate	Central Drug HousePvtLtd.
16.	Manganous sulfate	Central Drug HousePvtLtd
17.	Starch	Central Drug HousePvtLtd
18.	Potassium iodide	Central Drug HousePvtLtd
19.	Sulphuric acid	Central Drug HousePvtLtd

CHAPTER 7
RESEARCH METHODOLOGY

STEP 1

MAINTENANCE OF CULTURE

The microbial strain of *Enterobacter aerogenes* (MTCC 111) was collected from IMTECH, Chandigarh. The culture was revived and maintained in Nutrient Broth medium and sub-cultured after every 15 days (Marshall *et al*., 1993).

STEP 2

SAMPLE COLLECTION

The sample of fruits and dairy waste were collected from different source which were used as a substrate (Nazlina *et al.,* 2013).

STEP 3

SUBSTRATE CHARACTERIZATION

Both fruit and dairy sample were characterized by performing Reducing sugar test, BOD test, COD test, TDS test and TSS test (Miller *et al*.,1959; APHA 1995; Aneja *et al*., 2003; Howard *et al*., 1933).

STEP 4

PRETREATMENT OF SUBSTRATE

The samples were grounded into slurry with distilled. The slurry was pretreated by heating at around 90 °C for 30 minutes and the pH was adjusted to 6.5 (Guo *et al*., 2008).

STEP 5

BIO-HYDROGEN PRODUCTION

1L reactor serum bottle containing approximately 800ml slurry, sucrose 20g/l different nutrients (12.34 g/l) and 8% (64ml) inoculum of *E.aerogenes* was taken. 40%w/v KOH solution was also added in slurry for selective adsorption of CO_2. The serum bottle reactor was connected separately to 2 other bottles by gas pipes. One was used as the gas collecting chamber and the other as the reactor. The fermentation process was carried out in limited supply of air at 37^0C for 10 days (Azwar *et al*., 2014).

STEP 6

ANALYSIS

The estimation of H_2 gas was done by analyzing COD reduction and fermented byproducts were analyzed by FT-IR and HPLC analysis (Griffiths *et al*., 2007; Jong *et al*., 2006).

7.1 BACTERIAL CULTURE AND MAINTANENCE:

The microbial strain of *E.aerogenes* (MTCC 111) was collected from IMTECH, Chandigarh. The culture was revived and maintained in Nutrient Broth medium and sub-cultured after every 15 days. To check the viability and contamination, the microbial culture was inoculated on EMB agar medium. The EMB agar medium was prepared by adding 3.6 gm. EMB agar in 100ml distilled water. The media was autoclaved at 121 lbps for 15min. After autoclaving and cooling to approx. 55°C, antibiotic was added and poured into petridishes. Then plates were streaked with the inoculum of bacteria, the plates were inverted and stored at +4°C in the dark. The glycerol stock was prepared and the culture was stored at 4°C (Marshall et al., 1993).

For further studies, bacterial slants were prepared. 100ml EMB agar was taken and 15ml was poured in slants test tubes. Place test tubes without the caps on a test tube rack. It was kept tilt the rack holding the test tubes on a solid surface. After cooling, the caps of the test tubes were tightened back. The slants were inoculated by transferring cells with an inoculating loop from a single-colony microorganism on a plate to the slant's surface and stored in refrigerator (Levine *et al.*, 1918).

7.2 SAMPLE COLLECTION:

The samples of wastes were collected from different sources. The fruit waste was collected from fruit juice shop at lovely professional university, Punjab and dairy waste was obtained from Verka dairy plant at Amritsar, Punjab.

The main focus was on waste having high amount of carbohydrate content therefore it will convert to give desired moles of biohydogen gas. Fruit, food and dairy waste were great source of carbohydrate content and they are easily available.

7.2.1 FRUIT WASTES

The waste generated from fruit shop of college canteen contains peels of fruits such as bananas, apples, oranges and grapes were used as a substrate for fermentation. All these serve as a source of carbohydrate (Nazlina *et al.*, 2013).

7.2.2 DAIRY WASTES

The spoiled milk products generated by dairy plant contain whole milk products, cheese/whey/curd, milk solids, and milk wastes. Thus, these serves as good substrate and have high content of protein which were used in production (Nazlina *et al*., 2013).

7.3 SUBSTRATE CHARACTERISATION

7.3.1 DETERMINATION OF GLUCOSE CONCENTRATION (REDUCING SUGAR TEST)

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions (Miller *et al*.,1959).


```
                                    oxidation
aldehyde group                 ----------> carboxyl group

                                    reduction
3,5-dinitrosalicylic acid ----------> 3-amino,5-nitrosalicylic acid
```
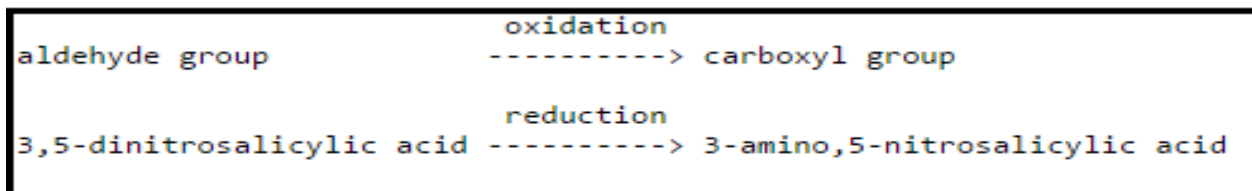
Figure 9: Oxidation reaction of aldehyde into carboxyl group.

3 ml of DNS reagent (3-5-Dinitrosalicylic acid(7.48 g), phenol (5.36ml),sodium meta-bisulphate (5.84gm), sodium hydroxide (13.92g) in l000 ml of distilled water) was added to 3 ml of glucose sample (5-30%) in a lightly capped six test tube. Then, the mixture was heated at 90º C for 15 minutes to from red brown colour. After that 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color. Test tubes were cooled at room temperature in a cold water bath and the absorbance was recorded by spectrophotometer at 540 nm (Miller *et al*., 1959)**.**

7.3.2 DETERMINATION OF BOD:

The biochemical oxygen demand is a way of expressing the amount of organic compound in sewage as measured by the volume of oxygen required by bacteria to metabolize it under aerobic conditions. The BOD of a water sample is generally measured by incubating the sample at 25^0 C for five days in the dark under aerobic conditions.

5litres dilution water was prepared by adding calcium chloride (5ml), magnesium sulphate (5ml), ferric chloride (5ml) and phosphate buffer (5ml) in 5litres fresh distilled water and incubated for 6hours at 25^0C. The pH of samples was brought to neutrality using 1N HCL acid or 1N NaOH solutions. 5 ml of sample and 5ml of distilled water was taken in 6 stopper bottles each and remaining volume was filled by dilution water. Out of six three bottles were incubated at 25^0C for 5 days. 2 ml of manganous sulfate and alkaline iodide-azide was added to remaining three bottles. Bottles were shaken upside down and allowed to settle the brown precipitate. 2ml of concentrated sulfuric acid was added and bottles were again shaken to dissolve precipitate. 200 ml of solution was taken in flask from three bottles respectively and titrated with sodium thiosulfate solution till the colour changes to pale straw. 1ml of starch solution was added which changes the colour of from pale to blue. Again the content in flask was titrated with sodium thiosulfate0 till the blue colour disappears. The mean of two readings of sample (D_1) was taken and mean of two readings of three sample bottles (D_5) which were incubated at 25^0 C for 5 days. (APHA 1995).

$$BOD\ (mg/l) = D_1 - D_5 - B * volume\ of\ titrant/volume\ of\ sample$$

Where, D_1 = initial DO in sample (mg/l)

D_5 = final DO in sample after 5 days incubation (mg/l)

7.3.3 DETERMINATION OF COD:

The chemical oxygen demand test is commonly used to indirectly measure the amount of organic compounds in water. It measures the amount of organic pollutants found in surface water making COD a useful measure of water quality (Aneja *et al*., 2003).

5ml of sample and 5 ml of distilled water was taken in two 100ml flasks respectively. 0.5ml of $K_2Cr_2O_7$ was added to each flask and heated to temperature of 100^0 C for 1 hr. flasks were allowed to cooled for 10 min. 0.5 ml of potassium iodide and 1 ml of H_2SO_4 were added and flasks were titrated with 1M sodium thiosulfate until pale yellow colour appears. 2 drops of starch solution was added to make solution turn blue colour. Again the solution was titrated with sodium thiosulfate and final reading of burette was noted down (Aneja *et al*., 2003).
COD of sample mg/l of sample by applying the formula

$$COD \text{ of sample } (mg/l) = 8*C*(B\text{-}A)/S$$

Where, C – concentration of titrant

A- volume of titrant used for blank

B- volume of titrant used for sample

S- volume of water sample taken

7.3.4 DETERMINATION OF TDS:

The total dissolved solid (TDS) refers to material that are completely dissolved in water. These solids are filterable in nature. It is defined as residue upon evaporation of filterable sample.

A evaporating dish of 100ml volume was taken and it weight was measured. 200ml sample was filtered through Whatman filter paper no. 4 and 100 ml of sample was poured in evaporating dish. Evaporating dish was heated to 100^0 C to evaporate all liquid content. The dish was cooled and weight of dish was noted again (Howard *et al*., 1933).

$$TDS \text{ mg/l} = (B\text{-}A)*10^6/V$$

Where,

A- initial weight of dish (g)

B- final weight of dish (g)

V- volume of sample taken (ml)

7.3.5 DETERMINATION OF TSS:

The TSS refers to materials which are not dissolved in water and are non-filterable in nature. It is defined as residue upon evaporation of non-filterable sample on a filter paper.

The weight of Whatman filter paper no. 4 was noted down. 200ml sample was filtered through and non-filterable portion of sample was collected on filter paper. Filter paper was heated to 100^0 C to evaporate all liquid content. The paper was cooled and weight of paper was noted again. (Howard *et al*., 1933).

$$TSS \text{ (mg/l) } = (B\text{-}A)*10^6/ V$$

Where,

A- initial weight of filter paper (gm)

B- final weight of filter paper (gm)

V- volume of sample taken (ml)

7.4 PRETREATMENT OF SUBSTRATE:

Firstly, 500gm of banana waste was grounded to form slurry by grinder. 400 ml of slurry was mixed with equal 400ml volume of water in order to reduce the effect of change in pH. The pH was adjusted by 1N HCL and 1N NaOH to 6.5. The slurry was pretreated by heating at around 121°C for 30 minutes to breakdown the substrate in order to release more organic compounds into the liquid phase (Guo *et al*., 2008)

7.5 EXPERIMENTAL SETUP

7.5.1 PRODUCTION INCUBATOR:

A hardboard box of area 15 inch * 30 inch was fully sealed with thermocol except one opening at top and walls of box were coloured black to maintain heat. A thermostat of temperature range 30- 90^{0}C was installed in series connection with 65 watt electric bulb. Incubator was set at temperature range 35-37^{0}C (Rob Ludlow 2014).

7.5.2 REACTOR SETUP

1000mL reactor flasks containing 800 ml of slurry and water mixture (1:1). The nutrient solution for bacterial growth containing Sucrose- 20g/l , NH_4HCO_3 -5.24 g/l, $NaHCO_3$- 6.72 g/l, K_2HPO_4- 0.125 g/l, $MgCl_2 \cdot H_2O$- 0.1 g/l, $FeSO_4 \cdot 7H_2O$- 0.025g/l, $MnSO_4 \cdot 6H_2O$- 0.015g/l, $CuSO_4 \cdot 5H2O$- 0.004gm, $CoCl_2 \cdot 5H_2O$ - 0.125 g/l was inoculated with 8% (64ml) overnight grown culture of *E.aerogenes*. 40%w/v KOH solution was also added in slurry for selective adsorption of CO_2.The reactor bottle was connected to other two bottles via pipe and kept inside incubation box at 37^0 C and pH 6.5 in presence of light and limited air inside reactor bottle for 10 days (Gopalakrishna *et al*., 2012).

7.6 GAS ANALYSIS

7.6.1 GAS ANALYSIS BY COD REDUCTION

The amount of gas production by fermentation of fruit and dairy substrates was analyzed by change in chemical oxygen demand of the waste. 50 ml of sample of both the wastes were collected and COD test was performed. The initial COD value i. e. before fermentation of waste and final COD value i. e. after fermentation of waste were calculated and this change determines the conversion of glucose into hydrogen gas. Therefore, the amount of hydrogen produced during process was calculated (Azwar *et al*., 2014).

7.7 BYPRODUCTS ANALYSIS

The fatty acids and alcohol which are byproducts of the glucose formed during fermentation process were analyzed by FTIR and HPLC ANALYSIS. After completion of process 15 ml of liquid sample of fruit and dairy waste were collected respectively. Samples were centrifuged at 5000 rpm for 20 minutes.

7.7.1 FTIR ANALYSIS:

The qualitative analysis of samples were done by fourier transform infrared spectroscopy (SHIMADZU 8400S, JAPAN) at lovely professional university, phagwara, punjab. Two drops centrifuged samples of both fermented waste was applied on potassium bromide pellet (KBr) and was run for analysis (Griffiths *et al*., 2007).

7.7.2 HPLC ANALYSIS:

The fatty acids or alcohol in samples were analyzed using high performance liquid chromatography (HP1100, Agilent Technologies, USA) at Sardar Swaran Singh National Institute Of Renewable Energy, Kapoorthala, Punjab. The HPLC was equipped with an Aminex HPX-87H packed column (φ300 x 7.8 mm, Bio-Rad, USA). The liquid samples were purified through a 0.2 μm disposable filter prior to injection. A refractive index detector (RID) was used to quantify the organic acids and alcohols. The injection volume of the samples was 1000μl and 0.01 N H_2SO_4 solution at 0.6 ml/min was used as the mobile phase and the column temperature was kept at 40^0C (Jong *et al*., 2006).

CHAPTER 8

RESULTS AND DISCUSSION

8.1 BACTERIAL GROWTH AND MAINTANENCE

The *E.aerogenes* stock culture was prepared from strain *E.aerogenes* (MTCC-111) order from IMTECH, Chandigarh. The nutrient broth was used as a medium, bacterial growth was seen after incubation period of 24 hours at 37^0C and further sub-culturing was routinely done in 15 days.
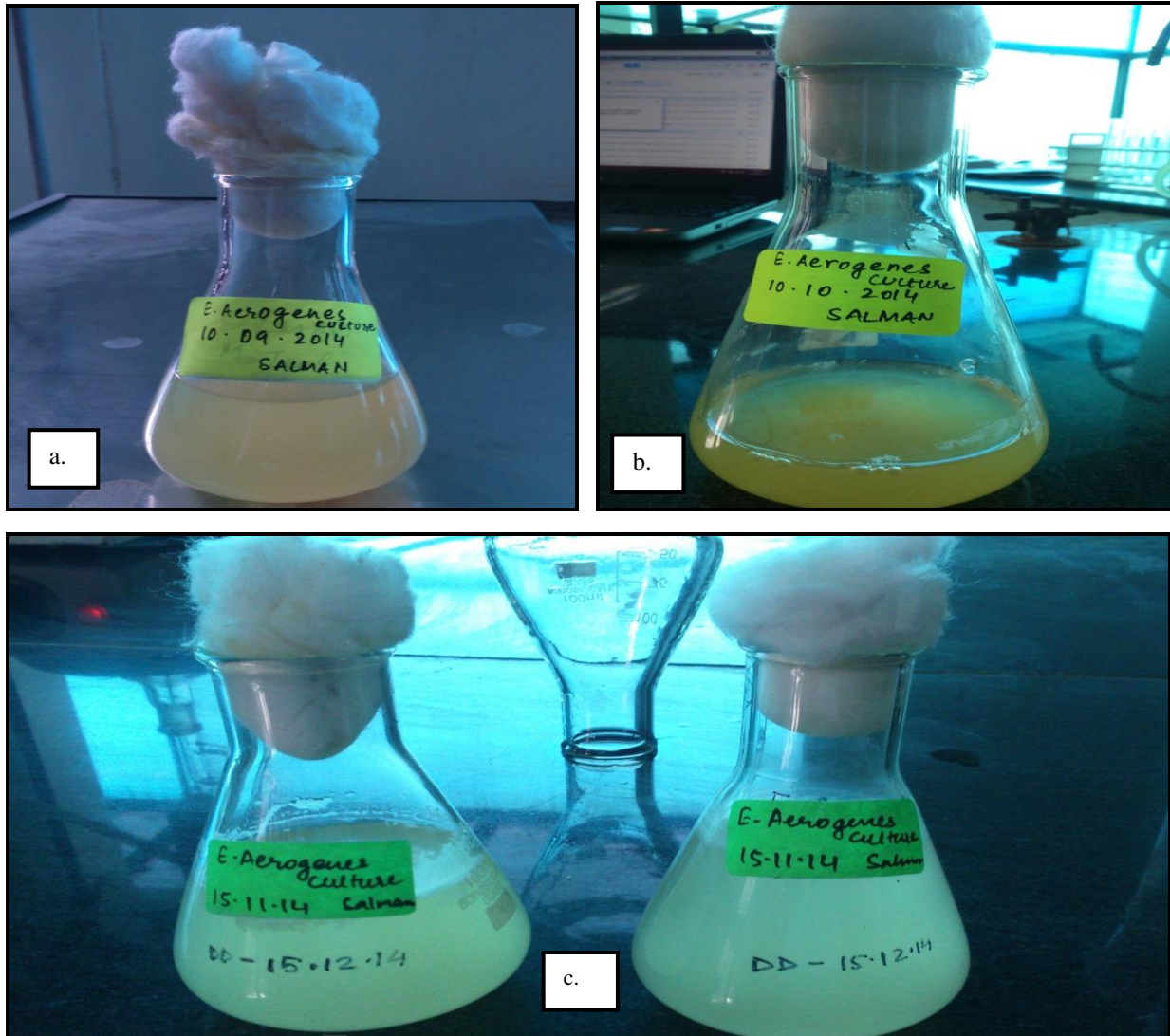

Figure 10: a, b and c showing *E.aerogenes* broth culture.

8.1.1. EMB AGAR PLATES:

After incubation of 24hours, *E.aerogenes* was inoculated from broth culture and streaked on EMB agar plates. The good growth of pinkish brown, dark-centered, mucoid colonies of *E.aerogenes* were seen after streaking .EMB agar plates were sub-cultured routinely within 15days.
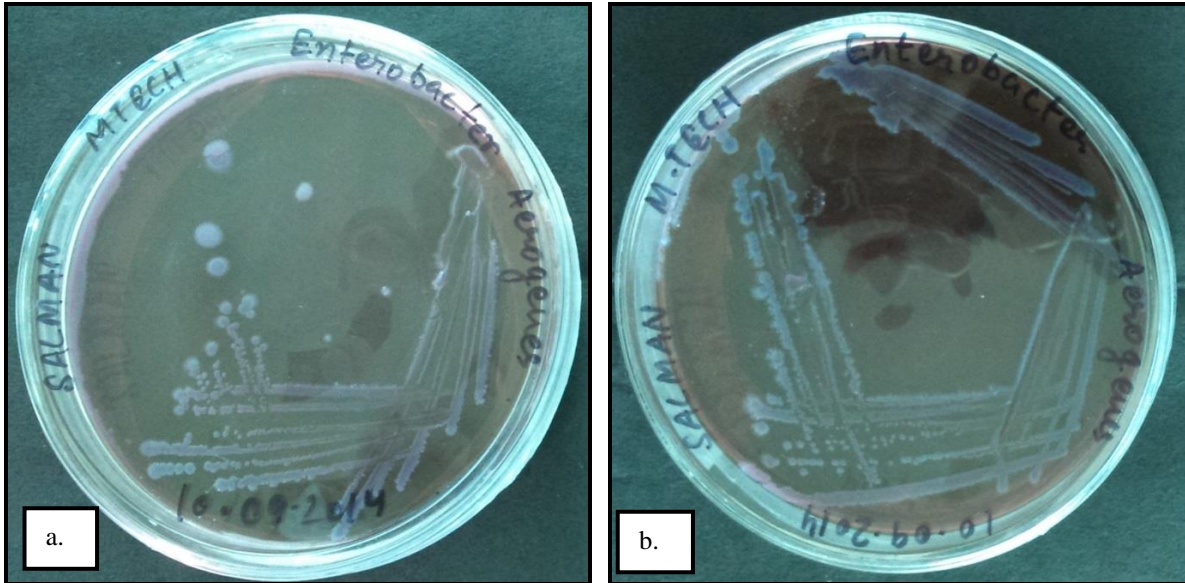
Figure 11: a and b showing colonies of *E.aerogenes* on agar plates.

8.1.2 PURE COLONIES
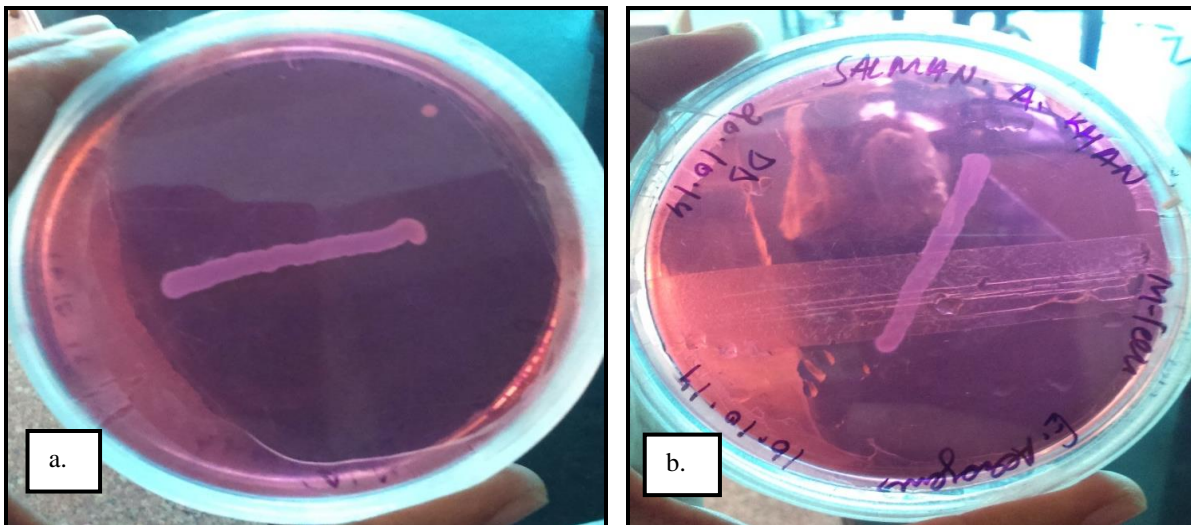
After two to three streak plating, the pure colonies were obtained.

Figure 12: a & b showing pure colonies of *E.aerogenes* on agar plates.

8.1.3SLANTS

The EMB agar slants were prepared from pure colonies of *E.aerogenes.* They were kept for long term storage after observing growth.
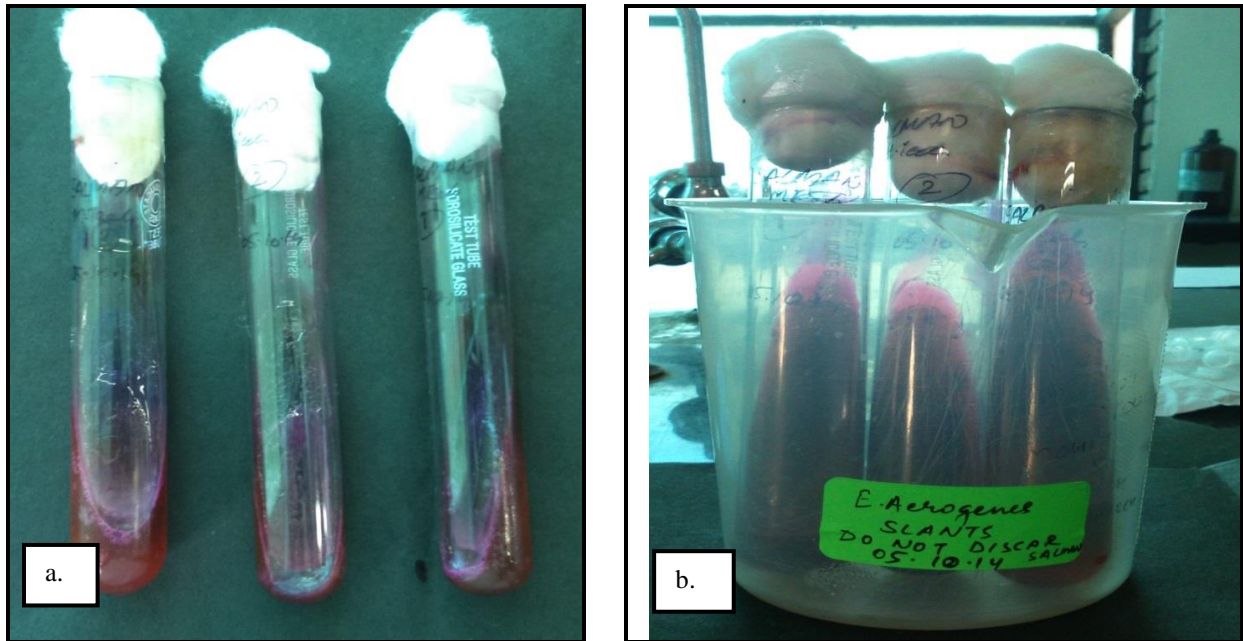

Figure 13: a and b showing *E.aerogenes* agar slants

8.2 SAMPLE COLLECTION

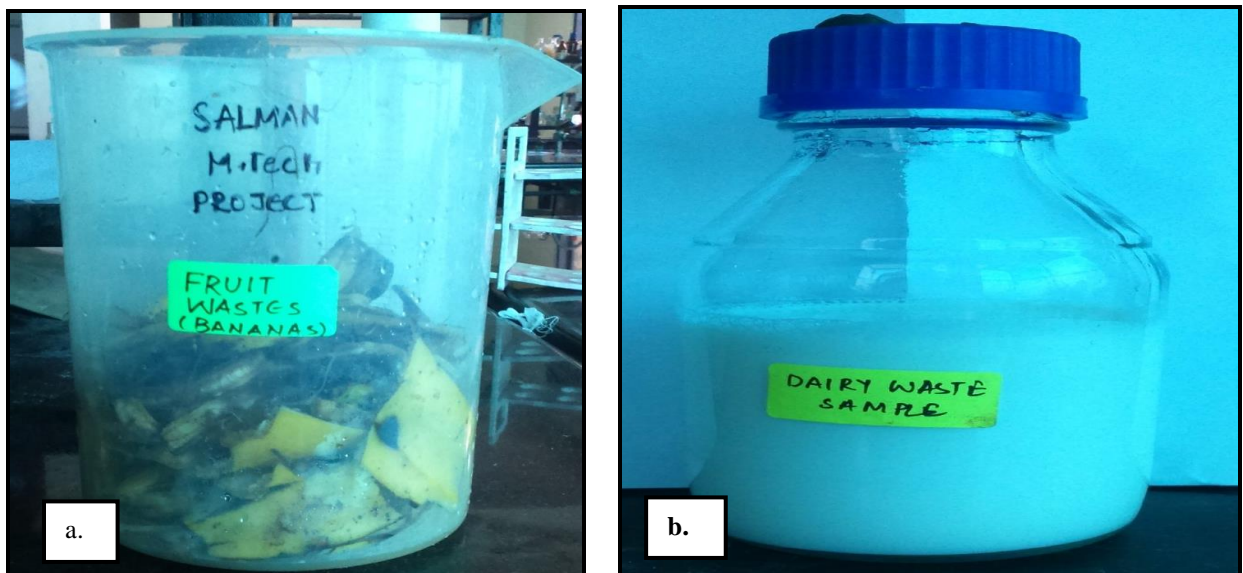From the fruit waste the peels of banana were collected and used as a sample.

Figure 14: a & b showing sample of banana waste and spoiled milk as substrate.

8.3 SUBSTRATE CHARACTERISATION

8.3.1. REDUCING SUGAR TEST:

The quantitative analysis was done by DNS method which tests the concentration of sugar present in substrate. Six test tubes containing (5-30) percent of glucose were prepared in Distilled water and DNS reagents.
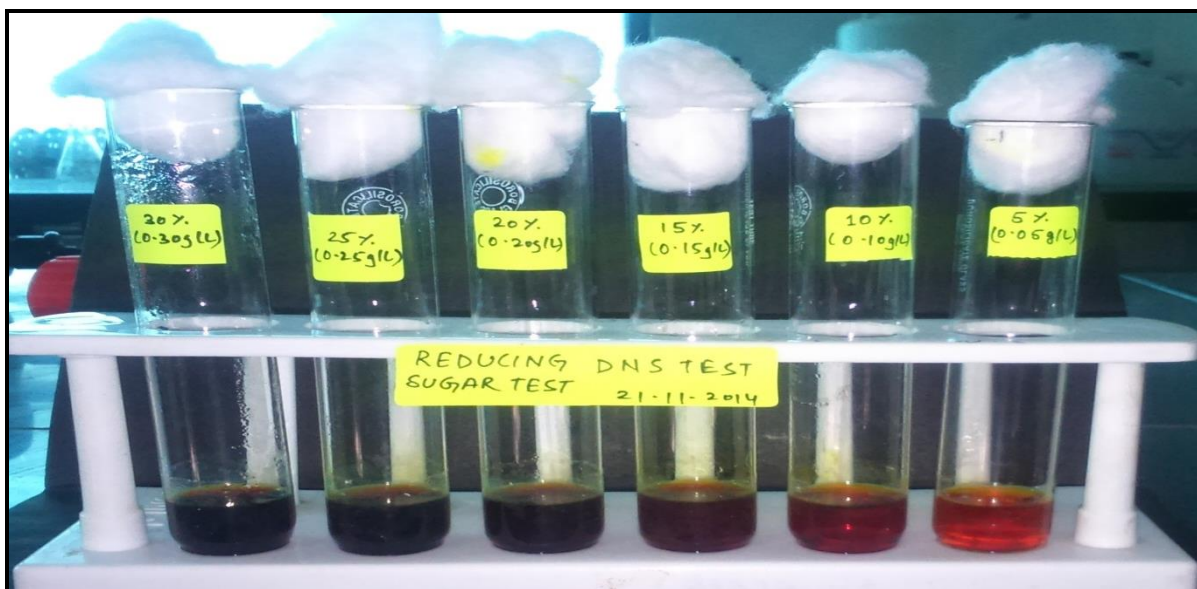

Figure 15: Showing six test tubes containing substrate and DNS test reagents.

FRUIT WASTES:

Glucose%	Concentration (mg/ml)	Substrate sample (ml)	DNS reagent(ml)	Distilled water (ml)	Potassium sodium tartrate (ml)	O.D. (540nm)
0	0.00	0.000	3.000	3.000	1.000	0.000
5	0.05	0.015	2.850	3.000	1.000	0.030
10	0.10	0.300	2.700	3.000	1.000	0.070
15	0.15	0.450	2.550	3.000	1.000	0.130
20	0.20	0.600	2.400	3.000	1.000	0.210
25	0.25	0.750	2.250	3.000	1.000	0.280
30	0.30	0.900	2.100	3.000	1.000	0.320

Table 4: Optical density of fruit waste sample at 540 nm
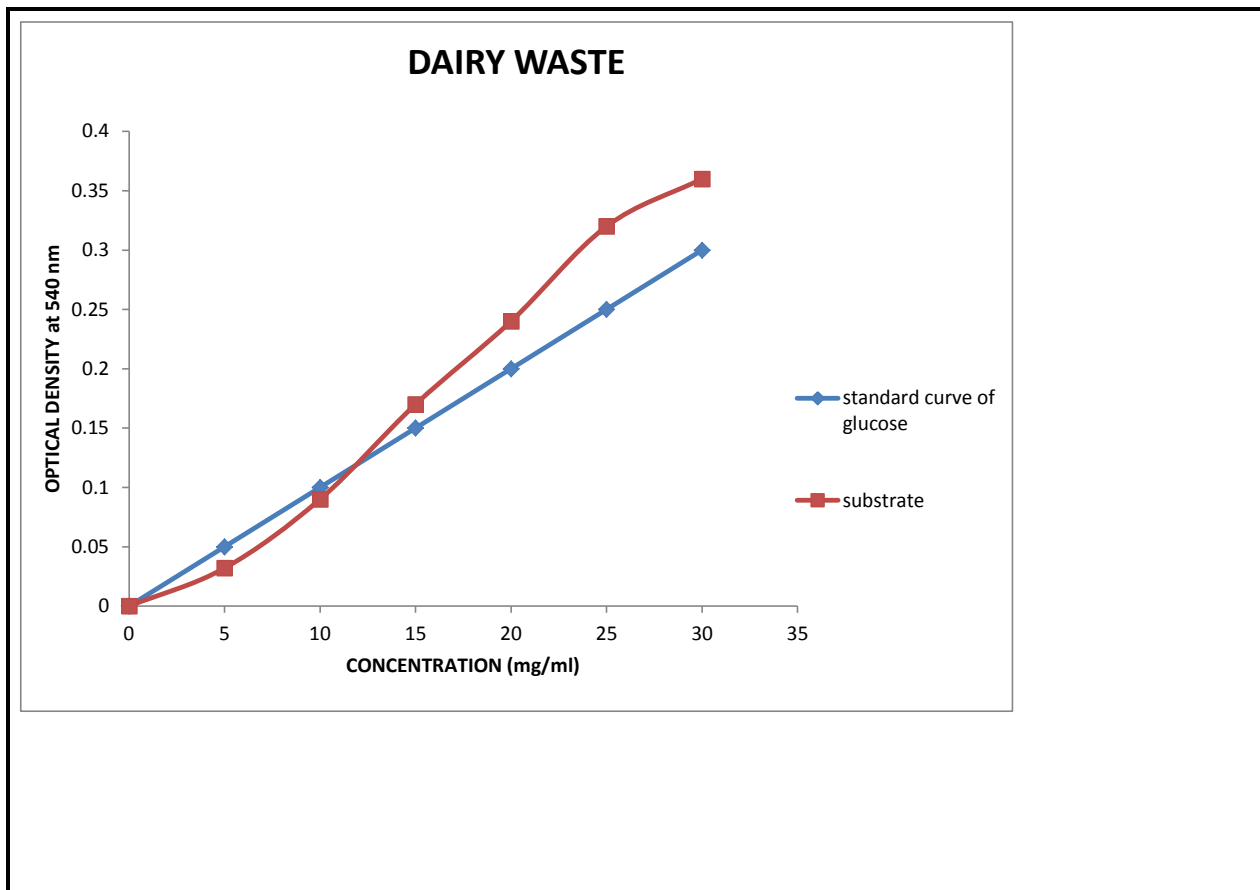
DAIRY WASTES:

Glucose%	Concentration (mg/ml)	Substrate sample (ml)	DNS reagent(ml)	Distilled water (ml)	Potassium sodium tartrate (ml)	O.D. (540nm)
0	0	0	3.000	3.000	1.000	0.000
5	0.05	0.015	2.850	3.000	1.000	0.032
10	0.10	0.300	2.700	3.000	1.000	0.090
15	0.15	0.450	2.550	3.000	1.000	0.170
20	0.20	0.600	2.400	3.000	1.000	0.240
25	0.25	0.750	2.250	3.000	1.000	0.320
30	0.30	0.900	2.100	3.000	1.000	0.360

Table 5: Optical density of dairy waste sample AT 540 nm
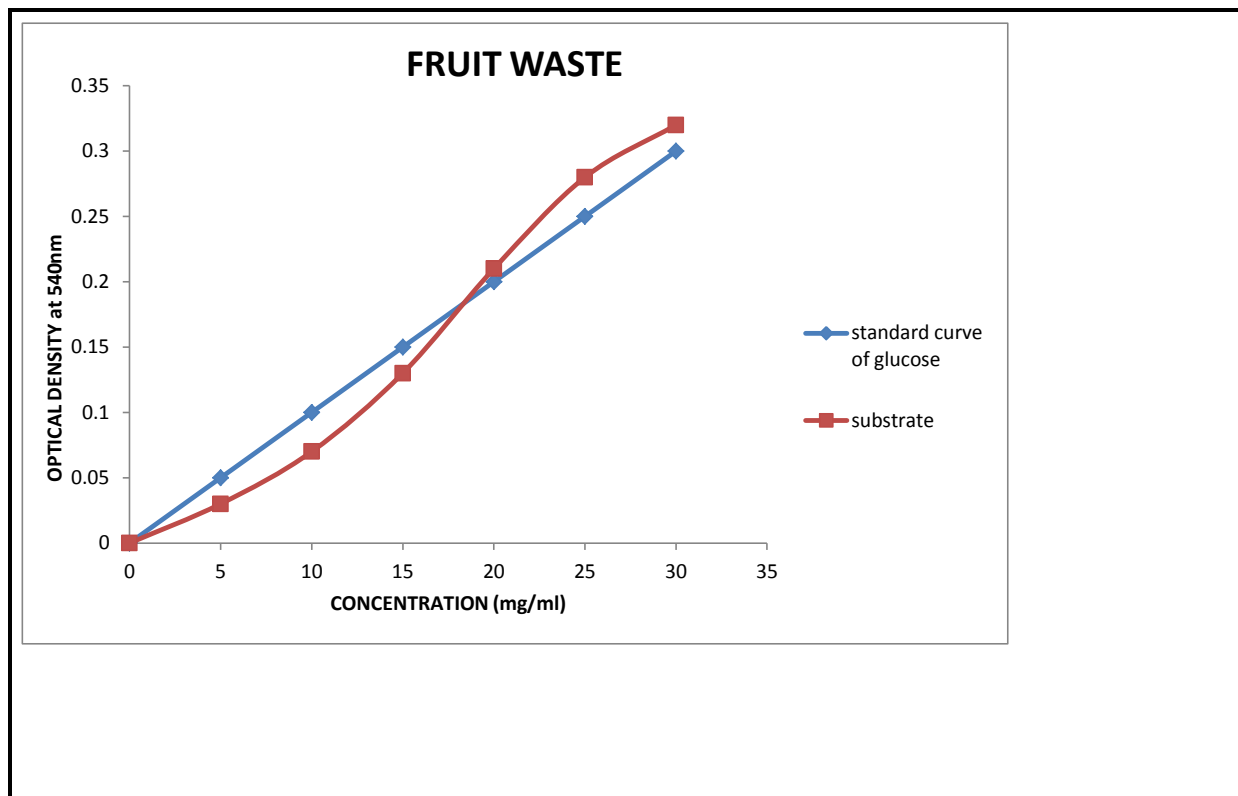
Graph 1: A graph between glucose concentrations of fruit waste sample versus optical density.

DAIRY WASTE

Graph 2: A graph between glucose concentrations of dairy waste sample versus optical density.

After taking optical density readings of both substrate samples, a graph was plotted between glucose concentration versus optical density and standard graph of glucose was used as a reference. As the standard curve of glucose and the substrate intersects, this determines the concentration of glucose present in substrate sample. Hence the concentration of glucose present in fruit wastes ad dairy waste was 17.5 mg/ml and 12.0 mg/ml respectively.

8.3.2 BOD TEST

Biological oxygen demand of fruit and dairy sample was estimated by calculating the dissolved oxygen at initial 1st day and final 5th day. By applying BOD estimation formula:

BOD (mg/l) = D_1- D_5- B*volume of titrant/volume of sample

Where, D_1 = initial DO in sample (mg/l)

          D_5 = final DO in sample after 5 days incubation (mg/l)

FRUIT WASTE:

TRIAL NO	DAY	VOLUME OF SAMPLE (ml)	BURETTE READING(ml) INTIAL	BURETTE READING(ml) FINAL	VOLUME OF TITRANT (ml)	DISSOLVED OXYGEN (mg/ml)
Blank	0	200	0	10.1	10.1	10.1
01	0	200	0	9.4	9.4	9.4
02	0	200	0	9.5	9.5	9.5
Blank	5	200	0	9.3	9.3	9.3
01	5	200	0	2.6	2.6	2.6
02	5	200	0	2.8	2.8	2.8

Table 6: Burette readings for calculating bod of fruit waste

DAIRY WASTE:

TRIAL NO	DAY	VOLUME OF SAMPLE (ml)	BURETTE READING(ml) INTIAL	BURETTE READING(ml) FINAL	VOLUME OF TITRANT (ml)	DISSOLVED OXYGEN (mg/ml)
Blank	0	200	0	10.1	10.1	10.1
01	0	200	0	8.6	8.6	8.6
02	0	200	0	8.5	8.5	8.5
Blank	5	200	0	9.3	9.3	9.3
01	5	200	0	4.0	4.0	4.0
02	5	200	0	4.0	4.0	4.0

Table 7: Burette readings for calculating bod of dairy waste

FRUIT WASTE:

Blank - D_1 =10.1 mg/l and D_5 =9.3 mg/l

Sample - D_1 =9.45 mg/l and D_5 =2.7 mg/l

BOD= 9.4-2.7-0.8*200/5= 228 mg/l

BOD of sample is 118 mg/l

DAIRY WASTE:

Blank - D_1 =10.1 mg/l and D_5 =9.3 mg/l

Sample - D_1 =8.55 mg/l and D_5 =4.0 mg/l
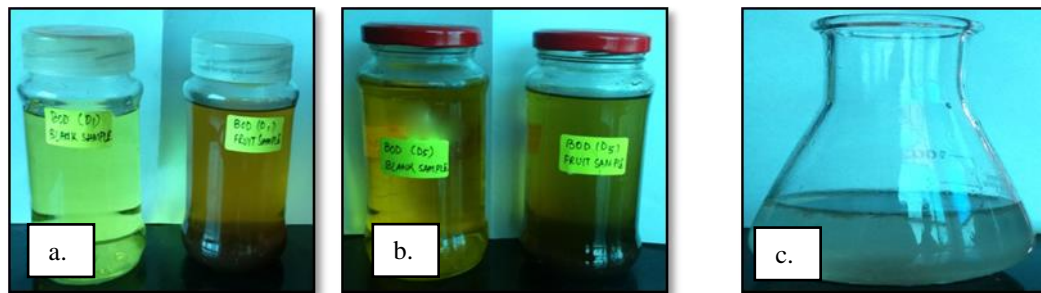

BOD= 8.55-4.0-0.8*200/5= 75mg/l

BOD of sample is 75mg/l

Figure 16: a & b representing bod bottles at day1 (D_1) and day5 (D_5) and c representing results after sodium thiosulfate titration of fruit sample.
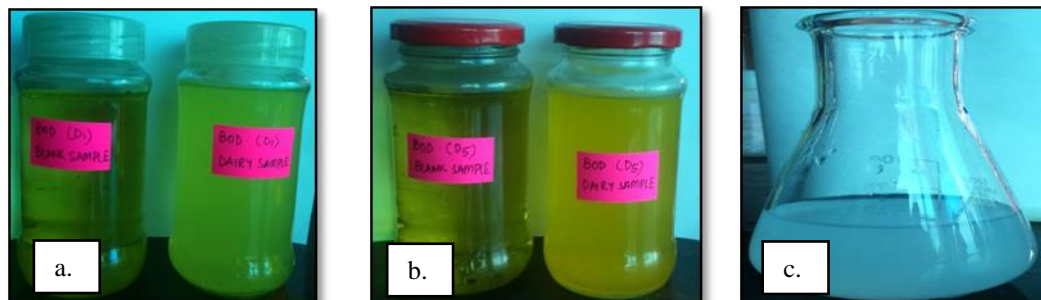

Figure 17 :a & b representing bod bottles at day1 (D_1) and day5 (D_5) and c representing results after sodium thiosulfate titration of dairy sample.

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8.3.3 COD TEST

The amount of organic matter in substrate sample is estimated by their oxidability by chemical oxidant.

COD of sample mg/lt of sample by applying the formula:

$$COD \ of \ sample \ (mg/l) = 8000*C*(B-A)/S$$

FRUIT WASTE

C=1M, S= 50ml, B=11.2ml, A=9.6ml

COD= 8000*1*11.2-9.6/50=256 mg/l

COD of sample is 256 mg/l

DAIRY WASTE

C=1M, S= 50ml, B=11.2ml, A=10.0 ml

COD= 8000*1*11.2-10.0/50=192 mg/l
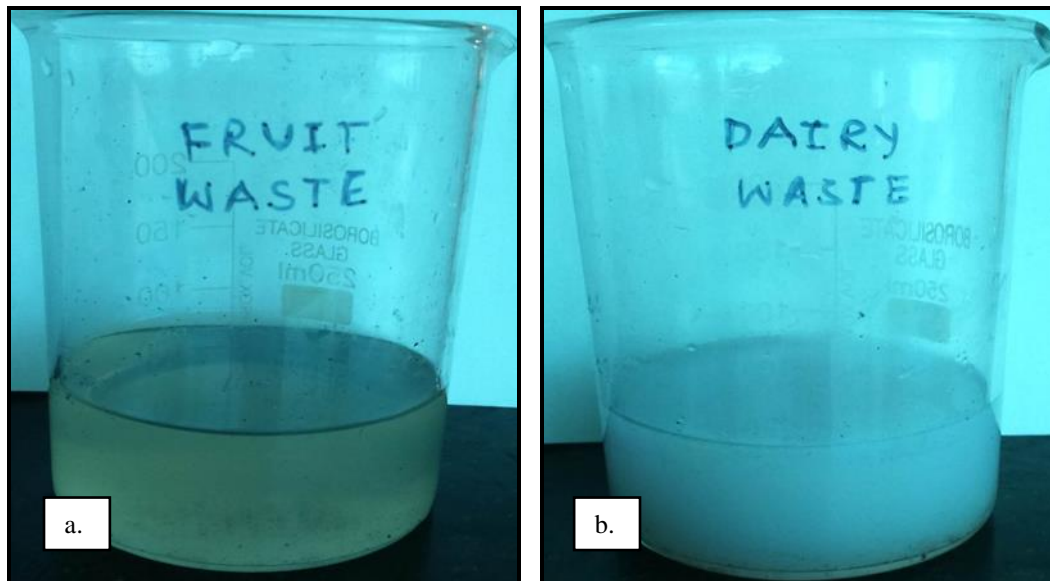
COD of sample is 192 mg/l

Figure 18: a & b showing results of cod test of fruit and dairy sample after sodium thiosulfate titration.

8.3.4 TDS TEST (TOTAL DISSOLVED SOLIDS):

TDS are determined as the residue left after evaporation of the filtered sample.

$$TDS(\ mg/l)\ =(B\text{-}A)*10^6/\ V$$

FRUIT WASTE:

A=18.180gm, B=18.270gm, V=100ml

TDS of fruit waste sample was 900 mg/l

DAIRY WASTE:

A= 18.180gm, B=18.242gm, V=100ml

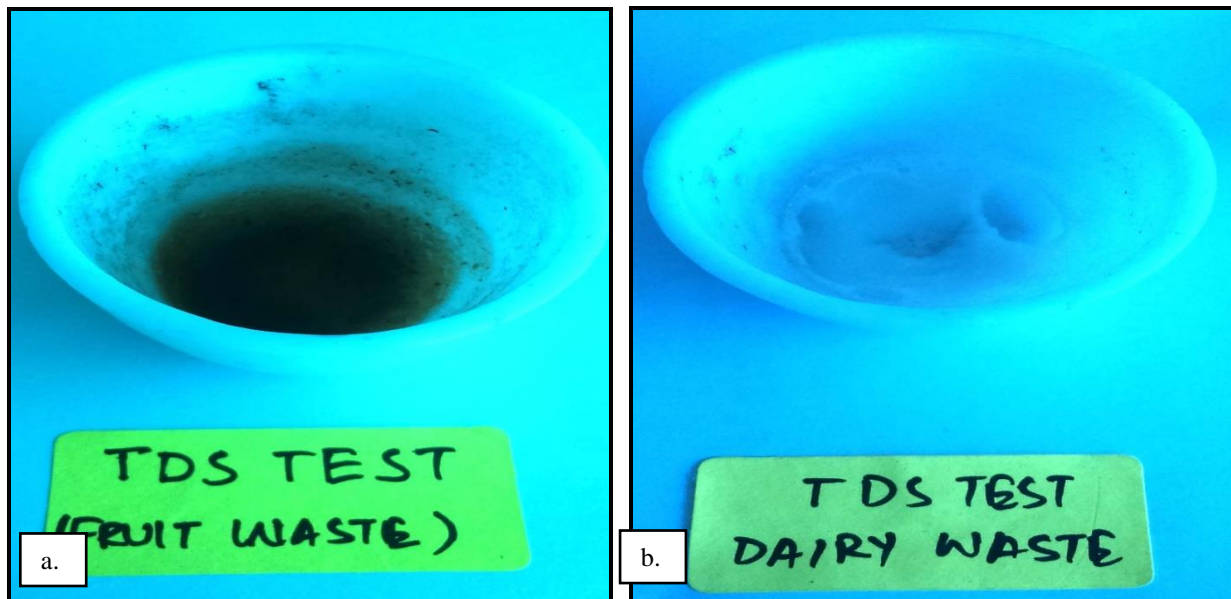**TDS of dairy waste sample was 620 mg/l**

Figure 19: a & b showing results of tds test of fruit and dairy samples.

8.3.5 TSS TEST (TOTAL SUSPENDED SOLIDS)

Total suspended solids are determined as residue upon evaporation of non-filterable sam-ple on a filter paper

$$TSS\ (\ mg/l)\ =(B\text{-}A)*10^6/\ V$$

FRUIT WASTE:

A= 0.600 gm, B=1.11gm, V=100ml

TDS of fruit waste sample was 510 mg/l

DAIRY WASTE:

A= 0.600 gm, B=0.985 gm, V=100ml
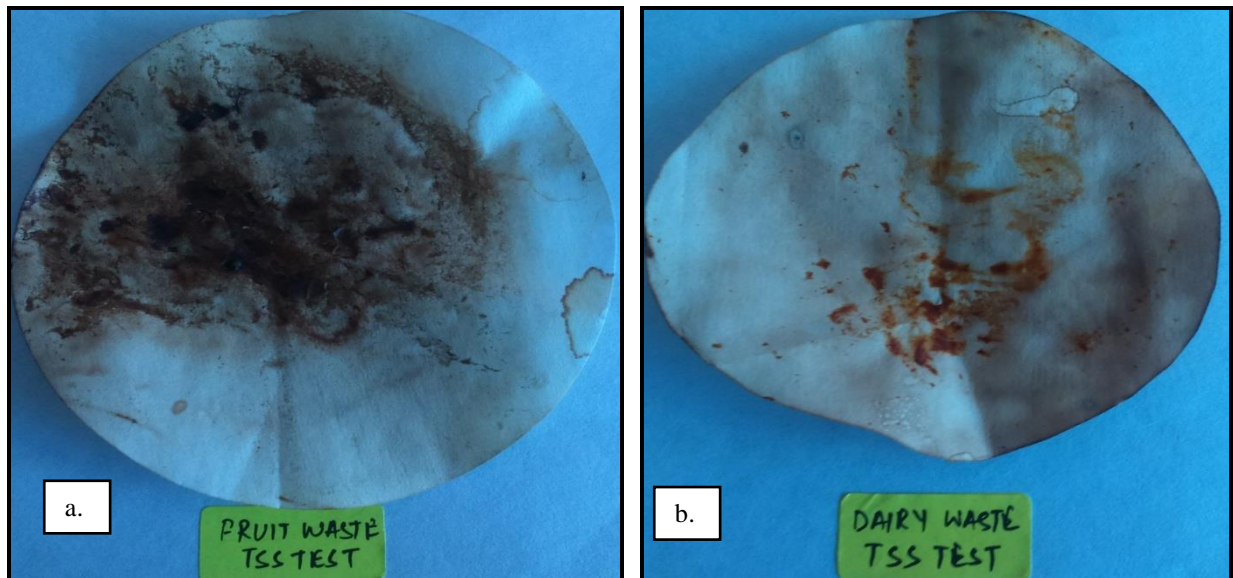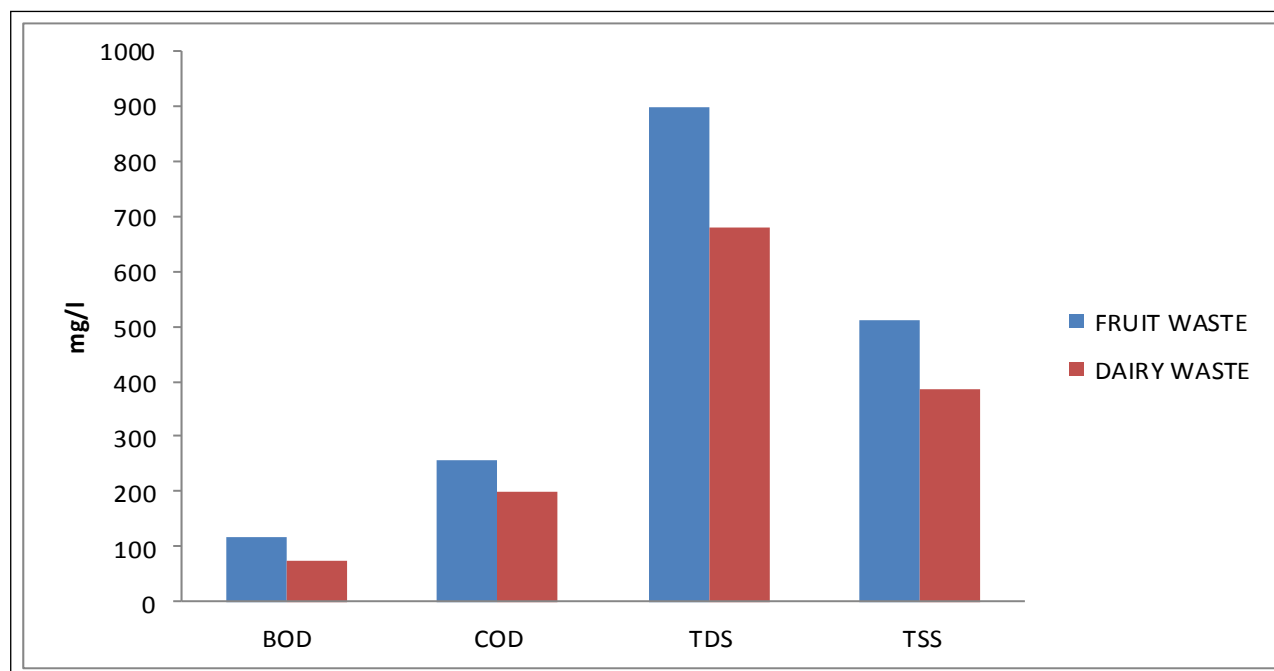
TDS of dairy waste sample was 380 mg/l

Figure 20: a & b showing results of tss test of fruit and dairy samples.

SUBSTRATE	GLUCOSE concentration (mg/ml)	BOD (mg/l)	COD (mg/l)	TDS (mg/l)	TSS (mg/l)	pH	Temperature
Fruit waste	17.5	118	256	900	510	6.3	27^0C
Dairy waste	12.0	75	198	680	380	5.8	27^0C

Table 8: Characteristics of organic waste.

Graph 3: Comparsion of charactersitics of fruit and dairy waste.

8.4 PRETREATMENT OF SUBSTRATE

FRUIT WASTE:

The banana peels were ground to produce slurry. From that 400ml of slurry was mixed with 400 ml distilled water was added (1:1) to make volume up to 800ml in 1000ml reactor bottle. Slurry was pretreated at 121^0 C for 30 minutes and pH was maintained to 6.5.

DAIRY WASTE:

400 ml of spoiled milk was taken as a substrate and distilled water was added (1:1) to make volume up to 800 ml in reactor bottle. The substrate was pretreated at 121^0 C for 30 minutes and pH was maintained to 6.5.
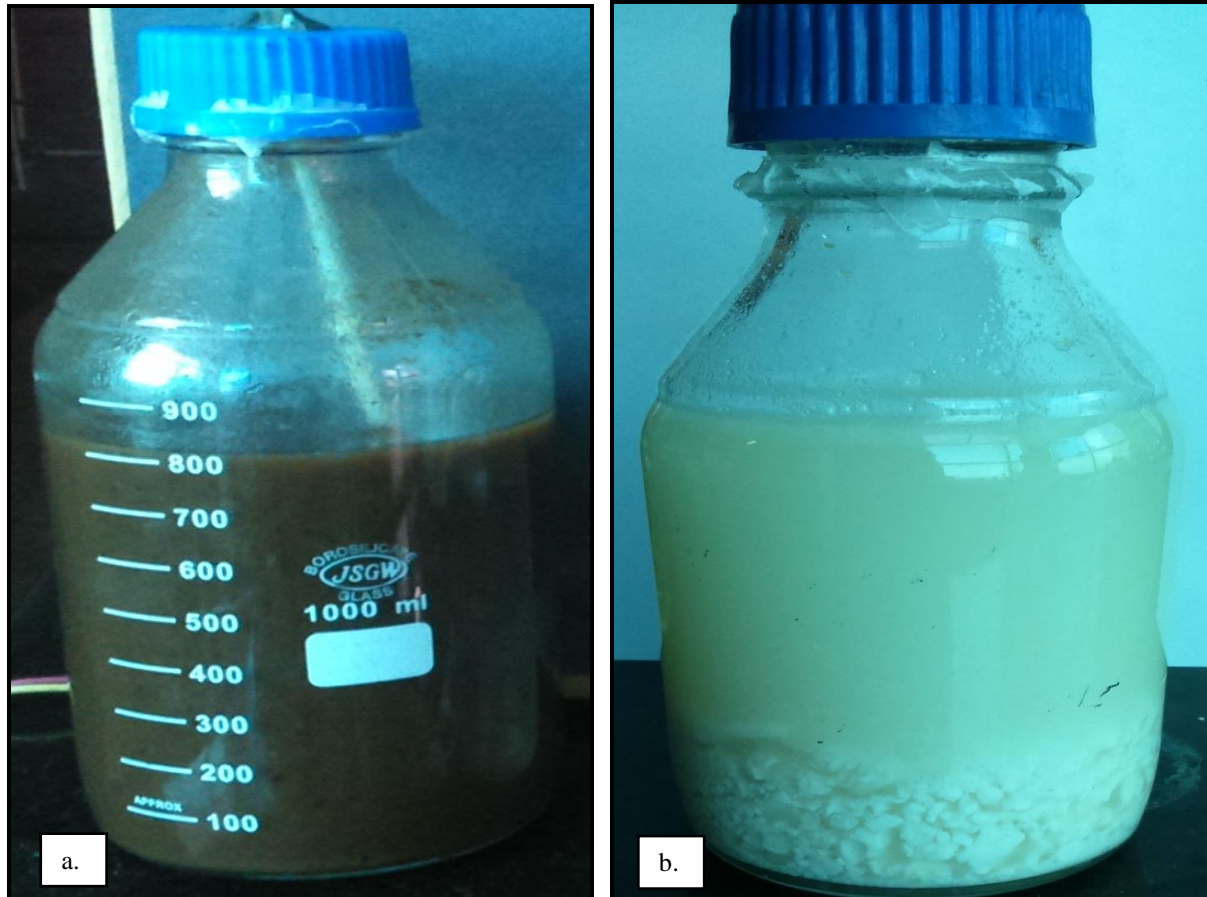

Figure 21: a and b representing pretreated fruit waste and dairy waste used as substrate in reactor bottles.

8.5 REACTOR SETUP

For production of gas, 1000 ml reactor serum glass bottles containing 800ml substrate slurry was inoculated with 8% (64 ml) inoculum of *E.aerogenes.* 40%w/v KOH solution was also added in slurry for selective adsorption of CO_2. Setup was connected via pipe system to gas collecting bottle which is based on principle of water displacement method. A 6 ml syringe is connected to pipe to collect gas
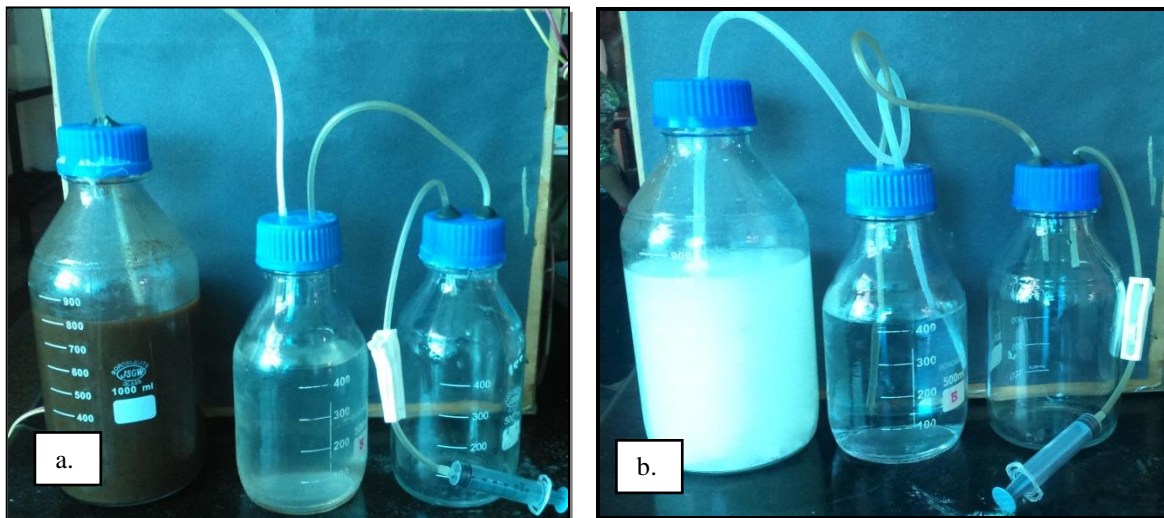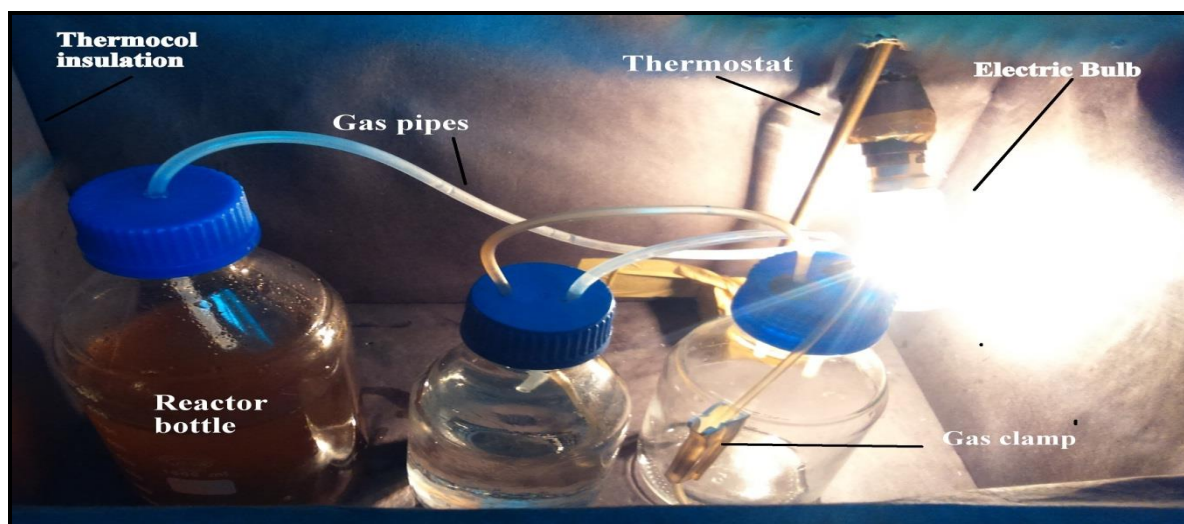
Figure 22: a & b showing reactor setup of fruit and dairy waste outside the incubator box.

Figures 23: Reactor setup inside the incubator box at 37^0C.

8.6 GAS ANALYSIS

After incubation of 10 days, it was observed that there was formation of some bubbles in reactor bottle. There was change in level of water from 2nd to 3rd bottle, 43ml change in case of fruit whereas 28ml change in case of dairy waste.
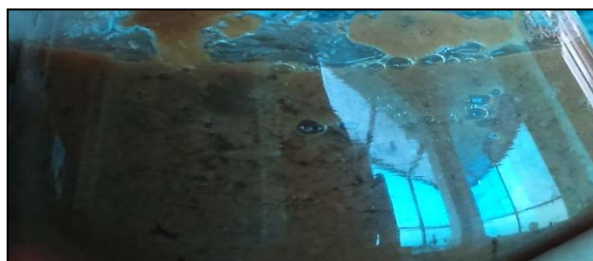

Figure 24: Gas bubbles formation inside reactor bottle.

8.6.1 GAS ESTIMATION BY COD REDUCTION

After fermentation the COD of fermented fruit waste and dairy waste was carried out. 50 ml of sample of fruit and dairy was collected respectively and COD test was performed (Aneja *et al*., 2003). The reduction in cod values gives the amount of hydrogen gas produced during the process (Bhavya *et al*., 2014).

SAMPLE	INITIAL COD BEFORE FERMENTATION (mg/l)	FINAL COD AFTER FERMENTATION (mg/l)	COD REDUCTION (%)	HYDROGEN PRODUCTION (H_2 ml/ mg COD reduced)
FRUIT WASTE	256	179.2	31.7%	76.8
DAIRY WASTE	194	135.8	26.4%	58.2

Table 9: Comparison of initial and final COD.
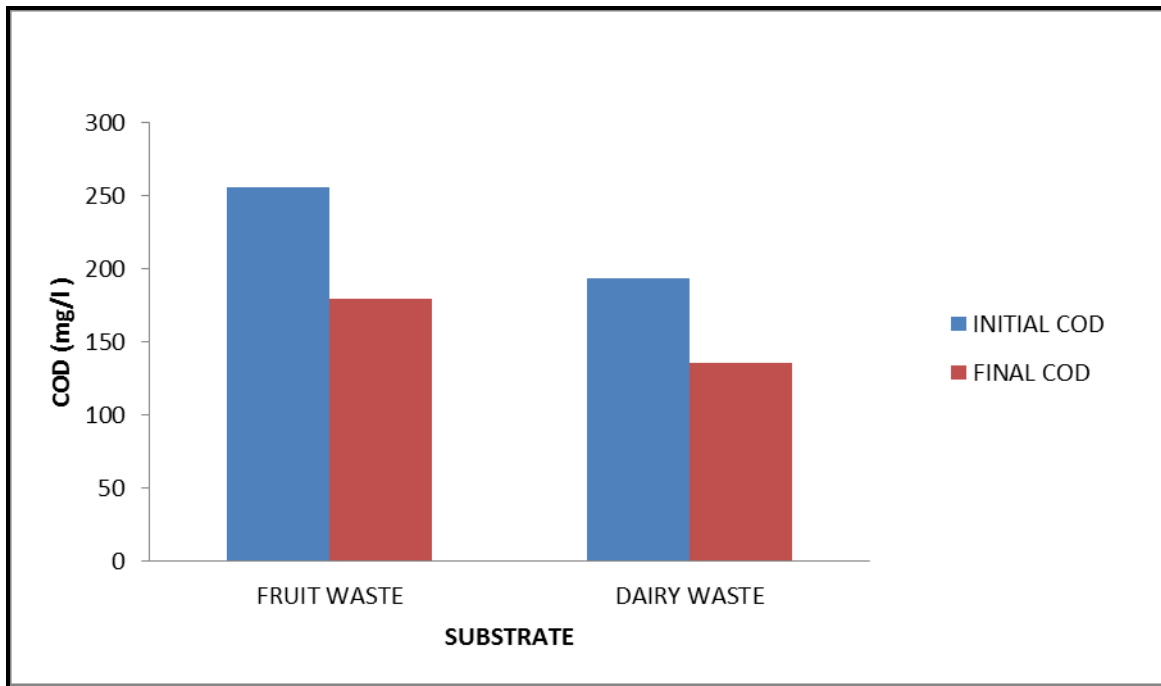
Graph 4: Comparison of initial and final COD reduction of fruit and dairy waste.

8.7 BYPRODUCTS ANALYSIS

15 ml of fermented products of both substrates was collected from reactor bottle which was further analyzed for the presence of fatty acids produced. FTIR and HPLC analysis were carried out which confirms the type of fatty acids and alcohol produced.

Figure 25: Samples collected after fermentation of fruit and dairy waste.

8.7.1 FTIR ANALYSIS: FRUIT WASTE
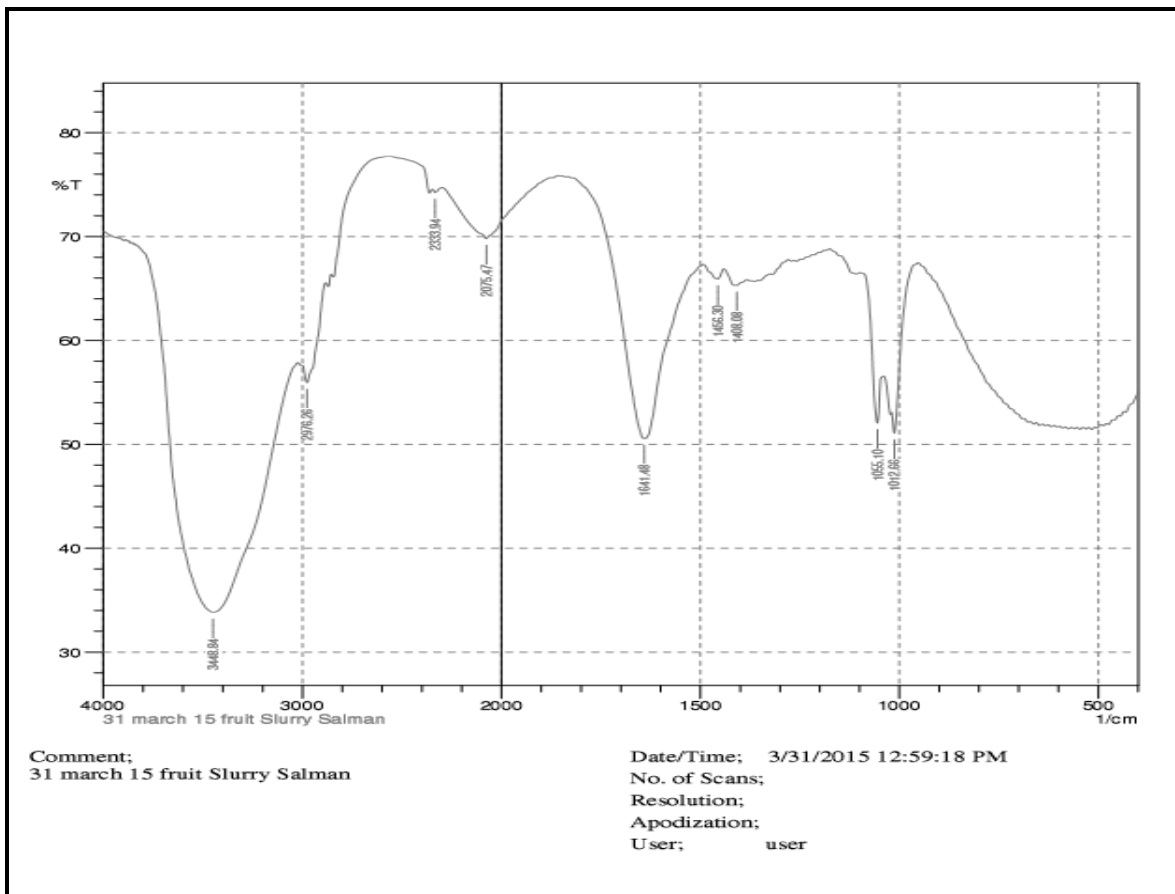
Figure 26: FTIR spectra for fruit waste.

FATTY ACID FREQUENCY	FTIR FREQUENCY	BONDS	FUNCTIONAL GROUPS	PEAK INTENSITY
~1645	1665-1760	C=O	CARBONYL	Strong
2976	2850-3000	C-H	ALKANES	Strong
3448	3200-3600	O-H	ALCOHOL	Strong, broad

Table 10: frequency result of FTIR spectra of fruit waste.

8.7.2 FTIR ANALYSIS: DAIRY WASTE
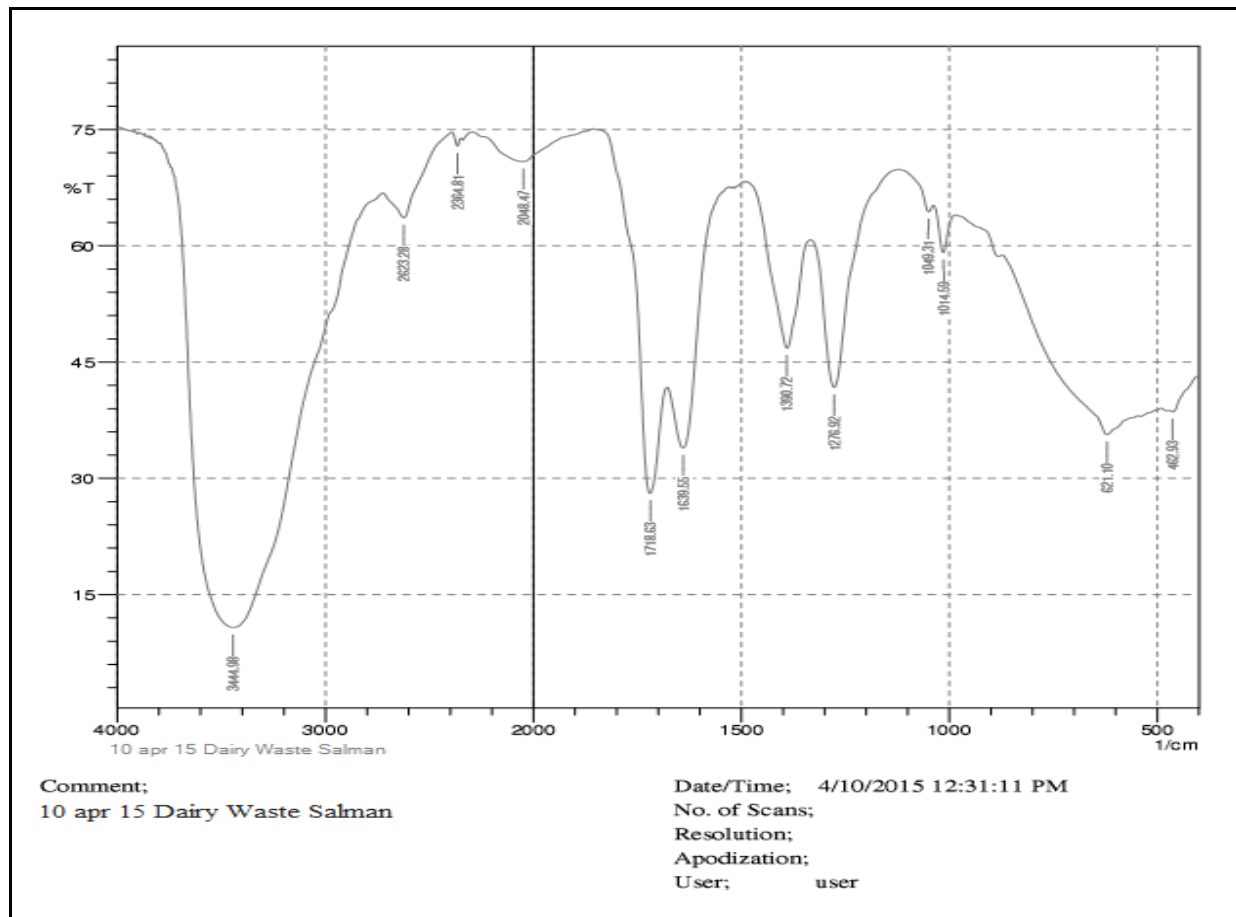
Figure 27: FTIR spectra for dairy waste.

FATTY ACID FREQUENCY	FTIR FREQUENCY	BONDS	FUNCTIONAL GROUPS	PEAK INTENSITY
1718.63	1665-1760	C=O	CARBONYL	Strong
2623.28	2850-3000	C-H	ALKANES	Strong
3444.98	3200-3600	O-H	ALCOHOL	Strong, broad

Table 11: frequency result of FTIR spectra of dairy waste

8.7.3 HPLC ANALYSIS: FRUIT WASTE
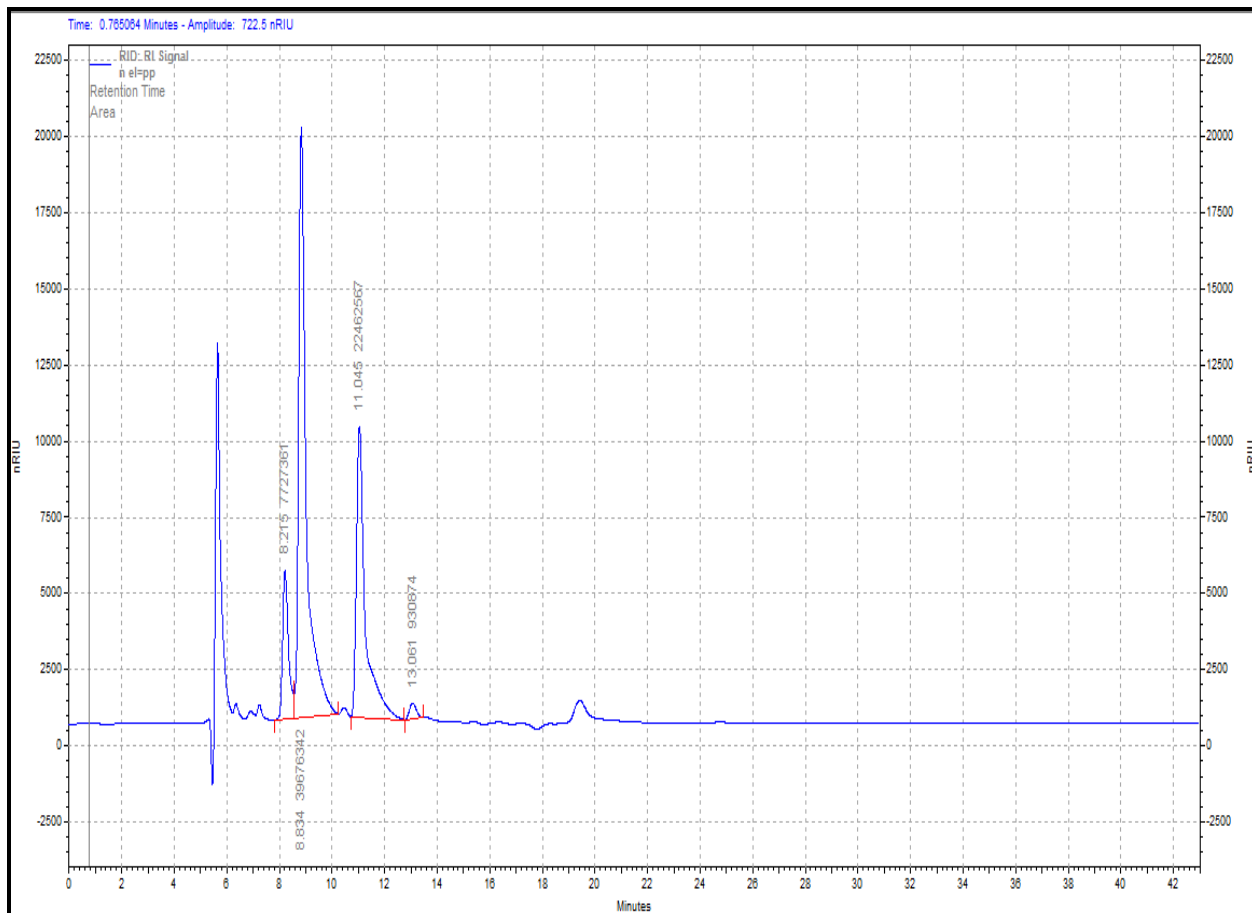
Figure 28: Chromatogram of fruit waste

PRODUCTS	RETENTION TIME (seconds)	PERCENTAGE (%)	CONCENTRATION (g/l)
GLUCOSE	8.215	0.28%	2.8
XYLOSE	8.834	0.56%	5.6
LACTIC ACID	11.045	0.03N	2.7
ACETIC ACID	13.061	0.07%	0.7

Table 12: Concentration of products in fruit waste sample.

8.7.4 HPLC ANALYSIS: DAIRY WASTE
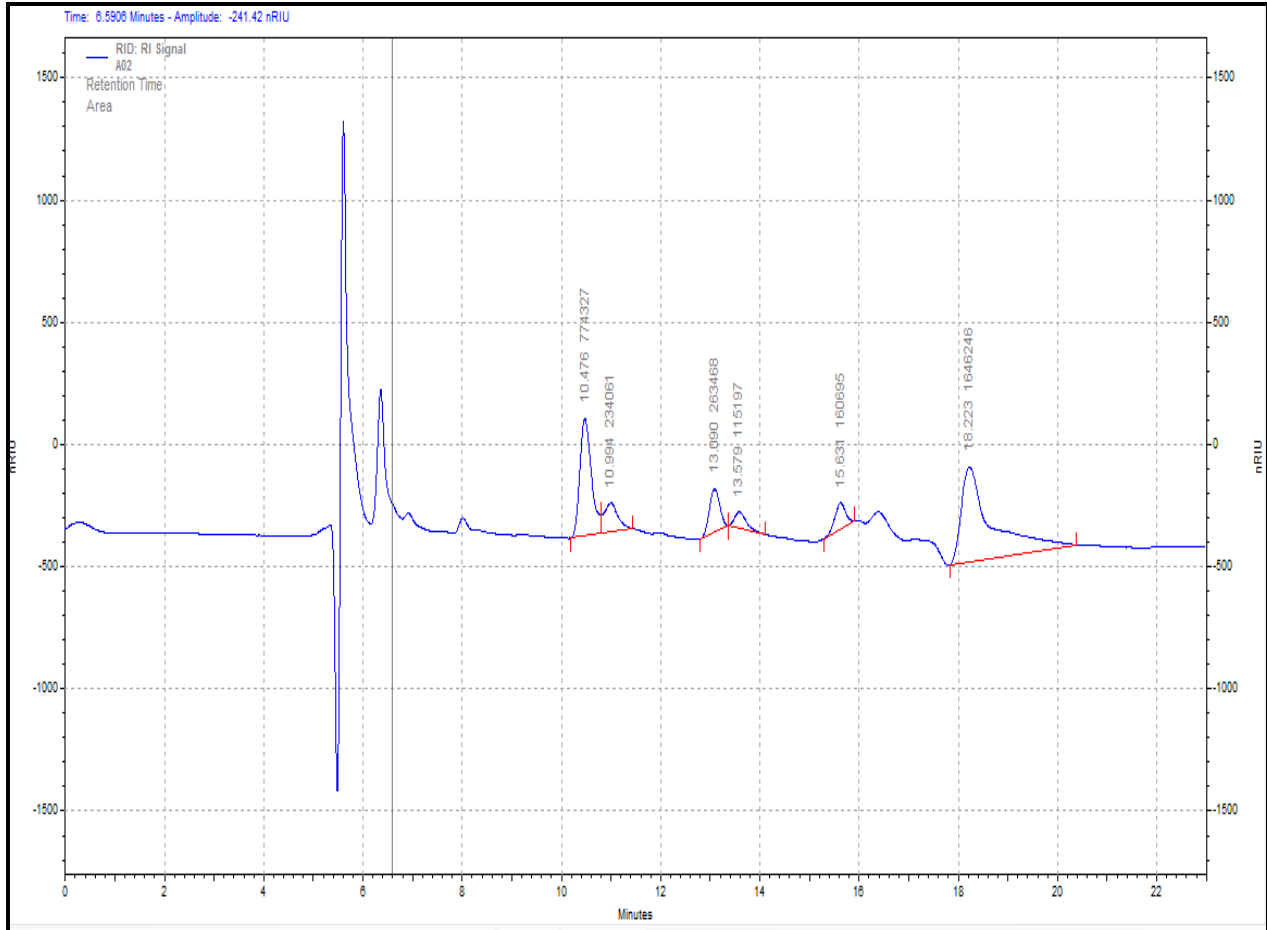
Figure 28: Chromatogram of dairy waste

PRODUCTS	RETENTION TIME (seconds)	PERCENTAGE (%)	CONCENTRATION (g/l)
GLUCOSE	10.47	0.16%	1.60
LACTIC ACID	10.99	0.0034N	0.030
ACETIC ACID	13.0	0.02%	0.20
PROPIONIC ACID	15.6	0.0002N	0.014
ETHANOL	18.2	0.14%	1.47

Table 13: Concentration of products in dairy waste sample.

CHAPTER 9
DISCUSSION

In this present study the substrates were characterized under different categories according to their characteristic. The concentration of glucose in fruit waste was found to be 17.5 g/l which was due to high carbohydrate content (glucose and xylose) of banana peel and grapes and whereas in dairy waste it was 12.0 g/l due to presence of more protein and lipids and low carbohydrate content (glucose and galactose).

It was observed that the BOD and COD value of fruit waste was higher than dairy waste. The data showed fruit waste was 36.4 % (BOD) and 24.21% (COD) high as compared to dairy waste. The TDS and TSS of fruit waste was also 23.07% and 25.49% more as compared to dairy waste. This difference is due to different composition of organic components present in fruit wastes. The characterization study was helpful in deciding the type of substrate chosen for better production.

The amount of gas produced was estimated by the reduction in COD value of waste before and after the fermentation process. There was decline in the COD value as the degradation of organic compound by micro-organism. In case of fruit waste the yield of hydrogen gas was 79.8 H_2 ml/ mg COD reduced, which was about 31.2% reduction in COD value. Whereas, the hydrogen yield from dairy waste was 50.68 H_2 ml/ mg COD reduced, which was about 26.4% reduction in COD value from pre-fermentation to post-fermentation. The high yield of hydrogen in fruit waste was due to more carbohydrate content as compared to dairy waste i.e. glucose concentration of waste. Thus, the greater the carbohydrate content more will be conversion of glucose into hydrogen gas. Ultimately the yield of hydrogen gas from fruit waste was 36.54 % higher than dairy waste.

The reactions of hydrogen production by fermentation of glucose show that the most desirable end-products is acetic acid ,lactic acid, propionic acid and ethanol, with production levels of four hydrogen per mole glucose. Theoretically, 33% of COD can be converted to hydrogen from glucose and the rest of the energy is released (Azwar, *et al*., 2014).

The hydrogen yield from this study was 79.87 ml H_2/ g COD reduced and 50.68 ml H_2/ g COD reduced has been found to be distinctively higher than by using heat –treated sludge which shown similarity to those of anaerobic spore forming bacteria, *Clostridum sp*., on food waste (Kim et al., 2004) and by the mixed consortium of *Enterobacter cloacae*, *Citrobacter freundii* and *Bacillus coagulans* on sewage sludge was 41.23 ml H_2/ g COD reduced (Das and Koaty, 2006). However, it was lower compared to the yield obtained from co-digestion of food waste and sewage sludge 122 ml H_2/ g COD reduced (Kim et al., 2004).

The byproducts formed during the process resulted in decline in pH from 6.5 to 5.2 which indicated the production of fatty acids and ethanol. The FTIR studies of the fruit and dairy sample showed the presence of functional groups of carboxylic acids. The information regarding the peaks for fruit and dairy sample were discussed in table 8 and table 9, which proves the presence of carbonyl and alcoholic group in byproducts.

The quantitative analysis of byproducts by HPLC showed the amount of acetic acid, lactic acid, propionic acid and ethanol were produced. The yield of acetic acid and lactic acid was 71.42% and 88.8 % more from fruit waste than dairy waste. In case of dairy waste, there was also production of propionic acid (0.014g/l) and ethanol (1.47g/l). These results show that the organic waste can be a potential source for the production of biohydrogen even on an industrial scale.

CHAPTER 10
CONCLUSION

Biohydrogen is the most desirable ultimate target to supply energy demand of mankind. However the bacteria is rather sluggish to produce biohydrogen and maintenance of optimum conditions in reactor is a delicate task. The yield of biohydrogen gas is fairly low and it needs scale up.

In this present study, all the factors affecting the yield has been discussed such as type of microorganism, type of substrate, temperature, pH and types of sugar present. The importance of biological conversion of organic waste into hydrogen is discussed. The historical view of nature of the present system clarifies its limitation and the necessity for a brand-new energy accumulation technology, replacing the industrial revolution based one.

Hydrogen holds out the promise of a truly sustainable global energy future. As a clean energy carrier that can be produced from any primary energy source, hydrogen used in highly efficient fuel cells could prove to be the answer to our growing concerns about energy security, urban pollution and climate change.

Considerably more research and development will be needed to overcome the formidable technical and cost hurdles that currently stand in the way of hydrogen. Large reductions in unit costs, notably in bulk transportation and storage, and in fuel cells, are needed for hydrogen to become competitive with existing energy systems.

CHAPTER 11
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