ETHANOL PRODUCTION FROM KITCHEN WASTE BY WILD TYPE YEAST ISOLATED FROM NATURAL SOURCES

By
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A Thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirement for the degree of
Bachelors of Science in Biotechnology

Department of Mathematics and Natural Sciences
Brac University
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

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Approval

The thesis titled “Ethanol production from kitchen waste by wild type yeast isolated from natural sources” submitted by Deepanwita Chakraborty (15136007) of Spring, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on **February 27,** 2020.

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Abstract

It is time we think about changing to alternate fuel and make energy production procedures greener. Bioethanol production by microbes is one step towards it. In this study, yeast from easily available food sources was used to produce bioethanol by using domestic food waste as substrate. This work was designed to bioconvert the lignocellulosic biomass to ethanol by the action of yeast and bacteria. Grapes, cow dung, rice water, vegetable peels and soil were chosen to isolate wild-type yeast for it to be the ethanol producing organism. The strain found went through isolation, identification, stress tolerance tests and detailed characterization and optimization before being used on inexpensive substrates to produce low cost ethanol. Considering all the characteristics it was assumed that the strains isolated belonged to *Saccharomyces* spp. The principal goal of this study was to produce ethanol at a low cost by the integration of highly efficient yeast strain into the substrate that is considered as lignocellulosic household waste. Optimization of fermentation was done at various temperature, pH, rotation per minute, substrate size and the optimum fermentation condition was found to be at pH 6, 30°C, 120 rpm having small cut substrates. An increase in the production was seen after the substrates were treated with starch hydrolyzing and cellulolytic bacteria where the highest increase of ethanol production was from 2.93 %v/v to 6.99 %v/v. These bacteria converted the complex carbohydrates to simpler ones which the yeast utilized to produce the maximum amount of ethanol.

The maximum ethanol produced by yeast using vegetable peels was 6.99 %v/v after 48 hours of incubation at 30°C, 120 rpm after bacterial treatment. This ethanol formed can be used as biofuel as well as disinfectant which will reduce our dependence on fossil fuel which are polluting our environment in a dangerous way.

Keywords: Bioethanol, substrate, lignocellulosic biomass, wild-type yeast, starch hydrolyzing and cellulolytic bacteria, biofuel, and disinfectant.

Dedication

This study is dedicated to

Researchers,

who are promoting the use of biodegradable resources,

Cleaners,

who are working to keep our environment clean and safe

and to my family and friends,

for inspiring me to proceed with my dreams.

Acknowledgement

First and foremost, I would like to thank Bhagban Sree Krishna for making me capable enough to successfully complete this project. I would also like to shower my overwhelming thanks to my parents for leading my path and constantly supporting me in my decisions.

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List of Acronyms

MT	Metric tone
CMC	Carboxymethylcellulose
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
DNS	Dinitrosalicylic Acid
YEPD	Yeast extract Peptone Dextrose
kg	kilogram
MHA	Mueller Hinton Agar

Chapter 1

Introduction

1.1 Background

The necessity for an environmentally friendly energy source is indisputable. The reduction of fossil fuels and the alarming effect their combustion causes to nature is increasing drastically which has compelled mankind to move to an alternative source to combust. Providing the situation, using biotechnology for creating a scope towards the implementation of ingenious processes by the usage of green energy sources has become inevitable. Moreover, due to exponential population growth and their needs to survive, now we need more and more energy sources which if can be only earned from non-renewable sources, it is going to run out of it real soon. Consequently, energy from renewable sources is happily welcomed. This conventional heart of energy can be replaced by petroleum and other chemical raw materials. However, using chemicals as a source of energy does surely reduce the use of nonrenewable materials and increase the efficiency and rate of production in many aspects but it might affect the organic life and environment. Use of natural resources like biomass is going to keep our nature cleaner, greener and safer.

Biomass is an abundant carbon-neutral, renewable resource for bioenergy and biomaterial processing, and its increased use would address many societal needs (Ragauskas et al., 2006). This is where bioethanol gets priority as a good alternative fuel. Vegetable waste is a biodegradable commodity that can be considered as an inexpensive biomass easily used for bioethanol production.

Bioethanol usage as a source of energy is advantageous from the use of fossil fuels in many ways (Singh et al., 2012). Fossil fuel availability is limited and it causes harmful effects on the environment. On the contrary, bioethanol is made from lignocellulosic waste which is limitless as it comes out of many houses every single day and its use reduces the effect it may cause when dumped out in open. This organic household waste generally goes into open fields to be decomposed into fertilizers that cause not only bad smell but visual pol lution as well. Overall, more than 30 % of the loss occurs to retailers and consumers where postha rvest and waste level of processing is the main culprit (Verma, Bansal, Kumar, 2011). Thus, forming environmental threats as these also have the potential to cause diseases as many different types of microbes work on it to decompose. Furthermore, reprocessing these waste products to produce bioethanol gives us an inexpensive, efficient and greener production of energy.

The people are becoming increasingly dependent on balanced and processed food items. It has been seen that the massive supply of food for human consumption puts waste of arou nd one-third worldwide (FAO, 2011). India, with its wealth of agricultural resources, accounts for 50 MT of vegetable waste, about 30% of its total production (Verma, Bansal, Kumar, 2011). Using these wastes produced at different levels of delivery starting from the agricultural farm, waste after harvest, storing, processing, and from distribution to consumers would be economically highly beneficial.

1.2 Ethanol as a biofuel

Fermentation derived ethanol is commonly known as bioethanol. Ethanol can be chemically produced from the fermentation of petroleum and of biomass or sugar substrates. This organic material is a liquid that is flammable, transparent and colorless, and can be used as biofuel.

Ethanol functions as a solvent and as an antimicrobial drug are mostly observed (AKPAN, ALHAKIM, Josiah, 2008).

Ethanol was used in Brazil the US and many other developing countries as a biofuel. The demand for ethanol is likely to increase in the US over the next 20 years. Bioethanol is produced in Europe and the USA from crops such as maize, which has increased from 7% to 25% in the last 10 years (Morrison and Boyd, 1992). This bioethanol is the main transport fuel for Brazil. About 19,534.99 million gallons of ethanol were produced worldwide in 2009, where US and Brazil produced 10,600.00 and 6577.89 million gallons, respectively (Mathewson, 1980). Ethanol produced through fermentation has been found to serve significantly as a transport fuel for cars, trucks and trains (Theomozhi and Victoria, 2013). Ethanol production is not constrained, but constantly replenished by growing plants, and is advantageous over petroleum as a source of fuel in which the use of petroleum is gradually declining (Hannon, 2010). Ethanol fuel has not been fully utilized, as petrol was available, affordable and easy to produce. Today's demand for fuel is increasing, however, and the price difference between ethanol and gasoline is getting smaller (Bhatia 2014). There are many other renewable fuels including methanol, gasoline, natural gas, propane, hydrogen, and so on.

Nevertheless, ethanol's impressive features make it the best substitute fuel for the car (Zappoli, 1991). It has high latent vaporization heat, high octane number and rating, and low emissions of toxic compounds when combusted. While ethanol has a lower heating value of about 60% of the average regular gasoline, this low heating value is compensated for by a high latent vaporization power of 839,686kJ / kg which is more than twice that of petrol, which is about 325,64kJ / kg. Therefore, when ethanol and gasoline are burned in proper stoichiometric ratios respectively, they have about equal volumetric efficiency. Pure ethanol, on the other hand, is burned to create

carbon dioxide, water, and much less carbon monoxide. Consequently, ethanol will be a stronger fuel substitute (Rural Industries Research and Development Corporation, 2002).

Ethanol from natural sources is better than ethanol from chemical reactions (Nan, 1994). Ethanol has been produced from crops in most countries but due to the growing demands this is going to be very less and would need alternate raw materials for ethanol production. Positively, waste usage would bring a drastic change in amounts of bioethanol produced. It might then become more widely used in other developed countries as well as in developing ones. Thus, ethanol is a vital source of biofuel and if it can be produced from wastage, equally waste management and low-cost durable fuel can be achieved.

1.3 Fermentation of yeast

Yeast is known to ferment carbohydrates to produce ethanol. This is a conventional way of producing ethanol for use as beverages. This technique has been used to ferment sugars in organic waste to produce ethanol needed for fuel and germicide. It can be fermented from cellulose, starch, glucose, sucrose, fructose, and other specified sugars. Yeast is a single, phylogenetically diverse one-celled fungus. As a member of Kingdom Fungi, yeasts are eukaryotes which digest their energy sources externally and absorb the nutrient into cell molecules. Sucrose is a sugar made up of fructose and glucose that can be obtained by fermentation hydrolysis, resulting in the creation of alcohol. Starch molecules are long chains of α-D-glucose monomers which can be transformed into ethanol if converted to glucose by enzymatic means or by hydrolyzing starch bacteria. Cellulose is the main structural sugar of lignocellulosic biomass. Cellulose is a homologous polymer where glucose units are linked by β-1, 4 glycosidic bonds. In order to ferment, cellulose has to be hydrolyzed into simpler form by cellulase directly or from cellulolytic bacteria.

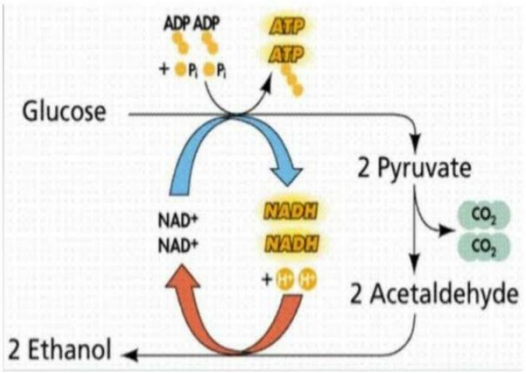


Figure 1. 1 Conversion of sugar to ethanol

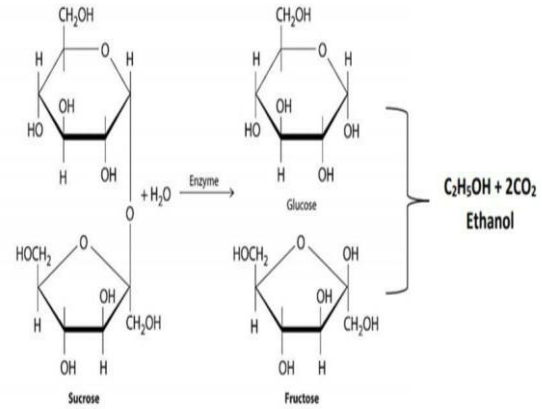


Figure 1. 2 Conversion of sucrose to ethanol

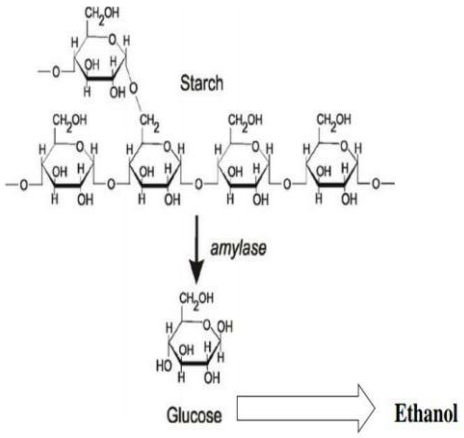


Figure 1. 3 Conversion of starch to ethanol

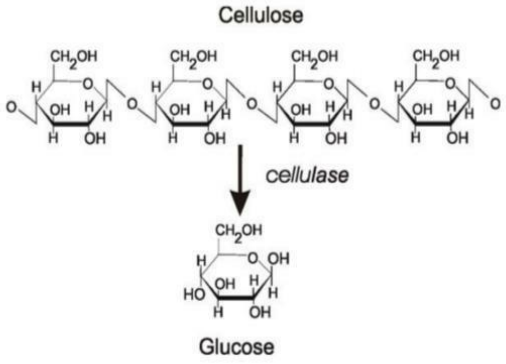


Figure 1. 4 Conversion of cellulose to ethanol

1.4 Bacterial pre-treatment

Yeast cannot break all forms of carbohydrates to produce ethanol. Thus, the amount of ethanol got may be of fewer amounts. To combat this problem, bacterial pre-treatment can be done where complex carbohydrates like cellulose and starch can be broken to glucose effectively (Raveedran et al, 2018). This can alternatively be done by cellulose and amylase enzymes, which would rather make the process expensive and decline the purpose of this study. One bacterium can be both cellulolytic and amylolytic of starch degrading. Using these bacteria would save complexity.

Cellulolytic bacteria are bacteria that can produce cellulase to break down cellulose. The previously discovered cellulolytic bacteria include Bacillus sp., Pseudomonas sp. and then Serratia sp. as well. Factors such as soil, municipal solid waste, and rice straw waste may isolate these bacteria (Kubicek, 1993).

All major life forms- animals, plants, and microorganisms-produce starch-degrading enzymes. Amylolytic capacity is normal in the microbial world; no specific starch degrading microbiota exists. Amylolytic bacteria are all but omnipresent in the environment. Soil contains many bacteria and fungi which actively produce enzymes that degrade starch. Waterlogged soil builds

an anaerobic environment for these amylolytic bacteria to survive (Ahmed, Sohag, Islam and Azad, 2016).

All in all, bacteria being able to break both cellulose and starch are vital to this pre-treatment of the substrate where more glucose is formed which can be converted to ethanol by the yeast.

1.5 Objectives

The prime purpose of this study is to find an inexpensive method of producing ethanol which can be utilized as biofuel as well as germicide. This has been done by the use of wild type yeast isolated from natural, easily available cheap samples and bacteria from the same samples to breakdown complex carbohydrates to glucose for the yeast to ferment. The substrate for yeast to utilize is taken from household organic garbage for which the cost of the substrate is reduced. Production of waste is reduced and as these wastes are not thrown in a free land, it is going to reduce pollution. The byproduct that comes out of this process that is the unused organic waste can be easily converted to fertilizers faster than it would have when thrown in soil. In addition, the process doesnot produce any harmful byproducts keeping the environment safe.

1.6 Hypothesis

Isolation of ethanol producing yeast and bacteria that hydrolyzes starch and is cellulolytic will be done and will observe them producing ethanol. This bacterium will be used as pre-treatment of the substrate so that the substrate when yeast is given has mostly simple sugar for it to ferment. Various yeast strains are to be isolated from natural sources from where few different strains of yeast will be selected to produce ethanol from inexpensive raw materials and the process will be optimized for them to produce maximum amounts of ethanol.

Chapter 2

Materials and Methods

2.1 Apparatus and Reagents

This research work was carried out at the Life Science Research Laboratory of the Department of Mathematics and Natural Sciences.

2.1.1 Equipment

1. Laminar airflow cabinet
2. Autoclave machine
3. Incubator
4. Shaker Incubator
5. Vortex machine
6. Spectrophotometer
7. Rotary evaporator
8. Glassware, falcon tubes, laboratory distillation apparatus- fractional distillation set up, microscope, pH meter, petri dishes, slants, micro-pipettes, Bunsen burner, hot plate, magnetic stirrer, clamp stands, electric balance, micro-burette, rotary evaporator, refrigerator(4^0C), Freezer (-20^0C) and some more.

2.1.2 Samples

1. Wild-type yeast strains isolated from different fruit peels, curd, rice and soil and cow dung.
2. *Bacillus subtilis* from laboratory stock.
3. Starch Hydrolysing and Cellulolytic bacteria isolated from cow dung.

2.1.3 Reagents

1. DinitroSalicylic acid (DNS)

2. Sulfuric acid
3. Sodium hydroxide
4. Sodium thiosulfate
5. Phenol red: phenolsulfonphthalein
6. Potassium Iodide
7. Potassium dichromate
8. 0.9% Sodium chloride solution
9. Soluble Starch
10. Hydrochloric acid
11. Urea.

2.1.4 Media

- **Nutrient agar medium -**

  NA is a common microbiological growth medium. Nutrient agar typically contains 0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl, 97.2% distilled water.

- **Yeast extract peptone dextrose medium (agar and broth)**

  YEPD is a complete medium for yeast growth. It contains 0.5% yeast extract, 0.5% peptone, 0.5% sodium chloride, 0.3% meat extract and distilled water. It can be used as a solid medium by including 2% agar.

- **Starch agar medium**

  Starch agar is used for the specific growth of microbes and observation of the starch hydrolyzing activity of the microorganism. It contains 0.3% beef extract, 0.5% peptone, 0.2% starch and 1.5% agar.

- **Carboxymethylcellulose agar medium**

  CMC is used to test bacteria (or fungi) for cellulolytic activity. It contains 0.05% NaCl, 0.001% $FeSO_4.7H_2O$, 0.001% $MnSO_4.H_2O$, 0.1% KH_2PO_4, 0.045% $MgSO_4.7H_2O$, and 0.037% NH_4NO_3, CMC: 2% and 1.3% agar.

- **Vegetable peels media**

  250 gm waste materials (chopped and ground peels) in 1000 ml water boiled, pH set at 6.

- **Defined sugar media**

  Different composition of glucose, fructose and molasses was used by boiling the solution with the addition of 0.10 gm urea for 250 gm molasses in 1000 ml of distilled water.

- **Skim milk, Tryptone, Glucose and Glycerol (STGG) medium**

  STGG media is used to store isolated strain of yeast in -20'C freezer. It contains 2% Skim milk, 3% tryptone soya broth, 0.5% glucose and 10% glycerol.

- **Trytone and NaCl (T1N1) medium**

  T1N1 media is used to store isolated strain of bacteria in room temperature for a month. It contains 1% tryptone and 1% sodium chloride. After incubation 0.1% glycerol added over the inoculums.

- **Biochemical test media**

  - Nitrate Broth

- Phenol red dextrose, lactose, sucrose, trehalose, maltose, starch, galactose and fructose broth
- Fermentation broth

2.2 Methodology

2.2.1 Sample collection, isolation and maintenance

Source samples for isolation of yeast strain were soil, cow dung, rice water, vegetable peels and grapes. Above mentioned sources were collected from local markets, cattle farms, houses and they were kept for 1 week at room temperature for yeast growth.

The sample was liquefied and 1ml from the source sample was serially diluted in sterile saline solution (0.9% NaCl) and inoculated onto YEPD agar plate by using the spread plate technique. Incubation was done at 30°C for 24 hours. Culture broth was prepared (0.5% yeast extract, 0.5% peptone, 0.5% dextrose, 0.5% sodium chloride and 0.3% meat extract) and autoclaved at 121°C and at 15 psi. Colonies from agar plates were inoculated into the broth. After 24 hours of incubation at 30°C, 0.2 ml suspension from the broth was cultured again by spread plate method on YEPD agar medium. This selective culture technique was used to isolate pure yeast strains.

Growths of microbes after 24-hour incubation was observed and colony forming units (CFU) were counted in each type of agar plate for specific dilution. Maintenance of yeast was done by sub-culturing on YEPD plates, incubating for 24hours at 30ºC and thereafter storing in a refrigerator at 4ºC. For long term preservation, -20 º C refrigerators were used with STGG storage media.

2.2.2 Characterization of Yeast

Yeast strains were characterized by both physiological and morphological characteristics. The isolated colonies were studied by using YEPD agar plates containing 24-hour culture. The size, texture, color, surface of the colonies were noted from direct eye view and the shape and budding was seen and noted from a compound microscope using 40X and 100X magnification.

Isolates were also characterized by using fermentation broth, nitrate broth and carbohydrate utilization broth (refer to appendix A) by using the respective media with inverted Durham tubes for understanding the amount of gas production.

Many different types of yeast were isolated according to their morphological characteristics from which few were selected by understanding their shape and budding ability from a microscope view.

The selected yeast isolates passed through optimization to seek their optimum pH, temperature, incubation time, rotation per minute and tolerance of salt and alcohol.

Each yeast isolates where kept for 24 hours in 12 different pH to get their optimum pH. The pH sets were pH2, pH3, pH4, pH5, pH6, pH7, pH8, pH9, pH10, pH11, pH12, pH13. The pH where their best was recorded.

Each yeast isolates were kept for 24 hours at 4 different temperatures to know the optimum one. The set temperatures were 20°C, 30°C, 37°C and 47°C. The temperature where they work best was logged.

Incubation time was also varied in order to detect the time it takes to produce the highest amount of ethanol. The strains were kept for 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours and a week. The optimum incubation time was jotted.

Rotation per minute was also optimized by keeping the strains in their optimum temperature at 0 rpm, 35 rpm, 80 rpm, 120 rpm, 150 rpm and the amount of rotation it needs to grow best was found.

In addition, salt tolerance of the yeast strains was also done to search for the amount of salt they can bear. Concentrations of salt were 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 6%, 7%, 8%, 9% and 10%.

The same method was also used to learn their alcohol tolerance. The concentrations used were 1%, 2%, 3%, 4%, 5%, 6%, 7%, 85, 9%, 10%, 11%, 12%, 13%, 14%, and 15%.

2.2.3 Bacterial colony isolation and characterization

Starch hydrolyzing and cellulolytic bacteria was isolated by dissolving 1-gram cow dung in 50ml autoclaved distilled water from which 1ml was serially diluted till 8 fold dilution. Each dilution was plated on nutrient agar media by spread plate technique and colony-forming unit (CFU) was calculated. Each different type of colonies has differentiated by their morphological and physiological characteristics. Those bacteria were plated on both Carboxymethylcellulose agar medium (CMC) agar plate and on starch agar plate by forming a circle. After 24 hours of incubation at 37°C, the plates were flooded with GRAM's iodine to spot any clear zone appearing around the circle. The ones showing clear zones for both CMC and starch agar was selected to be the starch hydrolyzing and cellulolytic bacteria. The selection was narrowed by running an experiment where the yeasts were used against the selected bacterial strains. Only the

one that showed no zone of inhibition was selected. The biochemical test was run on the selected bacterial strain (refer to Appendix A) for characterization. The selected strain was kept viable by subculturing it in CMC media plate and broth for every 2 weeks and for long term storage T1N1 media was used.

2.2.4 Glucose estimation-

Starch hydrolyzing and cellulolytic bacteria were used to convert complex cellulose and starch from the organic waste to a simpler, less complex version preferably monosaccharide like glucose. The amount of glucose they produced was computed by DinitroSalicylic acid (DNS). DNS assay can be used to measure the concentration of glucose in a particular sample. Here, DNS was made by dissolving 1 gram of DinitroSalicylic powder to 50 ml distilled water and stirred thoroughly. In the solution, 30-gram Potassium-Sodium Tartarate was added slowly. 20 ml of 2N Sodium Hydroxide was added, stirred and distilled water was added to make the volume up to 100 ml. The mixture was kept in an amber bottle for use. For estimation 1 ml of DNS was incorporated in a 2 ml sample and it was kept for 15 minutes incubation in 100°C water bath. After incubation, 9 ml distilled water was added and 2 ml from there was taken into a cuvette for UV-spectrophotometer. The readings were tabulated and the amount of glucose was estimated by matching the readings with standard glucose curve.

2.2.5 Ethanol production

Ethanol production was done from many different forms of fermentation medium. A loopful of freshly cultured yeast strain was inoculated in molasses, dextrose, fructose; different fruit peels and finally to different vegetable peels separately in separate conical flasks. They were plugged

with cotton and covered loosely with aluminum foil and set in a shaker incubator at 30°C 120 rpm and estimation were done after every 24 hours for a week.

Molasses media was taken in 30 different conical flasks of 100 ml volume. Each was inoculated with 30 different yeast strains and set for incubation in 30°C at 120 rpm. Two millimeters were taken from thereafter every 24 hours for ethanol assay.

Dextrose media was made for the same 30 yeast strains. For each strain, 1%, 3%, and 5% dextrose was taken to understand which yeast can produce high amounts of ethanol from a low concentration of glucose. The ethanol concentration was found by using the Conway method, titration method and colorimetric method. The residual amount of glucose was also measured by using the DNS method.

Fructose media was used in the same way as dextrose only with 1 concentration that is 2% fructose. The ethanol concentration was measured in the same way.

Various types and amounts of fruits were used for the experiment. Firstly, mixed fruit peel containing apple peels, orange peels, papaya peels, pears peels, guava peels, mango peels, pineapple and sugarcane bagasse.2 sets of media was used, 200 grams of mixed peels were taken into to 1000 ml conical flasks, one ground and the other cut into small about 1-inch pieces. Loopful of yeast strains were taken into autoclaved cooled distilled water till the water was turbid. The turbid yeast containing water was added to the media separately for each strain for both types of chopping ways. This was done after narrowing the yeast strains from 30 to 5 strains; these were the yeast which showed better alcohol production. Sequentially, the fruit peels were

used separately for the yeasts. Guava peels, papaya peels, mango peels were used separately in three 1000 ml conical flasks containing 200 grams of the peels ground and added to distilled water so that the final volume was 800 ml with the addition of 0.1gram urea, autoclaved and cooled. Inoculation was done in the same way as mixed fruits for all 5 yeast strains. They were set for incubation in 30°C at 120 rpm. Ethanol assay and glucose assay was done after 24 hours to a week at 24 hours interval.

Mixed vegetable peels were used primarily to detect the amount of ethanol each yeast can produce. Vegetable peels used were green papaya peels, potato peels, carrot peels, cabbage, cauliflower leaves, neem leaves, bottle guards' peels, pointed guards peel and other vegetables that are commonly used in our houses. Initially, only yeast was used to break the given vegetable peels and produce ethanol. All of the strains were individually kept at 30°C, 120 rpm for 48 hours before taking the reading. Peels of 62.5 grams were ground and boiled with 200 ml of distilled water for approximately 15 minutes to break the bond even further and autoclaved 121°C, 15 psi. The vegetable peel media was cooled and inoculated with 5 selected yeast strains separately and incubated for 48 hours to a week and ethanol estimation was done after every 24 hours. Consequently, individual vegetable peels and different combinations of those peels were used to do the same. The combination used was papaya and potato peel, carrot and potato, cabbage and cauliflower, carrot and pumpkin peel and separately papaya peels, potato peels, neem leaves and carrot peels were used under the given conditions.

After recording the ethanol percentage, bacteria were used prior to the yeast inoculation. Here, the isolated starch hydrolyzing and cellulolytic bacteria was used 8 hours, 18 hours, 24 hours, 48 hours, 72 hours and a week before the selected yeast was inoculated. After the incubation time of

bacteria at 37°C, 150 rpm glucose estimation was done to find the amount of glucose they could produce from utilizing the complex cellulose and starch from the organic waste. The desired incubation time for most glucose concentration was found and after that yeast was incorporated.

2.2.6 Ethanol estimation-

The amount of ethanol produced was calculated using several different types of estimation procedures. The most used method was the titration method by using acidified potassium dichromate, but the other methods used were colorimetric method, Conway method and alcohol meter after distillation.

2.2.6.1 Titration method

Procedure

1. Sample of 10 ml was taken and diluted to form 250 ml volume by incorporating distilled water.
2. Twenty milliliters from there was taken in a conical flask and covered.
3. Potassium dichromate (0.040 mol/l) of 20ml was mixed with the sample.
4. Addition of 10 ml 40% Sulfuric acid was done and it was mixed.
5. The solution then is set for heating in a water bath for 10 minutes at 47°C.
6. After heating, it was cooled.
7. Two grams of potassium iodide added and stirred to mix.

8. Two milliliters of 2% starch solution was added and titrated with 0.010 mol/l of sodium thiosulfate.
9. The initial and final titer was recorded after the color first changes from blue-black to green.
10. The method was repeated for 1%, 2%, 3%, 4% and 5% alcohol for forming standard.
11. The standard alcohol titration curve was formed with the titer found from the alcohol samples
12. The percentage of ethanol in the fermentation media was found by matching the titer with the standard as well as the calculations shown below.

Calculations: -

1. The average titer was noted.
2. Moles of sodium thiosulfate ($S_2O_3^{2-}$) used were calculated.
3. Moles of Iodine (I_2) present in the flask were found.
4. Calculations were done to find the moles of potassium dichromate ($Cr_2O_7^{-2}$) in the flask at the time of titration, added to the diluted sample at the beginning and that reacted with the ethanol.
5. Moles of ethanol (C_2H_5OH) in the diluted sample that reacted with potassium dichromate were calculated.
6. The concentration of ethanol in the original sample was finally achieved in v/v (%).

2.2.6.2 Alcoholmeter

After the incubation and estimation procedures were done, the broth was filtered and distilled by fractional distillation. The distilled product was taken in a volumetric flask and alcohol meter used to record the alcohol percentage.

2.2.7 Optimization of ethanol production-

Ethanol concentration found from each combination of vegetable peel was recorded. Production was optimized by varying the pH, temperature and rotation per minute (rpm). In addition, pre-treatment was also done to the peels to make the carbohydrate sources easily accessible for the yeast to ferment. The concentration of produced ethanol was recorded after 48hours of incubation in pH 3, pH 6, pH 7 and pH 8. The results were recorded. Then, keeping the same incubation time and pH 7 of the waste peels, the temperature and rpm were varied and the concentration of ethanol was measured. The temperature and rpm were as follows: - 37°C at 80 rpm, 37°C at 120 rpm, 30°C at 120 rpm, 30°C at 150 rpm and 25°C at 0 rpm.

Furthermore, the kitchen waste peels were used in different sizes as well. Whole large peels were used in one conical flask, in another, peels were cut into 1-inch cubes and used, a third way was to grind the peels to even smaller bits. The peels were then boiled for approximately 20 minutes and autoclaved at 121°C, 15 psi for 15 minutes. Lastly, one was taken where the peels were neither processed nor boiled nor were autoclaved.

2.2.8 Ethanol characterization

The produced ethanol was redistilled up to 80% and the affectivity of the bioethanol produced was evaluated in two different ways.

2.2.8.1 Antimicrobial capabilities-

Produced ethanol of 2ml was taken in 18 different autoclaved vials and 18 different bacterial strains were inoculated. The initial OD was measured by spectrophotometer at 540 nm, screw-capped loosely and kept at 37°C for 48hours. Later, the final OD was measured to identify whether the ethanol was able to stop or reduce microbial growth. For better comparison, the procedure was repeated using absolute alcohol taken from the laboratory.

2.2.8.2 Use as biofuel-

The bioethanol of 20 ml was poured in a fresh spirit lamp container and a new thread was used to absorb the fuel and light up. By this experiment, it was seen that whether the ethanol produced from yeast could be used alternatively to light up a spirit lamp.

Chapter 3

Results

3.1 Morphology of the strains

Morphology of selected yeast cells was detected by their growth on the YEPD agar plate and from their microscopic view.

3.2 Growth on YEPD agar plate-

The yeast strains found were narrowed to 5 different strains.

1. Alcohol tolerant yeast (T) - Hazy, oval white, a creamy colony with a dry consistency.
2. Yeast from Grapes (Gn1) - Smooth, creamy, white, big, oval with butter-like consistency.
3. Yeast from Cow dung (C2) - Smooth, small, oval-shaped, raised semi-white colony.
4. Yeast from Grape peel (Gn2) - Oval, small, oval-shaped, raised with a creamy texture.
5. Normal soil yeast (NS2) - Oval, Flat, hazy, translucent colonies.

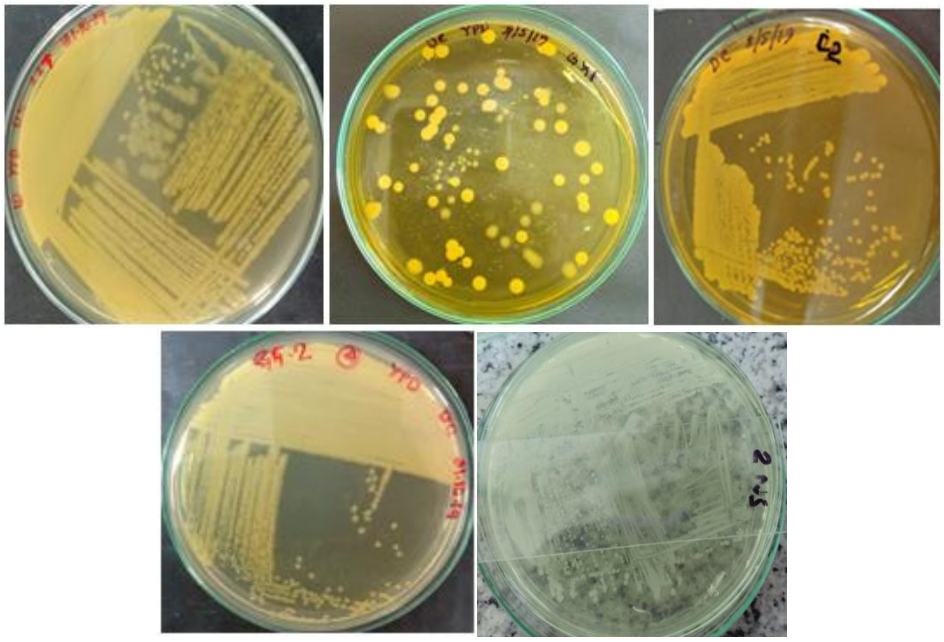


Figure 3. 1 Yeast colonies of 5 different strains in YEPD plate after 48 hrs of incubation

3.3 Microscopic view-

Compound microscopes were used in 100x magnification to observe the cell morphology of yeast. Budding was observed in them which showed vegetative reproduction. Alcohol tolerant yeast (T) is big pointed oval-shaped, yeast from grapes (Gn1) is oval-shaped, yeast from cow dung (C2) is round-shaped, yeast from the grape peel is thin rod-shaped and small, round-shaped yeast was the normal soil yeast (NS2).

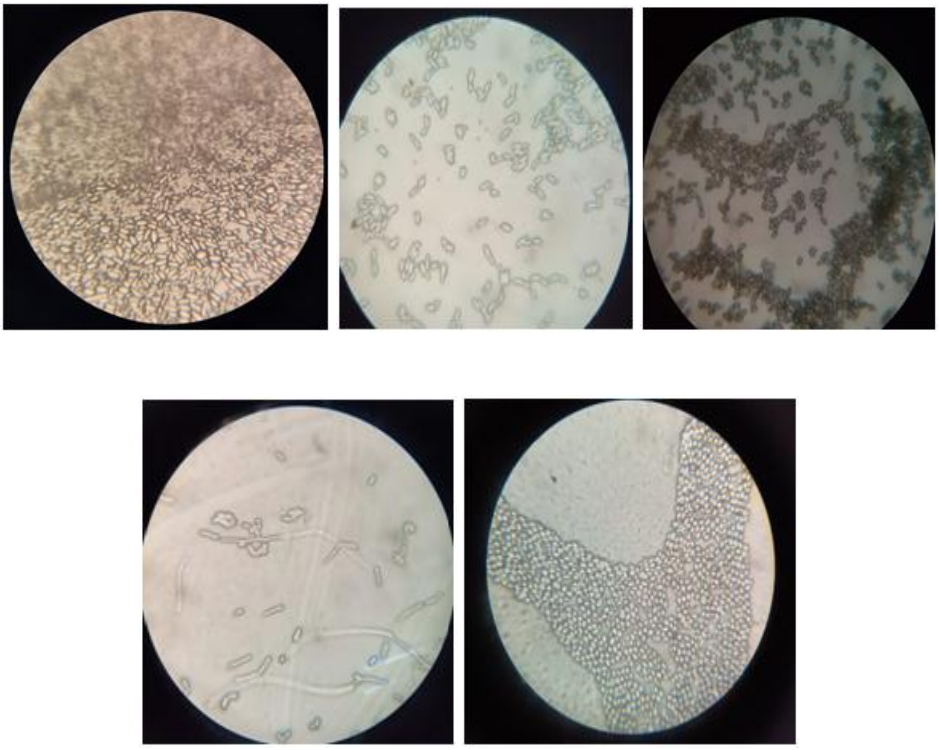


Figure 3. 2 (From top left to bottom right) Microscopic view of T, Gn1, C2, Gn2, NS2.

3.4 Physiological characterization

3.4.1 Fermentation of carbohydrates

Phenol red test was done to find which carbohydrates they are capable of utilizing and whether they form gas while fermenting. The culture was kept for 24 hours incubation at 30°C for the yeast and 37°C for the bacteria. NS completely utilized dextrose and sucrose forming gas, maltose and trehalose without gas, partially utilized fructose, galactose and lactose and cannot utilize starch. T completely utilized dextrose, sucrose, maltose, fructose, galactose, lactose and trehalose without forming gas but could not ferment starch. Gn1 completely utilized dextrose and fructose without forming gas, partially utilized sucrose, galactose and lactose and trehalose but

cannot utilize maltose and starch. Gn2 could not utilize dextrose, maltose, fructose, galactose, starch and trehalose without forming any gas, but partially could when sucrose and lactose were present. C2 could ferment all carbohydrates with forming gas except starch and lactose where it partially fermented lactose and did not at all utilize starch. SHC, Starch Hydrolyzing and Cellulolytic bacteria could only completely utilize starch and partially did all except sucrose and trehalose.

Yeast isolates / Carbohydrates	NS	T	Gn1	Gn2	C2	SHC
Dextrose	++(gas)	++	++	--	++(gas)	+-
Sucrose	++(gas)	++	+-	+-	++(gas)	--
Maltose	++	++	--	--	++(gas)	+-
Fructose	+-	++	++	--	++(gas)	+-
Galactose	+-	++	+-	--	++(gas)	+-
Starch	--	--	--	--	--	++
Lactose	+-	++	+-	+-	+-	+-
Trehalose	++	++	+-	--	++(gas)	--

Table 3. 1 The utilization of different carbohydrates where "++" means complete. "+-" means partial and "--" means no utilization

3.4.2 Nitrate reduction

Nitrate reduction test was done but none of the strains exhibited positive results after 24 hour of incubation at 30°C for the yeast and 37°C for the bacteria.

Strain	Nitrate Reduction test
NS	--
T	--
Gn1	--
Gn2	--
C2	--
SHC	--

Table 3. 2 The reduction of nitrate where "++" means complete. "+-" means partial and "--" means no reduction.

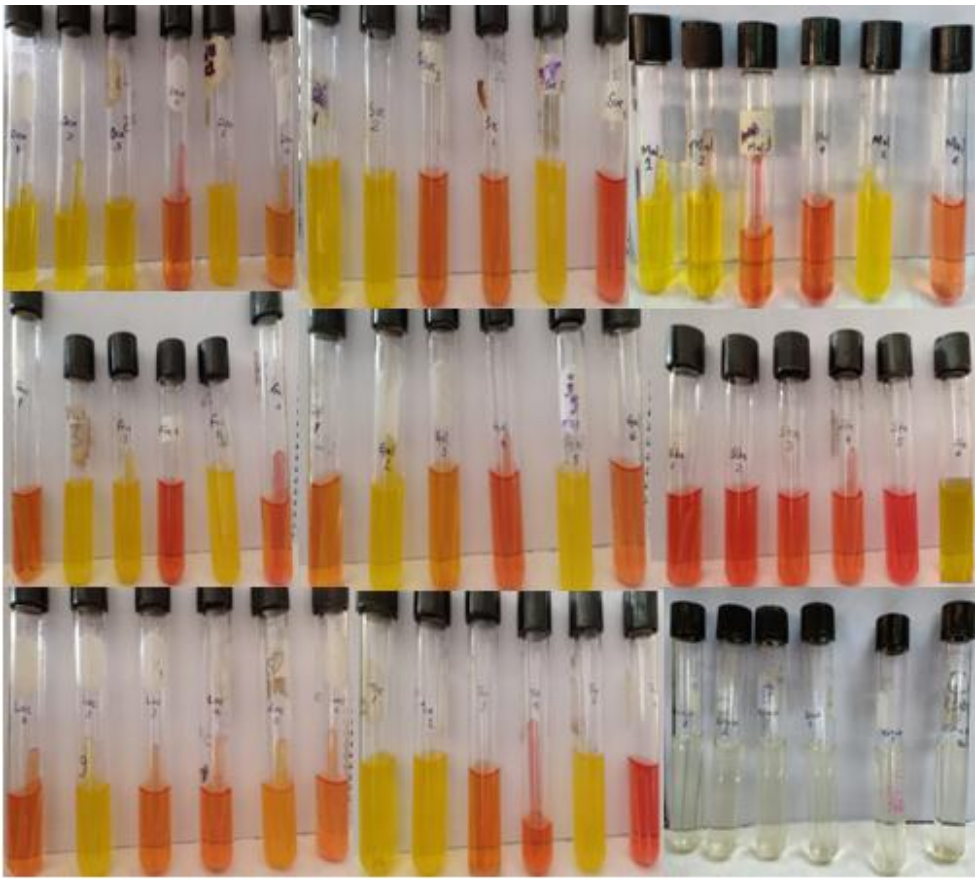


Figure 3. 3 Result of all physiological characterization

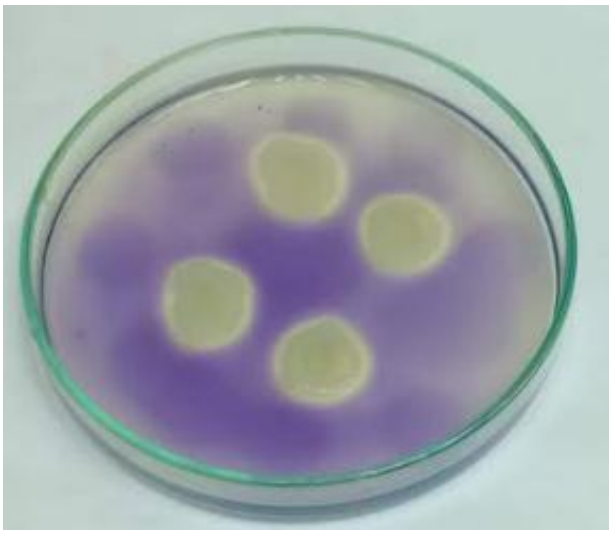


Figure 3. 4 Results of SHC on starch agar.

3.5 Stress tolerance characterization

3.5.1 Results pH tolerance test

Tolerance of pH was observed from its growth at 12 different pH. The value was recorded based on its optical density at 600 nm after 48 hours of incubation at 30°C. All the strains showed different amounts of growth. The highest growth for each strain was indicated as bold.

	Absorbance at 600nm				
pH \ Yeast isolates	**NS**	**T**	**Gn1**	**Gn2**	**C2**
2	0	0.0001	0	0	0
3	0.094	0.0001	0	0.012	0.0001
4	0.171	0	0.001	0.012	0
5	0.088	0.37	0.222	0.352	0.019
6	**0.544**	**0.496**	0.333	0.511	**0.523**
7	0.366	0.394	**0.345**	**0.516**	0.511
8	0.345	0.14	0.221	0.432	0.495
9	0	0.119	0.113	0.233	0.219
10	0	0.129	0.19	0.003	0.133
11	0	0.002	0	0.001	0.022
12	0	0	0	0.001	0.0001
13	0	0.433	0	0.001	0

Table 3. 3 pH tolerance for the 5 different yeast isolates.

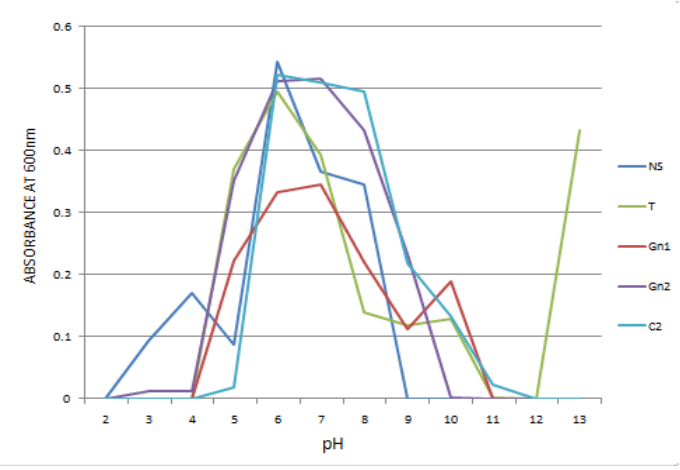


Figure 3. 5 pH tolerance for the 5 different yeast isoates.

3.5.2 Temperature Tolerance test

Tolerance of temperature was observed from its growth at 4 different temperatures. The value was recorded based on its optical density count at 600 nm after 48 hours of incubation at 30°C. All the strains showed different amounts of growth. The temperature having the highest growth is kept bold.

	Absorption at 600nm				
Yeast isolates / Temperature (°C)	**NS**	**T**	**Gn1**	**Gn2**	**C2**
20	0.019	0.002	0.192	0.085	0.819
30	**0.529**	**0.716**	**0.445**	**0.362**	0.524
37	0.425	0.524	0.028	0.333	**0.965**
47	0.072	0.122	0.012	0.089	0.056

Table 3. 4 Temperature tolerance for the 5 different yeast isolates.

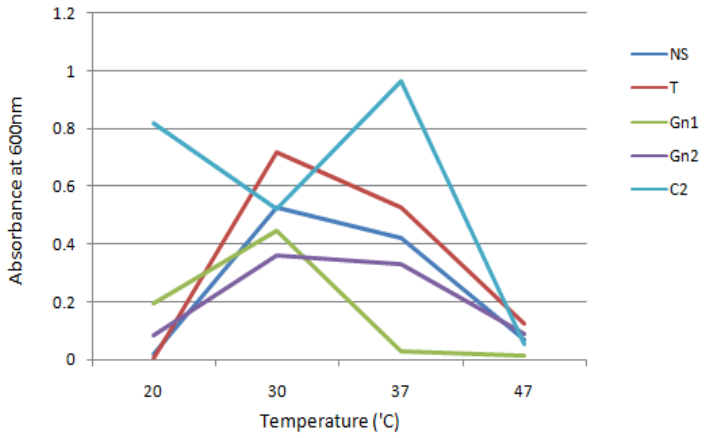


Figure 3. 6 Temperature tolerances for the 5 different yeast isolates.

3.5.3 Salt Tolerance Test

Tolerance of salt was observed from its growth at 5 different salt concentrations. The value was recorded based on its optical density count at 600 nm after 48 hours of incubation at 30°C. All the strains showed different amounts of growth from where 0.5 and 1 percent was the highest for all except for T strain, where 2.5% had the best growth. The highest values are indicated as bold.

	Absorbance at 600nm				
NaCl conc. / Yeast strains	**0.5**	**1**	**1.5**	**2**	**2.5**
NS	**0.135**	0.157	0.018	0.018	0
T	0.057	0.01	0.008	0.001	**0.026**
Gn1	0.014	**0.257**	0.02	0.023	0.024
Gn2	**0.187**	0.032	0.02	0.016	0.014
C2	0.285	**0.336**	0.293	0.217	0.184

Table 3. 5 Salt tolerances for the 5 different yeast isolates.

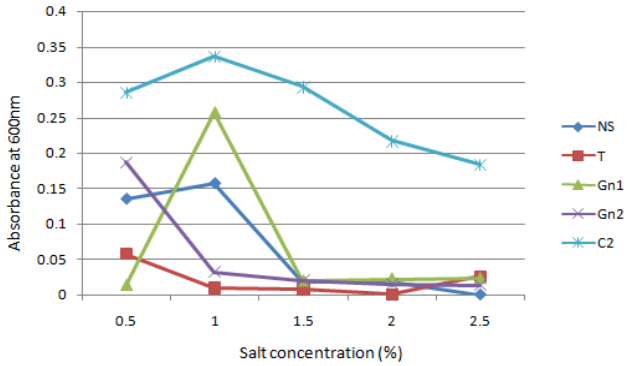


Figure 3. 7 Salt tolerances for the 5 different yeast isolates.

3.5.4 Results of Alcohol Tolerance

Tolerance of alcohol was observed from its growth at 5 different concentrations of alcohol. The value was recorded based on its optical density count at 600 nm after 48 hours of incubation at 30°C. All the strains showed different amounts of growth and the highest values are indicated as bold.

	Absorbance at 600nm				
% Alcohol / Yeast strains	**2**	**5**	**7**	**10**	**20**
NS	**0.738**	0.501	0.453	0.018	0
T	**0.958**	0.883	0.922	0.625	0.379
Gn1	**0.0023**	0.001	0.0021	0	0.0008
Gn2	**1.331**	0.68	0.99	0.007	0.005
C2	**1.391**	1.156	0.928	0.003	0.009

Table 3. 6 Alcohol tolerances for the 5 different yeast isolates.

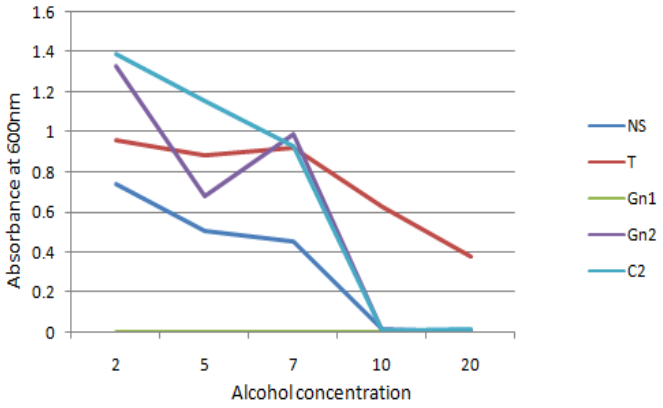


Figure 3. 8 Alcohol tolerances for the 5 different yeast isolates.

3.6 Glucose estimation

Glucose estimation was done by the DNS method. This was used to find the amount of glucose in the substrate that the bacteria would form from the complex carbohydrates in the solution. It was seen that glucose liberation varied from time interval. Glucose in the solution increased with the incubation time which was understood by comparing the value to the standard curve.

3.6.1 Glucose standard curve

To estimate the amount of glucose in a solution it has to be compared with a fixed value. The standard curve was made from making glucose concentration from 0.1mg/ml to 1mg/ml where each absorbance was recorded at 540 nm and a curve was plot. This graph was used to match the absorbance of the unknown sample to find the corresponding concentration of glucose.

CONCENTRATION of Glucose (mg/ml)	**ABSORBANCE at 540nm**
0.1	0.109
0.2	0.15
0.3	0.198
0.4	0.263
0.5	0.315
0.6	0.452
0.7	0.529
0.8	0.6639
0.9	0.7
1	0.731

Table 3. 7 Glucose standard curve

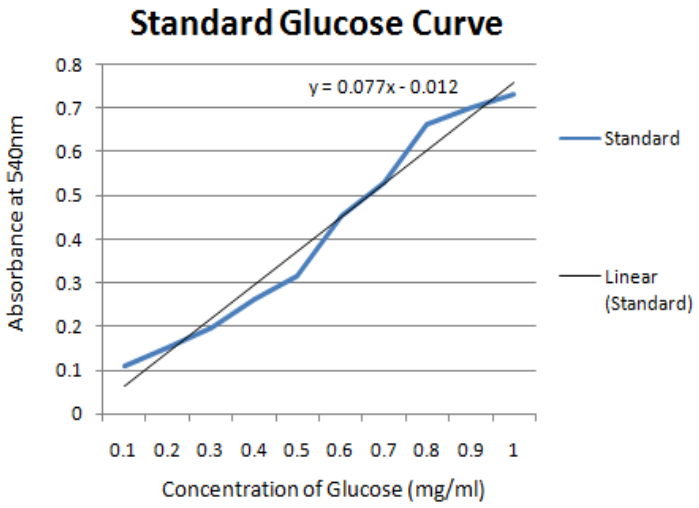


Figure 3. 9 Glucose standard curve.

3.7 Ethanol production results

The efficiency of the yeast isolated was seen by the amount of ethanol they could produce. The substrate was prepared according to the optimum conditions found for each yeast strains and allowed to ferment. Substrate constituents were varied to find in which state they can produce ethanol better.

3.7.1 Ethanol production from refined sugars

Initially, defined sugars were used as the substrate for ethanol production. Here, Molasses, which have been found to have 25% glucose, dextrose which was used in three concentrations (1%, 3%, and 5%) and 2% fructose was used for the 5 selected strains for fermentation. The results varied for each strain and each sugar and the highest values are indicated as bold.

	Alcohol percentage				
Yeast strains / Refined sugar	NS	T	Gn1	Gn2	C2
Molasses	4.97	5.32	4.63	3.7	**6.99**
1% Dextrose	2.33	**2.82**	2.66	1.08	2.22
3% Dextrose	**2.92**	2.88	2.01	1.9	3.1
5% Dextrose	3.55	3.02	2.84	2.92	**4.72**
2% Fructose	0.02	1.65	1.69	0.001	**3.32**

Table 3. 8 Alcohol concentrations from defined sugars by 5 yeast isolates.

3.7.2 Alcohol from fruit peels

Fruit peels were next used as a raw material. Different types of fruits were selected, both sweet and sour, in different forms. Mixed was taken in two parts one ground and the other cut in small pieces. There Gn1 gave maximum for ground and C2 for the other. Then guava, papaya, and mango peels were used for separate fermentation. There, guava was not a good substrate but for the other two, ethanol production was good enough. The highest values are indicated as bold.

	Alcohol percentage				
Yeast strains / Fruit peels	NS	T	Gn1	Gn2	C2
Mixed fruit peel (grinded)	2.45	2.3	**2.91**	1.99	2.93
Mixed fruit peel (1 inch cube)	2.25	2.1	2.6	2	**2.81**
Guava peel (grinded)	1.64	**1.92**	2	1.32	1.91
Papaya peel (grinded)	2.51	2.41	1.33	2.01	**3.02**
Mango peel (grinded)	**3.33**	2.53	1.97	2.25	2.99

Table 3. 9 Alcohol from fruit peels by 5 isolates.

3.7.3 Alcohol from vegetable peels without bacteria

Vegetable peels from household waste were incorporated and separated to have 2 mixed sections and others were individualized. The different mix up is portrayed in the table below. There it has been seen that carrot peels gave most ethanol by the usage of T strain and the highest values are indicated as bold.

	Alcohol percentage				
Yeast strains / Vegetable peels	**NS**	**T**	**Gn1**	**Gn2**	**C2**
Mixed peels (grinded)	1.439	1.211	0.991	**1.999**	1.732
Mixed peels (1 inch cube)	1.116	1.501	1.51	**2**	1.521
Papaya+Potato peel	**2.013**	1.92	1.675	2.012	1.91
Carrot+Potato peel	1.003	**1.45**	0.921	0.332	1.011
Cabbage+cauliflower	0.923	0.658	0.31	0.224	**1.092**
Papaya peel green	1.566	2.313	1.61	1.293	**2.65**
Potato peel	1.31	**2.88**	2.31	1.604	1.56
Neem leaves	0.62	1.58	0.256	0.798	**1.92**
Carrot peel	2.65	**2.93**	1.65	2.01	2.81

Table 3. 10 Alcohol percentages for 5 isolates of yeast.

Figure 3. 10 Fermentation of vegetable peels without bacteria.

3.7.4 Alcohol production after treated with bacteria

Ethanol production from just the peels means that the simplified sugars present there is only converted to ethanol. Thus, to maximize the production even further, a bacterial colony was incorporated in the media prior to yeast inoculation. First, the time taken for the bacteria to produce the maximum amount of monosaccharide and disaccharides was recorded. Following treatment with bacteria, yeast was inoculated for 48 hours at 30°C, 120 rpm and alcohol

percentage measured by titration method. Highest came to be for C2 strain after 168 hours of incubation with cellulolytic and starch hydrolyzing bacteria. The highest values are indicated as bold.

Treatment time (Hrs)	Glucose concentration (mg/ml)	Alcohol concentration (%)				
		Yeast Isolates				
		NS	T	Gn1	Gn2	C2
24	2.62	**1.42**	1.32	0.85	0.88	1.41
48	3.1	1.45	2.01	1.68	1.43	**2.54**
96	13	3.99	**4.61**	3.67	3.01	4.51
168	37	5.13	5.02	4.88	4.15	**5.49**
336	38	5.11	5.05	4.88	4.24	**5.42**

Table 3. 11 Glucose concentration and alcohol percentage after varying incubation time of bacteria, keeping yeast for 48 hours.

3.7.5 Alcohol percentage from varying peels with bacteria.

Different combinations of vegetable peels were taken and SHC bacteria incorporated and kept for a week at 37°C 150 rpm. Then 5 different yeast strains were inoculated separately and incubated at 30°C for 48 hours at 120 rpm giving a maximum of 6.99% alcohol concentration for C2 strain in substrate constituting carrot and pumpkin peel. The highest values are indicated as bold.

Figure 3. 11 Titration of the fermented samples.

Figure 3. 12 Fermentation of Vegetable peels by pre-treatment with bacteria.

	Alcohol percentage				
Yeast strains / Vegetable peels	**NS**	**T**	**Gn1**	**Gn2**	**C2**
Mixed peels grinded	4.99	5.3	4.25	5.01	6.52
Mixed peels 1-inch cube	4.13	5.37	**5.39**	6.12	5.93
Papaya+Potato peel	**6.11**	5.47	5.173	**6.88**	6.19
Carrot+Potato peel	4.29	4.46	3.37	3.13	5.64
Cabbage+cauliflower	4.068	4.172	5.14	5.81	5.78
Carrot+Pumpkin peel	5.22	**6.123**	5.02	5.36	**6.99**
Papaya peel green	3.27	3.99	3.649	5.21	5.91
Potato peel	2.77	3.29	3.33	2.98	4.96
Neem leaves	2.13	5.58	4.17	4.32	4.92
Carrot peel	5.95	5.93	4.01	5.01	5.93
Non autoclaved	4.925	4.36	4.258	5.01	4.11

Table 3. 12 Alcohol percentages from varying peels at 30°C for 48 hours at 120 rpm, by pre-treatment with bacteria for a week.

3.8 Distillation

Distillation is a process of separating solvents by their difference in boiling points. Ethanol has a boiling point of approximately 78°C and if heated to this temperature, ethanol is separated from the remaining solvent. This process can be used to not only separate but also concentrate the separated ethanol to the desired percentage.

Figure 3. 13 Distillation done by Rotary Evaporator

Estimation Technique	After First Distillation (%)	After Re-distillation (%)
Titration method	21.6	74.36
Alcoholmeter	35	80

Table 3. 13 Alcohol percentages after distillation.

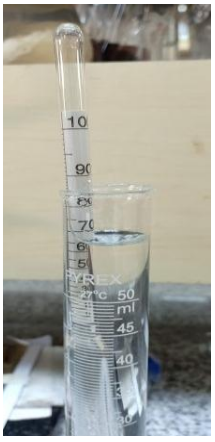
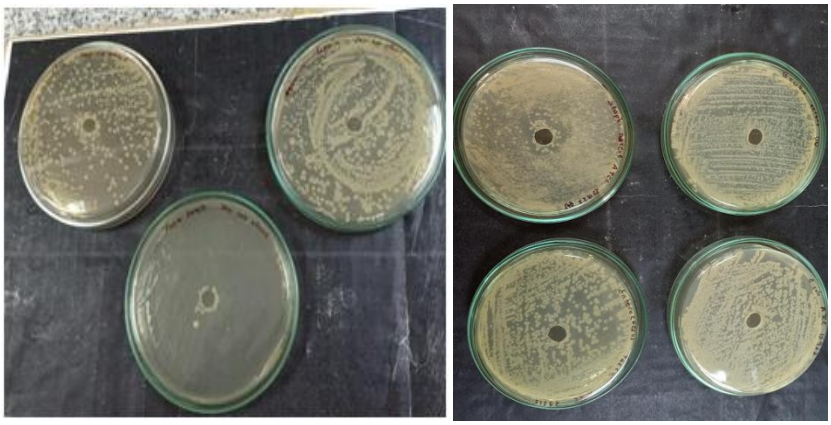


Figure 3. 14 Alcohol percentage after distillation measured by an alcoholmeter.

3.8.1 Germicidal activity of the ethanol

The ethanol has been concentrated to 80% according to the alcohol meter and used to detect its germicidal activity. Agar well diffusion was done where the well was filled with 60 microlitres of the produced ethanol and the plate was spread with various bacteria obtained from laboratory samples. The result was compared with that of 70% lab ethanol.



(a) (b)

Figure 3. 15 (a) Plate with 70% alcohol (b) Plate with Fermented ethanol

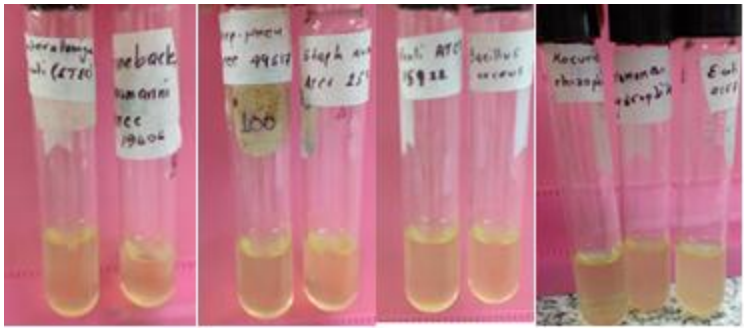


Figure 3. 16 Comparison of bacterial normal groeth to the growth with bioethanol.

Bacterial Strains	Absorption at 600nm			
	Presence of Bioethanol		Absence of Bioethanol	
	0 hour	48 hours	0 hour	48 hours
Acinobacter baumonni ATCC 19606	0.112	0.289	0.456	1.272
Aeromonas hydrophila	0.0.178	0.271	0.399	1.138
Bacillus cereus	0.251	0.290	0.327	1.170
Enterococcus faeces ATCC 15922	0.299	0.303	0.392	0.790
Enterotoxigenic Escherichia coli (ETEC)	0.211	0.300	0.320	1.330
Escherichia coli 0157.H7	0.234	0.274	0.517	1.310
Escherichia coli ATCC 15922	0.299	0.303	0.392	0.790
Hafnia alvei	0.219	0.301	0.412	0.972
Kocuria rhizophila	0.199	0.279	0.237	0.955
Proteus vulgaris	0.200	0.301	0.462	1.113
Pseudomonas sp.	0.111	0.213	0.379	0.981
Staphylococcus aureus ATCC 25923	0.197	0.295	0.466	1.196
Streptococcus agalactiae	0.154	0.209	0.221	1.317
Streptococcus pneumoniae ATCC 49619	0.277	0.310	0.371	1.211
Vibrio cholerae	0.200	0.285	0.426	0.829

Table 3. 14 The reduced growth due to ethanol compared to their normal growth.

3.8.2 Use of ethanol as biofuel

The concentrated ethanol was then poured in an empty spirit lamp and lit. This proves that the ethanol they produced from organic waste can be used as fuel for burning.

Figure 3. 17 Bioethanol spirit burning

Chapter 4

4.1 Discussion

Bioethanol production is better with wild type yeast than with industrial yeast as these yeast strains are more adapted to any given environment (Bell, Higgins, Attfield, 2001). This is the reason yeast was isolated from natural sources where they did not have their desired environment. These strains found were screened and only the ones having better results were selected which is an important strategy towards forming more ethanol. Based on the white and creamy appearance on solid media of all the selected colonies, their texture, oval shape and polar budding, it can be believed that all the yeasts are from *Saccharomyces* spp (Boekhout and Kurtzman, 1996). Fermentation was done by different defined sugars where NS completely utilized dextrose and sucrose forming gas, maltose and trehalose without gas, partially utilized fructose, galactose and lactose and cannot utilize starch. T completely utilized dextrose, sucrose, maltose, fructose, galactose, lactose and trehalose without forming gas but could not ferment starch. Gn1 completely utilized dextrose and fructose without forming gas, partially utilized sucrose, galactose and lactose and trehalose but cannot utilize maltose and starch. Gn2 could not utilize dextrose, maltose, fructose, galactose, starch and trehalose without forming any gas, but partially could when sucrose and lactose were present. C2 could ferment all carbohydrates with forming gas except starch and lactose where it partially fermented lactose and did not at all utilize starch. SHC, Starch Hydrolyzing and Cellulolytic bacteria could only completely utilize starch and partially did all except sucrose and trehalose (Table 3.1 and Figure 3.3). According to (Vaughan-Martini and Martini, 1993), *Saccharomyces* types of species portray similar types of ability to ferment defined sugars which suggest, these yeast isolates can be recognized as *Saccharomyces* spp.

Stress tolerance was done in case of pH, temperature, salt and alcohol and it has been seen that pH 6-7 (Table 3.4 and Figure 3.5), temperature 30°C (Table 3.5 and Figure 3.6), salt concentration below 1% (Table 3.6 and Figure 3.37) and alcohol concentration of below 7 % (Table 3.7 and Figure 3.8) gave the best results. For strains NS, T and C2 it was seen that pH6 was optimum for growth and for Gn1 and Gn2 pH5. Strain T could grow in even pH13 though growth was slower but they grew. All the temperature was tolerable for all 5 strains but 30°C was optimum for all except C2 which grew most at 37°C. Strains T, Gn1, Gn2, and C2 could tolerate salt of 2.5% as well but T has the most growth in this concentration whereas other growth was reduced above 1% salt. The most tolerable was strain C2 as it grew better than others. Alcohol tolerance is the most important as if they could not tolerate a high amount of alcohol then product inhibition would have happened. Luckily, this was not a problem as all the strains could tolerate a maximum of 7% alcohol having little growth above that as well whereas the highest amount produced ethanol less than that. Similarly, *Saccharomyces* species are known to be tolerant to extreme pH, temperature, salt and alcohol (Ogunremi, Oboh and Ogbe. 2013), in this paper they found that pH of 4-6 and salt concentration of 5-20% and alcohol up to 16% was tolerable for *Saccharomyces cerevisiae*. According to Arachchiga, Yashida, and Toyama, *Saccharomyces* spp could tolerate salt up to 10% (2019).

Screened yeasts were then subjected to fermentation. First, refined sugar was used where for molasses, 5% dextrose and 2% fructose, C2 worked well producing 6.99%, 4.72%, and 3.32% (% v/v) alcohol respectively (Table 3.9). It came to be 76g/l in molasses, cabbage and cauliflower waste in the work of Thenmanzhi and Victoria (2013). Fruit peels were used then where most alcohols were produced with ground mango peels with a percentage of 3.33 (Table 3.10). Mango peels with yeast extract had 60g/l of ethanol after 48hrs of incubation in Reddy, Lebaka &

Obulam, Vijaya Sarathi & Wee, Young-Jung. (2011). Vegetable peels were then used where 2.88% was found from strain T and for strain NS papaya and potato were good substrates, for Gn1 gave the highest potato peel which was less than others. Gn2 gave good amounts for the mixed peels and C2 worked well for cabbage, cauliflower, papaya peels and neem leaves (Table 3.11). N. Goriwale and S. Khan (2018) have found from 1-6% by varying acid and base to cure the leaves.

Starch Hydrolyzing and Cellulolytic bacteria SHC was used for the breakdown of complex carbohydrates to simpler ones preferably glucose where the glucose concentration was found to be the highest after having incubated for a week and after that, it did not further rise which might be because the bacterial growth was reduced. (Table 3.12). (Milala et al., 2005) found how they produced cellulase for the breakdown of substrates to glucose.

After isolation and characterization of SHC bacteria, the fermentations were repeated but this time with prior treatment with these bacteria for a week at 37°C and 120 rpm. Strain NS gave best for papaya and potato peels with 6.11%, strain T for carrot and pumpkin peels with 6.123%, Gn1 colonies produced 5.39% alcohol for mixed peels of 1-inch cubes and Gn2 was best in papaya and potato peels as well with 6.88% alcohol. Strain C2 showed remarkable results forming 6.99% of ethanol when carrot and pumpkin peels were used as substrates (Table 3.13). Whereas, with potato, 31.5 mg/ml was found after incubating in its optimum condition (S.Yamada et al., 2009). Paddy and husk, on the other hand, produced 16.22g/l of bioethanol (Ramachandran and Karthikeya, 2013). (Demiray et al., 2016) stated that 6.91g/l when from carrot pomace was used as substrates.

The alcohol found from the experiment was separated by fractional distillation. Distillation deals with a mixture, a solution composed of two or more elements, that when boiled, will cause each element to vaporized at different temperatures, (Williamson, Minard & Masters. 2007). The temperature was set at 78°C which is the boiling temperature for ethanol. When the solvent reached the desired temperature, ethanol from the solvent, filtered from the fermentation media, evaporated and came to the receiving bottle at the end of the machine. After completion of distillation, the distillate was brought out and measured via both titration method and by dipping alcohol meter. Alcoholmeter is a graduated tube where according to the density, the ethanol is measured, Durasens, (2018). The result was 21.6% by the titration method and 35% with alcohol meter. After re-distillation, this percentage increased to 74.36% according to the titration method and 80% was found by dipping an alcohol meter (Table 3.14 and Figure 3.10). This distilled ethanol was subjected to germicidal tests and tests to confirm its usage as biofuel. The bacterial count did not increase much when it was inoculated in bioethanol portraying its effectiveness as a germicide. Later, the bioethanol was lit in a spirit lamp proving its usage as biofuel.

4.2 Conclusion

Microscopic view and morphological features were used to select the yeast strains. These five selected strains were seen to produce ethanol in good amounts. The highest ethanol found in this study is 6.99%. Five yeast isolates in Bangladesh were reported to use for similar experiments where those isolates TY, BY, GY-1, RY, and SY produced alcohol 12.0%, 5.90%, 5.80%, 6.70%, and 5.80%, respectively at 30°c after 48hours of incubation. The primary purpose of the present study was to increase the ethanol production rate from the fermentation of starch hydrolyzing and cellulosic kitchen waste (vegetable peels) using cellulose-degrading bacteria. The final highest percentage of ethanol found was 6.99% by strain C2 and the lowest was 2.13% by NS strain after

pre-treatment with SHC bacteria. Therefore, yeast isolate from cow dung showed the highest percentage of alcohol production from cellulosic kitchen wastes. Vegetable peels pretreated by cellulolytic bacteria are determined as a suitable fermentation substrate.

This study has shown that the ethanol produced from household waste after pre-treatment with starch hydrolyzing and cellulolytic bacteria can be a good source as biofuel as well as germicide.

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Appendix A.

Preparation of Nitrate reagent

The nitrate reduction test is based on nitrite detection and its ability to form a red compound when reacting with sulfanilic acid to form a complex (nitrite-sulfanilic acid) that then reacts with α-naphthylamine to give a red precipitate (prontosil), which is a water-soluble azo dye. Nevertheless, red color will only be produced when the nitrate is present in the medium. If the medium does not have a red color after you have added sulfanilic acid and α-naphthylamine indicates only that nitrite is not present in the liquid (Aryal, 2019).

Solution A, Sulfanilic acid (1gm of sulfanilic acid was dissolved in 125 ml of 5N acetic acid). Solution B, Alpha-naphthylamine (0.625 gm of α-naphthylamine dissolved in 120 ml of 5N acetic acid.)

Preparation of Dinitrosalicylic acid (DNS)

The DNS method is a colorimetric technique that consists of a redox reaction between the 3, 5-dinitrosalicylic acid and the reducing sugars present in the sample. The reducing power of these sugars comes from their carbonyl group, which can be oxidized to the carboxyl group by mild oxidizing agents, while the DNS (yellow) is reduced to 3-amino-5-nitrosalicylic acid (red brown) which can be quantified by spectrophotometer at 540 nm, wavelength of maximum absorbance. The intensity of the color is proportional to the concentration of sugars. The reaction is carried out in an alkaline medium (Garriga, Almaraz and Marchiaro. 2017).

About 1g of DNS was dissolved in 50 ml of distilled water. To this solution, 30 grams of sodium potassium tartarate tetrahydrate was added. Then 20 ml of 2N NaOH was added, which turns the

solution to transparent orange yellow color. The final volume was made to 100 ml with the distilled water. This solution was stored in an amber colored bottle.

Preparation of Phenol red indicator broth

Phenol red indicator solution was formed by dissolving 0.1 g of phenol red in 14.20 ml of 0.02 N NaOH and diluted to 250 ml with deionized water.

This test is used to observe whether or not a microorganism can utilize a particular carbohydrate as an energy source. The ability of the microorganism to breakdown pyruvate into Hydrogen and Carbon dioxide is also tested.

In order to perform this test, the carbohydrate of choice is added to a solution of phenol red broth. In case the microorganism can utilize the provided carbohydrate, acid is released as a by-product. Phenol red acts as an indicator and turns yellow in the presence of acid. Thus, carbohydrate fermentation can be observed. Furthermore, whether or not the microorganism produced gas as a by-product can also be observed with the help of a Durham tube. If the microorganism is able to ferment pyruvate - the end product of glycolysis - into Hydrogen and Carbon dioxide, the gas will be trapped inside the Durham tube. A bubble that forms in the Durham tube will be an indicator of gaseous by-product formation (Payne et al. 2015).

In this experiment, eight different carbohydrate sources - namely, Dextrose, Lactose, Sucrose, Maltose, Starch, Galactose, Trehalose and Fructose- were used for testing fermentation capabilities of the microorganisms.

Preparation of Starch Hydrolysing media

Some bacteria, present in starch, can hydrolyze amylose and amylopectin. We are therefore able to use starch as a source of energy. This is possible because of the development of the oligo-1, 6-glucosidase and α-amylase enzymes. The enormous size of amylopectin and amylose molecules makes it difficult for them to traverse the bacterial cell wall. Thus, unless they are broken down into glucose, they cannot be utilized. Flooding the culture plates with Gram's iodine gives us the results. If the microorganism is able to hydrolyze starch, clear zones can be observed after flooding with Gram's Iodine. A clear zone formed around the colonies is an indicator that starch hydrolysis has taken place (Aryal. 2017).

Preparation of Mueller Hinton agar media

Mueller Hinton Media includes Casein, Starch, and Agar's Beef Extract, Acid Hydrolysis. Casein Beef Extract and Acid Hydrolysate contain oxygen, vitamins, oil, amino acids, sulphur and other nutrients of vital value. Starch is added so as to absorb any produced toxic metabolites. Starch hydrolysis produces dextrose which serves as an energy source.

It is important that an effective medium is used to test the sensitivity of micro-organisms to sulfonamides and trimethoprim. Paraminobenzoic acid (PABA) and its analogs show antagonism to the sulfonamide activity. Reduced trimethoprim development, resulting in smaller inhibition zones and intrazonal colonies, is shown on unsuitable Mueller Hinton medium with high levels of Thymid. Both the content of PABA and thymine / thymidine in Mueller Hinton Agar is reduced to a minimum, thereby significantly reducing sulfonamide and trimethoprim inactivation when the media is used to test the susceptibility of bacterial isolates to these antimicrobials (Aryal. 2020).

Suspend 38 gm of the medium in one liter of distilled water. Heat with constant agitation and boil 1 minute to dissolve the medium completely. Autoclave at 121°C for 15 minutes. Cool to room temperature. Pour the cooled Mueller Hinton Agar on a smooth, horizontal surface into sterile petri dishes to provide an even depth. Enable temperature to cool to space. Check for the final pH 7.3 ± 0.1 at 25ºC.