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Production of vinegar from pineapple peels using *Acetobacter* species isolated from soil sample and its antibacterial activity

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ABSTRACT

Fruit waste has become one of the main sources of municipal solid wastes, which have been an increasingly tough environmental issue. Vinegar is very important food preservative in food industry. The vinegar can be obtained various raw materials such as various fruits and fruit peels. The present study investigates production of vinegar from pineapple peels using *Acetobacter* species isolated from soil sample and its antibacterial activity. *Acetobacter spp.* and *Saccharomyces cerevisiae* was isolated from soil samples. Identification was done by performing morphological, biochemical and cultural characteristics of bacteria and fungi. Firstly, wine was produced by *Saccharomyces cerevisiae,* confirmed by performing CO_2 production and Iodoform test. The wine produced was now inoculated with *Acetobacter spp.* and it is incubated for 11 days for aerobic fermentation at 37^0C it was calculated that 4.60% of vinegar was produced. The antibacterial activity of vinegar was tested against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella paratyphi* and *Pseudomonas aeruginosa*. The results of antibacterial activity of vinegar against *Escherichia coli* - 16mm, *Staphylococcus aureus* - 20mm, *Salmonella paratyphi* -19mm and *Pseudomonas aeruginosa* - 19mm. Subsequent upon this research, materials like pineapple peel considered generally as waste can be bio converted into important value-added materials thus aiding environmental safety.

Keywords : *Acetobacter*, Pineapple Peels, Vinegar, *Saccharomyces cerevisiae*

INTRODUCTION

In the past decade, along with the rise of the middle class and fast economic growth in world, different varieties of fruits produced in all countries are increasingly consumed. Due to the high consumption and industrial processing of the edible parts of fruit, fruit wastes such as citrus

fruit skins, pineapple residues, sugarcane bagasse and other fruit residues (principally peels and seeds) are generated in large quantities in big cities. Fruit waste has become one of the main sources of municipal solid wastes (MSW), which have been an increasingly tough environmental issue. At present, the two main techniques to dispose MSW are landfill and incineration. However, inappropriate management of landfill will result in emissions of methane and carbon dioxide (Qdais *et al.* 2010), and incineration involves the subsequent formation and releases of pollutants and secondary wastes such as dioxins, furans, acid gases as well as particulates (Buekens, 1998), which pose serious environmental and health risks. For these reasons, there is an urgent need to seek resource and value-added use for fruit wastes. In fact, inexpensive and readily available use of agri-food industry waste is highly cost-effective and minimizes environmental impact. The generated waste is growing at increasing rate and the municipal council is not able to provide service for solid waste collection due to the rapid increase in the population and urbanization. A large amount of the solid waste generated by the market areas during working hours produced different types of waste which is not being properly managed, and might have detrimental effects on the environment, health and ecosystem (Abdulrasoul, 2016).

Waste from pineapple cannery has been examined for ethanol production. Then by further fermentation of ethanol and with the help of *Acetobacter* species the alcohol produce can be converted into vinegar. The high increase in food deterioration is due to the contamination of food microorganism since the microorganisms colonize the entire environment in which we live. These microorganisms include bacteria, yeast, and mould. Their beneficial aspect can be noted in the production of ''vinegar'', spirit, wine and some antibiotics. Also, the pineapple waste has therapeutic application. In the tropics, pineapple is grown and used as medicinal plants. Pineapple contains the enzyme bromelain (protease) which has therapeutic properties including malignant cell growth, thrombus formation, inflammation, control of diarrhoea, dermatological and skin debridement (Tochi, 2008). Processed pineapples are consumed worldwide and processing industries are trying out or using new technologies to retain the nutritional quality of the pineapple fruit. This is to meet the demand of consumers who want healthy, nutritious and natural products. Pineapple wastes can be utilized to produce methane, animal feed, phenolics, bromelain and by fermentation ethanol and "vinegar" can be produced.

Saccharomyces cerevisiae is an eukaryotic microbe. More specifically, it is a globular-shaped, yellow-green yeast belonging to the Fungi kingdom, which includes multi-cellular organisms such as mushrooms and molds (Martini, 1993). Most often it is found in areas where fermentation can occur, such as the on the surface of fruit, storage cellars and on the equipment used during the fermentation process. It is the critical component in the fermentation process that converts sugar into alcohol, an ingredient shared in beer, wine and distilled beverages. It is also used in the baking process as a leavening agent; yeast releasing gas into their environment results in the spongy-like texture of breads and cakes (Mortimer, 2000).

The vinegars produced by this traditional system are generally considered of high quality because of their organoleptic complexity. In fact, the product quality results from (i) the raw material (wine or other substrate), (ii) the metabolism of the acetic acid bacteria, which produce some additional transformations (mostly oxidation reactions, but also ester formations) on top of the basic transformation (ethanol to acetic acid), (iii) the interaction between the vinegar and the wood from the barrels, and (iv) the aging process, which integrates all of the previously mentioned characteristics. However, the characterization of wine vinegar as a byproduct means that its production is often inadequately performed and includes many unnecessary risks (Albert, 2013).

Vinegar fermentation is essentially a two stage process, firstly the anaerobic conversion of fermentable sugars to ethanol by yeasts, usually *Saccharomyces* species, and secondly the aerobic oxidation of ethanol to acetic acid by bacteria, usually *Acetobacter* species. Acid yield improvements can be achieved using high rates aeration during continuous production**.** *Acetobacter* is a genus of acetic acid bacteria characterized by the ability to convert alcohol, C_2H_5OH, (ethanol) to acetic acid CH_3COOH, in the presence of air by oxidation. (Adams, 1999).

Vinegar was used earlier as a preservative for other fruits and vegetables. Vinegar is an inexpensive commodity, therefore economic considerations requires that a relatively low- cost raw material like pineapple peel be used in its production. All commercial vinegars

are used primary in the food processing industry. It also found to have the potent bioactive effects which may benefit human health. The therapeutic properties of vinegar include antibacterial activity, blood pressure reduction, antioxidant activity, reduction in the effects of diabetes, and prevention of cardiovascular disease. Vinegar is very important food preservative in food industry. The vinegar can be obtained various raw materials such as various fruits and fruit peels. Various agricultural waste materials can also be used as raw materials. It was observed during various investigations that enzymatic treatments of pineapple wastes had a significant effect on the saccharification process. Also review indicated that Pineapple peel vinegar had comparatively high total phenol content and antioxidant activity. Vinegar is commonly used as food ingredient but also for its medicinal properties and for its physiological effects such as invigorating, regulator of blood pressure, diabetes mellitus regulator, appetite stimulator, digestion and absorption of calcium (Bitange, 2008).

It is anticipated that discarded fruit as well as the waste material can be utilized for further industrial processes like fermentation, bioactive component extraction and production and industrial important products. This study emphasized the production of vinegar from pineapple fruit waste by *Acetobacter* spp. isolated from soil samples which has industrial importance.

MATERIAL AND METHODS

1) For Production of Wine

Isolation of *Saccharomyces cerevisiae* from wet yeast

For culture medium preparation, the wet yeast was first weighed and added to the sterile distilled water in a test tube. Then, after few minutes the suspension was used to inoculate sterilized SDA (Sabaraud Dextrose Agar) medium and it was kept for 24 hrs at 37^0C.

Identification of Bacteria and yeast

Morphological characteristics

1) Gram staining: A well isolated colony was selected by picking the colony with inoculating loop and then smear was prepared on clean slide. A staining technique was performed as per standard procedure. Shape, size and gram character was then observed.

2) Cotton blue staining

Place a drop of 70% ethanol on a clean glass slide. Immerse the specimen of yeast in the drop of alcohol. Add one or two drops of Lactophenol cotton blue stain. Place a cover slip gently avoiding air bubbles. Then slide was observed microscopically under oil immersion objective.

Collection of pineapple peels

Substrate: Pineapple was obtained from local market in Nagpur, India. The pineapple was selected according to the degree of ripeness needed at the time of purchase. Occasionally, the pineapples continued to ripen at room temperature until the proper degree of ripeness was obtained.

Preparation of the peels juice: The pineapple was washed thoroughly with water and peels were hand chopped aseptically and kept in a sterilized container. These peels were weighed and then blended thoroughly in blender.

Experimental set up for wine production

In a Erlenmyer conical flask (500ml), the blended mixture of pineapple peels weighing 44.75 g was added with 1.5 g of yeast nutrient (ammonium dihydrogen phosphate) and 200 ml of distilled water were added. Finally, a loopful of inoculum obtained after inoculation on SDA was added in the conical flask. Then, the conical flask was corked with tight cotton plug and adhesive tape was held around it and kept for 7 days for fermentation at 37^0C.

Wine production

Test for CO_2 production

After 7 days, the adhesive tape was removed very carefully and then the residue was filtered with the help of muslin cloth and then some amount of filtrate was removed separately in another conical flask and the rest was kept for vinegar production.

For the detection of presence of CO_2, prepared lime water was used. Some amount of lime water was taken out in a beaker and then the gases present in the filtrate was allowed to pass into the lime water with the help of a pipe. (Ndoye,2007).

Iodoform test

Some amount of the filtrate was taken in a test-tube. Add small amount of potassium iodide and after that small amount of calcium hypochlorite was added. Then, the tube was placed in hot water bath for few minutes until formation of yellow crystals. (Ndoye *et al.* 2007).

Production of Vinegar

Isolation of *Acetobacter* species from soil sample

Collection of soil sample: Soil sample was collected from garden of P. G. Department of Microbiology, RTM Nagpur University, Nagpur. The soil samples were at a depth of 5-10 cm.

Primary screening: For isolation of Acetobacter species, serial dilution method and spread plate technique was performed as follows:

1 gm of soil was weighed and dissolved in 10 ml of distilled water making the stock solution. 9 test tubes were taken and it was labelled as 10^{-1}, 10^{-2} upto 10^{-9}. In these 9 test tubes, 9 ml of distilled water was added. In the next step, 1 ml from the stock solution was pipette out and added in the test tube labelled as 10^{-1} . Similar procedure was carried out upto last dilution tube 10^{-9}. Then, 0.1 ml of the solution was transferred from the dilution tubes of 10^{-2}, 10^{-3}, 10^{-4} to the respective plates of Acetobacter agar. Then, the solution was spread evenly on the above plates using spreader. These plates were incubated at 37° C for 24 hours. Then, a single isolated colony was picked up and streaked with the help of sterilized inoculating needle on Acetobacter agar plates to obtain pure culture. The colonies obtained were further characterized with the help of morphological, biochemical and cultural characteristics.

Identification of *Acetobacter* species

The isolated colonies were identified on the basis of morphology by performing Gram staining and motility, biochemical by testing sugar fermentation using Glucose, Lactose, Mannitol , Maltose, Sucrose, IMViC Test, Catalase test, Oxidase test, Triple Sugar Iron (TSI) test, Urease test and cultural characteristics by inoculating bacteria on Acetobacter agar.

Production of Acetic acid by *Acetobacter* species

Preparation of culture inoculum

10 ml nutrient broth was prepared and it was sterilized. Then, after autoclaving, tube containing nutrient broth was cooled. Then, the broth was inoculated with loopful of isolated organism and was incubated at 37^{0}C for 24 hours.

Experimental set up for aerobic fermentation

The filtrate i.e. the wine produced is now inoculated with the culture inoculum and it is incubated for 11 days for aerobic fermentation at 37^{0}C.

Extraction of vinegar from fermentation medium

After 11 days, the flask was opened for extraction of vinegar. The fermented medium is filtered and then some amount of the filtrate was taken out in 10 Eppendorf tubes. And then it was centrifuged at 10,000 rpm for 10 minutes. The, the supernatant was removed out and the extract was identified as crude vinegar.

Qualitative test for acetic acid

Some amount of crude extract was taken in a test tube and sodium bicarbonate also known as baking soda was added in the test tube. Positive result indicates formation of effervescence due to formation of carbon dioxide gas which is the product of reaction of vinegar and sodium bicarbonate. Negative result indicates absence of effervescence.

Determination of amount of acetic acid (%)

The amount of acetic acid was measured using titration method and the steps are as follows:

Fill the burette with 0.1 N NaOH solution. Take 20 ml of the extract in a conical flask. Add 2-3 drops of phenolphthalein indicator and stir the extract. Titrate the extract with NaOH by adding drop by drop and simultaneously stir the extract till the color changes to pink which remains constant for 10-15 seconds. Note down the burette reading. (Bellankimath, 2017)

To calculate the number of grams of acetic acid neutralized by the sodium hydroxide

Number of grams of acetic acid = Molarity of NaOH x 1 mole of CH_3COOH x 60 g CH_3COOH x Vol. NaOH litres

Antibacterial Activity of Acetic Acid (Vinegar) By Well Plate Method:

The antibacterial activity was carried out by Kirby-baurer agar well plate diffusion method. The extract was tested against four pathogenic bacteria isolated from clinical sample. These organisms were *Escherichia coli*, *Staphylococcus aureus*, *Salmonella paratyphi* and *Pseudomonas aeruginosa*. Different concentrations of the extracts were added into the wells. The concentrations varies from 100%, 75%, 50% and 25% for each organisms. Then, the plate was incubated at 37°C for 24 hours. After incubation period, the zone was measured with the metric ruler placing it across the zone of inhibition and measuring it from one edge to the other edge of the zone. (Sossou 2009).

RESULTS AND DISCUSSION

Isolation of *Saccharomyces cerevisiae* from wet yeast

On Sabouraud Dextrose agar plates, after incubation of 24 hours creamish color colonies were obtained. Blue color observed shows the presence of chitin around the yeast by Cotton blue staining.

Wine production

Test for CO_2 production

After passing the filtrate into the prepared lime water, the lime water turned milky. This is because of the presence of CO_2 that turned the lime water milky.

Lime water is prepared by mixing distilled water with calcium hydroxide and allowing it to settle for 24 hours. The supernatant liquid that separates out is lime water. Thus, when CO_2 reacts with $Ca(OH)_2$, it results in formation of calcium carbonate which turns the lime water milky.

$$CO_2 + Ca(OH)_2 \longrightarrow CaCO_3 + H_2O$$

Test for alcohol production:

The test was performed and after addition of potassium iodide and calcium hypochlorite, the tube was kept in water bath and there were formation of yellow crystals and it was detected by pouring the solution onto whatmann's filter paper. This confirms the formation of alcohol.

Isolation of Acetobacter species from soil samples:

On Acetobacter Agar (glucose) plate, after incubation of 24 hours, creamish white colonies were obtained. These colonies were streaked on Acetobacter agar slants and from these slants, the isolates were further inoculated on nutrient agar slants.

Bellankimath *et al*. (2017) studied isolation and characterization of the indigenous Acetic acid bacteria from Western Ghats soil sample.

Identification of *Acetobacter* species:

Morphological characteristics

Pink colored gram negative short rods bacteria were observed in microscope under oil immersion. The motility test was performed by hanging drop method and the organism was found to be highly motile.

Cultural characteristics

On YPG agar plates after incubation for 24 hours at 37°C, pale yellow color colonies were obtained and the medium also turned pale yellow from pale green color. This indicates that *Acetobacter species* isolated from the soil sample are capable of producing acetic acid. Similar work were performed by these scientists. Tahsina *et al*. (2017) and Farzana *et al*. (2015) worked on screening of Acetic acid bacteria producing microorganisms from decomposed fruits for vinegar production.

Biochemical characteristics:

1) Sugar fermentation characteristics:

Table 1: Sugar fermentation characteristics of *Acetobacter* species

Bacterial isolates	Glucose		Sucrose		Mannitol		Lactose		Maltose	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
Sample	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

2) IMViC test, TSI agar test, Urease test, Catalase test, Oxidase test :

Table 2: Biochemical characteristics of *Acetobacter* species:

Sr.No	Test	Observation	Result
1.	Indole	No cherry red color ring was observed.	Negative
2.	Methyl red	No red colored complex was observed	Negative
3.	Voges-Proskauer	No brick red precipitation was observed	Negative
4.	Citrate utilization	No color change of the slant to blue color from green color	Negative
5.	Triple sugar iron (TSI) agar	No color change, no upliftment of butt and no blackening of slant	Negative
6.	Urease agar	No pink color formation	Negative
7.	Catalase	Formation of bubbles within 10 sec	Positive
8.	Oxidase	No purple color formation	Negative

Table 3: Qualitative test for acetic acid (sodium bicarbonate test)

Sample	Observation	Result	Interpretation
Crude extract	Effervescence was observed	Positive	Presence of Acetic acid

Table 4: Antibacterial activity of vinegar against tested bacteria

Sr. No.	Concentrations (%)	Diameter of Zone of inhibition (mm)			
		Escherichia coli	***Staphylococcus aureus***	***Salmonella paratyphi***	***Pseudomonas aeruginosa***
1	Vinegar(synthetic)	17mm	19mm	20mm	20mm
2	100%	16mm	18mm	19mm	19mm
3	75%	15mm	16mm	16mm	18mm
4	50%	12mm	14mm	13mm	16mm
5	25%	10mm	11mm	11mm	14mm

Vinegar production:

After addition of sodium bicarbonate to the crude extract, effervescence was observed. This indicates formation of acetic acid, because acetic acid and sodium bicarbonate when reacts results in formation of carbon dioxide which is detected by formation of effervescence.

Determination of amount of acetic acid (%):

Acetic acid (vinegar) was extracted through centrifugation process at 10,000 rpm for 10 minutes. Estimation of percentage of acetic acid was carried out by titration method. The amount of acetic acid was calculated using following formula:

To calculate the number of grams of acetic acid neutralized by the sodium hydroxide:

Number of grams of acetic acid = Molarity of NaOH x 1 mole of CH_3COOH x 60 g CH_3COOH x Vol. NaOH litres

= 0.1 × 1 × 60 × 0.02 L

= **0.12 g CH_3COOH**

To calculate the % of acetic acid in vinegar:

$$\% \text{ acetic acid} = \frac{\text{mass of acetic acid}}{\text{mass of vinegar}} \times 100$$

= (0.12 / 5.2) × 100

= 2.30% acetic acid for 200 ml

The amount of acetic acid (%) was found to be **4.60%** acetic acid.

Umaru *et al*. (2015) produced vinegar from pineapple peels using *Acetobacter* species and % of acetic acid estimated was 3% and Farzana *et al.* (2015) studied screening of Acetic acid bacteria producing microorganisms from decomposed fruits for vinegar production estimated that % acetic acid was 3% supported the present investigation. Tharinee *et al*. (2015) worked on isolation and characterization of acetic acid bacteria from fruits and fermented fruit juices for vinegar production estimated 3.70% of acetic acid which is close to the observation of the present study. Wang (2006) also produced vinegar from ripe pineapple and percentage of acetic acid estimated was 4.77% which may be because of difference in experimental procedure.

Antibacterial Activity of Acetic Acid (Vinegar) By Well Plate Method:

The crude extract which was obtained by the process of centrifugation was tested for antibacterial activity against four pathogenic bacteria isolated from clinical sample by agar well plate diffusion method. The microorganisms used are *Escherichia coli*, *Staphylococcus aureus*, *Salmonella paratyphi* and *Pseudomonas aeruginosa*. The results for zone of inhibition obtained for four different concentrations i.e, 100%, 75%, 50% and 25% and also for purchased vinegar against above our pathogenic bacteria are shown in table 4.

Also, in the present study antibacterial activity of vinegar was performed by agar well plate diffusion method against four pathogenic bacteria i.e, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella paratyphi* and *Pseudomonas aeruginosa.* This study was to show the activity of vinegar produced on both gram positive and gram negative bacteria. Different concentrations of the crude extract were prepared and tested for antibacterial activity. The concentrations prepared were 100%, 75%, 50% and 25% and the one was control which was synthetic vinegar. The zone of inhibition were ranked

according to 2.30% of the acetic acid of vinegar produced. For *Escherichia coli,* the diameter of zone of inhibition were 100% (16mm) , 75% (15mm), 50% (12mm), 25% (10mm) and control (17mm) respectively. Comparing with the zone of inhibition of control the best results were shown by 100% and 75% concentrations. For *Staphylococcus aureus*, the diameter of zone of inhibition were 100% (20mm) , 75% (19mm), 50% (14mm), 25% (11mm) and control (19mm). Comparing with the zone of inhibition of control the best results were shown by 100% and 75% concentrations.

Figure 1

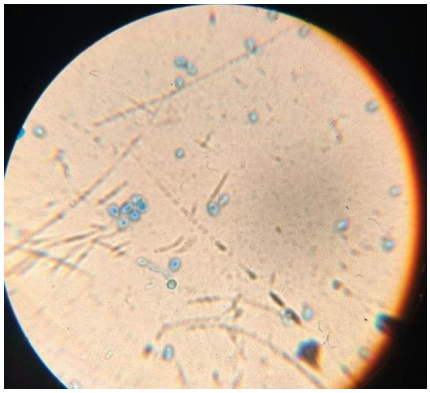


Figure 2

Figure 1: Cultural characteristics of *Saccharomyces cerevisiae* on Sabouraud Dextrose Agar
Figure 2: Morphological characteristics of *Saccharomyces cerevisiae* (Cotton blue staining)

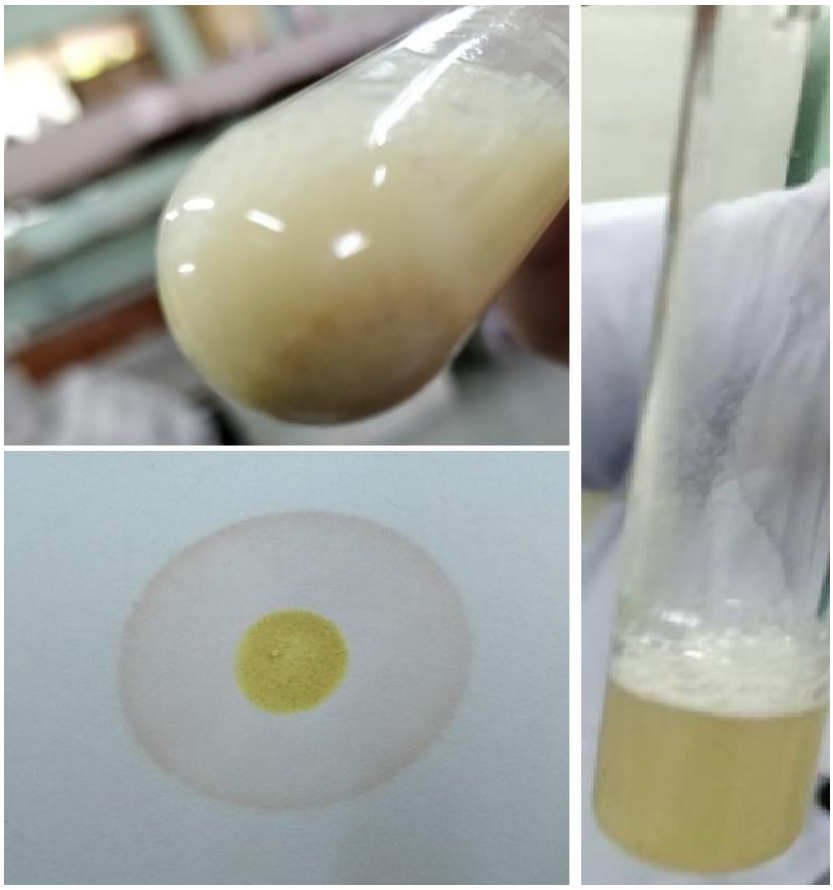
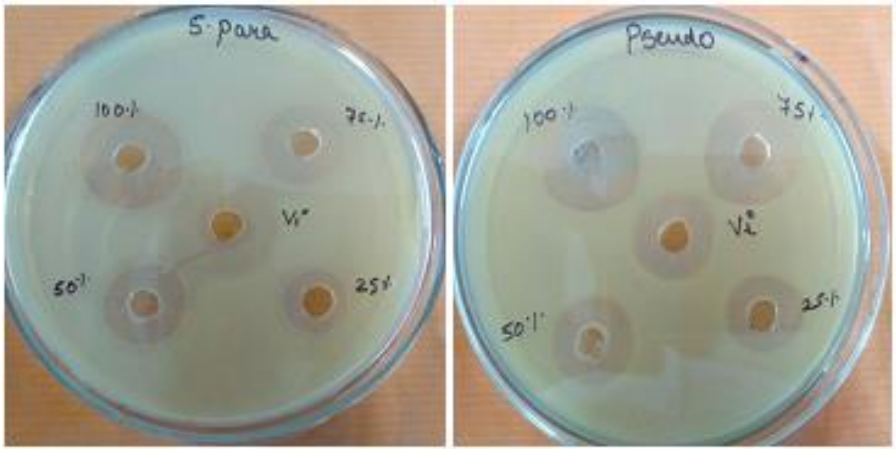


Figure 3: Alcohol production by Iodoform test and Qualitative test for acetic acid (presence of effervescence**)**

(a) **(b)**

Figure 4: Antibacterial activity of vinegar (acetic acid) against a) *E.coli & b) S. aureus*



(a) **(b)**

Figure 5: Antibacterial activity of vinegar (acetic acid) against a) *Salmonella paratyphi & b)Pseudomonas aeruginosa*

For *Salmonella paratyphi*, the diameter of zone of inhibition were 100% (19mm), 75% (16mm), 50% (13mm), 25% (11mm) and control (20mm). Comparing with the zone of inhibition of control the best result was shown by 100% concentration. For *Pseudomonas aeruginosa*, the diameter of zone of inhibition were 100% (19mm), 75% (18mm), 50% (16mm), 25% (14mm) and control (20mm). Comparing with the zone of inhibition of control the best result was shown by 100%. Therefore, it can be interpreted that the vinegar produced is effective against the above four pathogenic bacteria and can be used as antibacterial agent.

Mufeed *et al.* (2013) also done similar kind of work on the ability of 2-(2-hydroxy phenylimono) to inhibit growth of different types of pathogenic bacteria was investigated. It was found that this chemical compound have the ability to inhibit the growth of *E. coli, Staphylococcus aureus and Streptococcus agalactiae,* and the best inhibitory concentration was determined as 70 mM. The mean optical density OD750 reading of *E. coli, S. aureus and St. agalactiae* were subjected to [2-(2- HP-AA)] in different concentrations.The presence of this compound in the growth of *K. pneumoniae, P. mirabilis, St mutaris, St. pneumoniae* ,did not cause substantial inhibition of growth. However {2-(2- HP-AA)} nearly completely inhibited growth of *E. coli , S. aureus and St. agalactiae*. 40 mM and 50 mM did not show an inhibitory effect on all bacterial isolates. While 70 mM considered as minimum inhibitory concentration of [2-(2- HP-AA)].

Asma (2017) studied antimicrobial activity of apple cider vinegar (ACV). The results of the anti-microbial efficacy ACV are for *S. aureus* 31.60±0.548, for *E. coli* 26.60±0.548, for *S. paratyphi* 15.80±0.447 and results for standard ciprofloxacin are 21.40±0.894, 20.40±0.894 and 21.40±0.548 respectively. ACV exhibit potent

antibacterial activity against Gram positive and Gram negative bacterial strains. whereas sensitivity compared to reference standard Ciprofloxacin is less. As far as the Gram negative organisms are concerned, ACV showed less activity against *Salmonella paratyphi*. The vinegar had characteristic pineapple bouquet, a harsh taste and a light yellow colour implying that pineapple peel which conventionally could be regarded as waste, can be converted into value-added commodity, thus, facilitating environmental safety.

CONCLUSION

The present study successfully achieved vinegar production from pineapple peels. Alcoholic fermentation within one week with *Saccharomyces cerevisae* and acetous fermentation during 12 days using two *Acetobacter* spp isolated from soil samples. 4.60% of acetic acid degree vinegars were yielded with 90.9% fermentation efficiency for alcoholic and acetous fermentations respectively. The semi-continuous fermentation method of Pineapple peels wine was well experimented and consequently, the acetic strains are really empowered for industrial vinegar production.

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