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Fruit peels: A potential substrate for acetic acid production using *Acetobacter aceti*

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Abstract
Acetic acid is known to be ethanoic acid. When free of water molecule it is termed as glacial acetic acid. In this study, fruit peels are used to generate acetic acid using *Acetobacter aceti*. Banana, screened from different fruit peel produces maximum acetic acid with the media components of medium 3, best medium for acetic acid production. Banana hydrolysate with oats and coconut oil cake as carbon and nitrogen source respectively compiles a perfect media for the production of acetic acid. The produced acetic acid was confirmed with GC/MS analysis. The acetic acid produced showed effective antibacterial activity against *Bacillus sps.* with zone of inhibition of 21 mm with 20µg/ml concentration and showed effective antioxidant activity.

Keywords: Acetic acid, *Acetobacter aceti*, fruit peels, Banana, antibacterial activity, GC/MS

1. Introduction
Organic compounds with acidic properties are termed as organic acids which possess a single carbon atom. They have vast usage in food industries such as vinegar in fermentation process. Vinegar can be produced by the transformation of ethanol to acetic acid involving a particular group of bacteria held by the genus *Acetobacter and Gluconobacter.* It is a traditional method in cooking and used as food preservatives. During continuous fermentation, the yield of acetic acid can be achieved with aeration in high rates from fermented sugars with other sugar metabolites which is either converted into other compound or lost its volatilization (Ghommidh *et al*, 1986) [1]. It has noteworthy attention towards potential applications in soft drink bottles, Photographic films, Synthetic fibres, Fabrics, Desiccating agents, Acidity regulators, Condiment, Medicinal applications. It plays an notable role in hot sauce, salad dressings, ketchup, and other sauces which demands industrial fermentation system that should be well maintained for its performance and should provide perfect abode for the growth of acetic acid bacteria (Ory *et al.,* 1999) [2]. Techniques have developed for massive production by increasing the transfiguration of ethanol into acetic acid with the existence of acetic acid bacteria (Tesfaye *et al*, 2002) [3]. Most often, submerged culturing technique is employed (Hormatka and Ebner, 1951) [4]. AAB occurs in substrates such as beer, wine, cider etc., which are oxidized into organic acids. If the ethanol presence is confirm in the substrate, the acid produced is acetic acid. There are three different biotechnological processes employed in producing vinegar (Greenshields, 1978) [5]: the Orleans method, the German method and the submerged method. The need for vinegar hires the industrial fermentation of acetic acid production (Ory *et al* 1999) [2]. Fruit and vegetable peels can be utilized as raw materials in the production which is economical and helps in reduced allergens to the society. They have appreciable amount of carbohydrates which could be utilized by the microorganisms producing organic acids.

2. Materials and methods
2.1. Collection of raw material for fermentation
A total of five different fruit waste substrates (orange, banana, mango, pomegranate and papaya) were collected from the fruit market at Coimbatore and screened for the amount of total sugar content by preparing their hydrolysate and used for Acetic acid production by *Acetobacter aceti*.

2.3. Analysis of nutritive parameters in fruit peels
Moisture content and ash content of different fruit peels were analyzed using standard A.P.H.A, 2005 protocol. Lipid content and crude fiber content of the fruit peels were analyzed using A.O.A.C, 2005 method and the Protein content was assessed using Lowry's method. The total sugar content after hydrolysate preparation was done using Anthrone method (Hedge *et al.,* 1962) [6]. The reducing sugar content post acid hydrolysis was analyzed using DNS method (Miller, 1959) [7].

2.4. Inoculum preparation
The lyophilized culture was activated by sub-culturing in liquid synthetic YPM medium and different test hydrolysates separately. The culture media containing hydrolysates were prepared by adding components of synthetic medium (Hesham *et al.,* 2011) [8] (Yeast extract 0.5g, Peptone 0.3g, Mannitol 2.5g for 100 ml YPM medium)in100mlofeachhydrolysate instead of distilled water. These media were kept for incubation at ambient temperature on rotary shaker at 120 rpm for 2days. This was used as inoculum for further studies.

2.5. Screening of media for acetic acid production
Six different media were used in this study for primary evaluation for primary selection of the highly productive medium. All these media were reported before for their ability to support cell growth and acetic acid production by *A. aceti.* The composition of these media were as follows in (g/L): Medium (1): Glucose, 10; K_2HPO_4, 0.1; KH_2PO_4, 0.9; $(NH_4)_2SO_4$,1.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.01; $FeSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.01; Yeast extract, 10;0.1M Citric acid, 50 ml, pH 5.0 14; Medium (2): Yeast extract, 5; Peptone, 2; Glycerol, 30,pH 6.3; Medium (3): Yeast extract, 5; Peptone, 2; Glucose, 30. pH 6.3; Medium (4):Ethanol, 47.4; Glucose, 1; Peptone, 2; Yeast extract, 5; Acetic acid, 10. pH 6.3 16; Medium(5): Glucose, 2; Yeast extract, 3; Polypeptone, 2.; Glycerol, 3, pH 6.5 ; Medium (6):Glucose, 100; Yeast extract, 3; Polypeptone, 2; Glycerol, 3 at pH 6.5. The carbon source ofeach medium was sterilized separately and added to the fermentation medium before inoculation. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 28 °C.

2.6. Preparation of hydrolysates for fermentation
2.6.1. Steam explosion
The modified method of Pumiput*et al.,* (2008) [9] was used for substrate hydrolysate preparation. About 8gram of dried powder of fruit peels was steam exploded in an autoclave at 121 °C for 20min.Sterile water was added to the wet pretreated material to make the volume of 200ml and boiled at 80 °C for 30 min. Later the hydrolysate was recovered by filtration with cheese cloth.

2.6.2. Acid hydrolysis
Acid post hydrolysis of hydrolysate was carried out to cleave the oligosaccharides in to monomeric sugars by autoclaving at 121 °C with concentration of 1% HCl v/v for 30min (Pumiput, 2008) [9].

2.6.3. pH adjustment
The hydrolysate from acid post hydrolysis was adjusted with NaOH to pH 6-6.8 and the precipitate was removed by filtration with Whatmann filter paper No.1 (Pumiput, 2008) [9].

2.7. Estimation of acetic acid production
The fermentation broth with produced acetic acid un wavered by transferring 25 ml of *Acetobacter aceti* broth culture in to 100ml flask and appended with 1ml phenolphthalein as indicator. The titration with 1N NaOH against it appraise of the amount of acetic acid (g/L) produced with the appearance of pink colour and it was calculated using the formula:

$$\text{Amount of acetic acid (g/L)} = \frac{\text{Normality}_{\text{NaOH}} \times \text{Vol}_{\text{NaOH}} \times \text{Eq.Wt}_{\text{Acetic acid}}}{\text{Volume of sample.}}$$

2.8. Optimization of acetic acid production
The optimization of media and the fermentation condition for Acetic acid production was done by physical optimization method. The production based on pH was carried out with varying pH and was adjusted using 0.1N HCl and 0.1N NaOH. On the basis of varying temperatures, the production of acetic acid was studied and estimated. The incubation period and the NaCl concentration was noticed for the maximum production of acetic acid. The cheap carbon and nitrogen source were screened with the optimized conditions. The acetic acid produced was estimated by titration against standardized 1N NaOH using phenolphthalein as indicator.

2.9. Mass production and estimation of acetic acid
With the optimized conditions, the media was prepared and inoculated with starter culture and incubated on a shaker (180rpm) at 37 ^{0}C for 5 days. The production of acetic acid was observed with titration against 1N NaOH using phenolphthalein.

2.10. Calcium acetate precipitation
The fermentation broth was subjected to centrifugation to avoid the presence of viable cells and media components after the time of fermentation. Equal volume of $CaCl_2$ was added to 10ml of the supernatant and the precipitated calcium acetate can be collected by filtration using Whattman filter paper, dried and stored.

2.11. Analysis of antibacterial activity
The well diffusion method was performed pouring 20ml of nutrient agar in petriplates and allowed to set which was inoculated with broth culture of bacterial species of common gram positive and gram negative (Jack *et al*.,1995) [10] in a uniform manner. Then, well was punctured in the solidified media. After fermentation period, 5ml of centrifuged fermentation broth was transferred to free tubes and made into different dilution concentrations, were loaded on to the wells. Distilled water was used as control. The plated were incubated at 37 ^{0}C for 24 hrs. The diameter of the zone was measured after incubation.

2.12. Total antioxidant activity
The total antioxidant activity was determined by phosphomolybdenum method after acetic acid fermentation (Preito *et al*., 1999) [11] 1ml of sample was mixed with 2ml of reagent solution [0.6 M sulfuric acid: 28mM sodium phosphate: 4mM ammonium molybdate (1: 1: 1 v/v/v)] was incubated in a water bath at 95 ^{0}C for 90 mins. Absorbance was measured at 695 nm. Total antioxidant activity (µg/ml) was expressed as that of ascorbic acid equivalents in the cell free supernatant.

2.13. DPPH radical scavenging activity

The DPPH radical scavenging activity (Szabo *et al* 2007) [12] states the effect of cell free supernatant. Mixture of sample (1ml) and 0.1mM methanol solution (5ml) was vortxed followed by incubation at 27 ^{0}C for 20 mins. O.1mM methanol solution of DPPH served as control, methanol as blank and absorbance was measured at 517nm. The radical scavenging activity was calculated by

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]}{(\text{Abs}_{\text{control}})} \times 100$$

3. Results and discussion

3.1. Analysis of nutritive parameters

Table 1: Analysis of physical parameters

Fruit Peel Type	Moisture	Total sugars	Reducing sugars	Ash content	Crude fat	Crude protein	Crude fibre
Banana	6.13	53.32	10.98	8.52	1.62	0.98	31.03
Orange	11.19	22.4	5.12	2.08	3.12	1.48	11.08
Mango	54.3	14.5	4.2	1.08	2.73	1.18	1.32
Papaya	10.02	15.43	2.10	14.03	1.20	13.13	1.62
Pomegranate	74.3	13.9	4.92	5.43	2.16	3.80	11.84

Moisture content is the loss in weight of water when heated under specified condition. The highest moisture content was observed in pomegranate (74.3%) whereas, the least moisture content was found in banana (6.13%). Total ash is the inorganic residual remaining on incineration in a muffle furnance. This reflects the quantity of mineral matter present in the fruit peels. The highest ash content was present in papaya (14.03%) whereas, the lowest ash content was present in mango (1.08%). The protein content in the peel was determined using Lowry's method which employs intensity of colour change for quantification of crude protein. The highest protein content was found in papaya (13.13%) and the lowest protein content in banana (0.98%). Petroleum ether was used to extract the total lipids present in various fruit peels. The highest and the lowest lipid content were observed in orange and papaya as (3.12%) and (1.20%) respectively. Crude fibre consists largely of cellulose and lignin along with some mineral matter. During the acid and subsequent alkaline treatment, oxidative hydrolytic degradation of native cellulose and lignin occurs. The highest crude fibre was observed in banana (31.03%) and the lowest crude fibre was observed in mango (1.32%). The total sugar content in the fruit peels were analysed using anthrone reagent. The high level of total sugar content was determined in banana (53.32%) and the least amount of total sugar content was determined in pomegranate (13.9%). The reducing sugar content was estimated using DNSA method which involves the oxidation of aldehyde and ketone functinal groups present with the simultaneous reduction of DNS to 3- amino, 5-nitro salicylic acid under alkaline condition. The reducing sugar content was high in banana (10.98%) and low in papaya (2.10%).

3.2. Screening of best media for acetic acid production

Table 2: Different screening media for acetic acid production

Different Media	1st day	2nd day	3rd day	4th day	5th day
Medium 1	0.212	0.347	0.703	0.606	0.545
Medium 2	0.378	0.381	0.562	0.431	0.352
Medium 3	0.789	1.113	1.432	1.023	0.981
Medium 4	0.203	0.321	0.467	0.451	0.398
Medium 5	0.316	0.392	0.576	0.423	0.351
Medium 6	0.321	0.765	0.893	0.798	0.712

Medium 3. (Lasko DR *et al*., 2000) [13] was found to be the best medium for maximizing the production of acetic acid bacteria amount of acetic acid was produced in medium 3 by *Acetobacter aceti* during 3rd day of incubation. Thus, medium 3 composition was selected for further studies.

3.3. Estimation of acetic acid production

The acetic acid generated in the fruit peel was estimated by titration of the supernatant of fermented broth against 1N NaOH. The end point was noticed with the pink colour appearance due to phenolphthalein indicator. The maximum amount of acetic acid was generated by banana (2.41 g/L) on the 3rd day of fermentation.

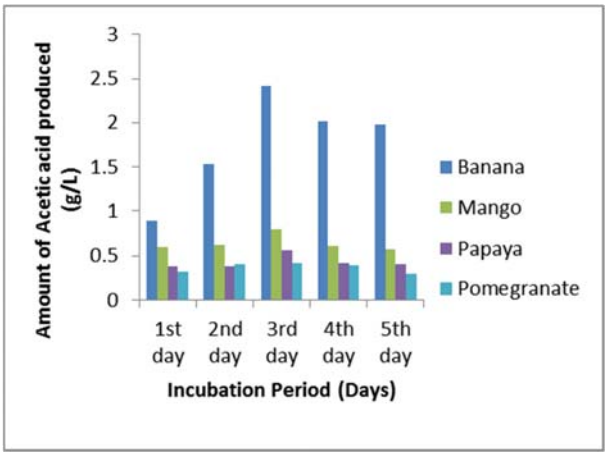
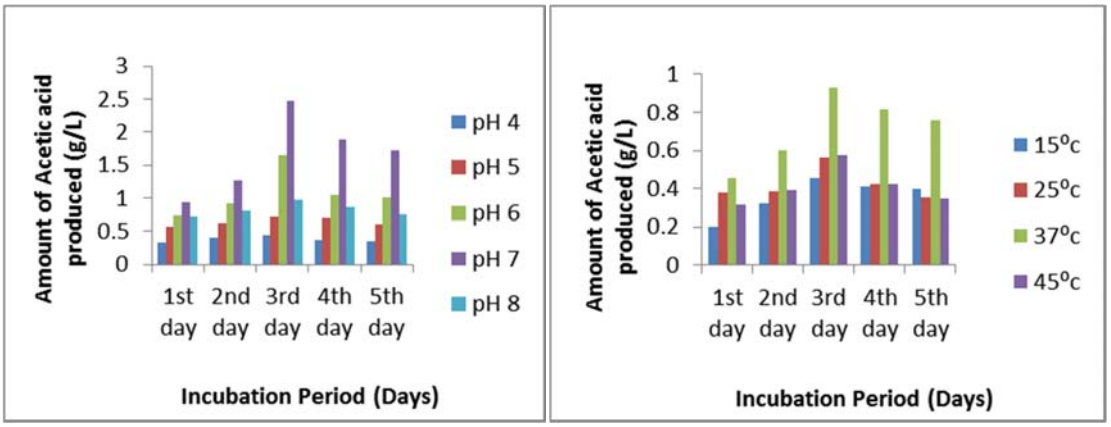


Fig 1: Estimation of acetic acid

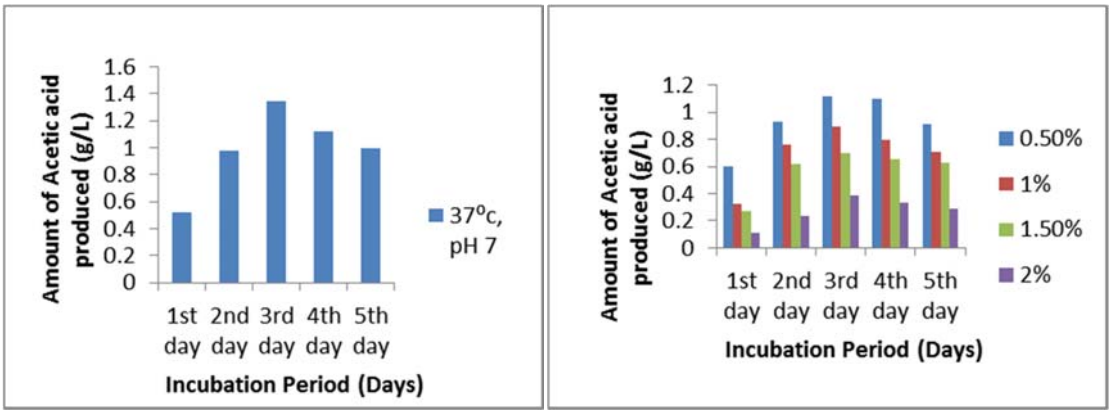
3.4. Optimization of ideal substrate for acetic acid production

The acetic acid bacteria in optimized conditions can able to utilize the carbohydrate in the fruit peel and generate high yield of acetic acid at pH 7.0, temperature at 37 ^{0}C, 0.5% NaCl concentration. The oats and coconut oil cake are cheap substrates utilised as carbon and nitrogen source which yield high in acetic acid production.



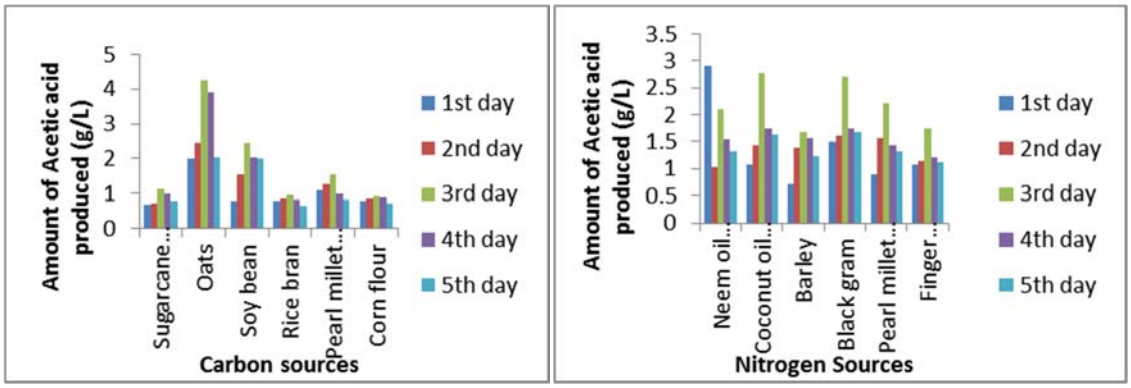
a. pH optimization

b. Temperature optimization



c. Optimization of incubation period

d. Optimization of NaCl concentration



e. Carbon sources

f. Nitrogen sources

Fig 2: (a-f) Optimization conditions

3.5. Mass production and estimation of acetic acid

The acetic acid generated before and after optimized conditions was plotted on the graph. By implying optimized conditions, the maximum acetic acid was found to be 5.11g/L on the 3rd day of fermentation. After 3rd day due to decline in the reducing sugar content, the acetic acid production decreases.

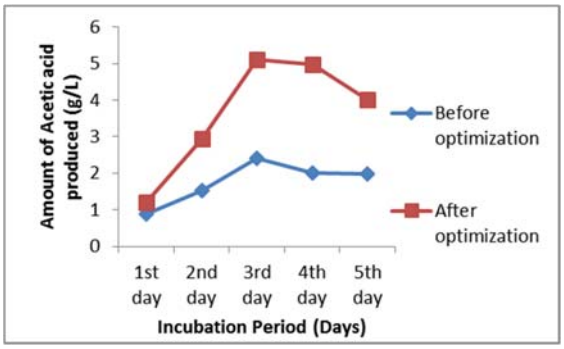


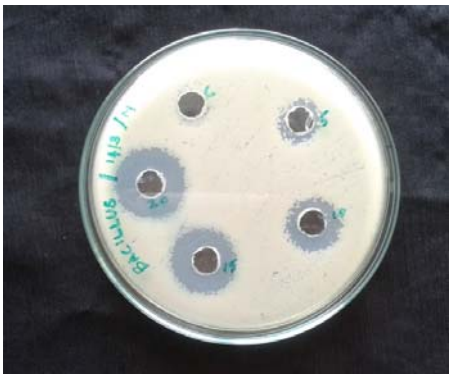
Fig 3: Mass production of acetic acid

3.6. Antibacterial activity

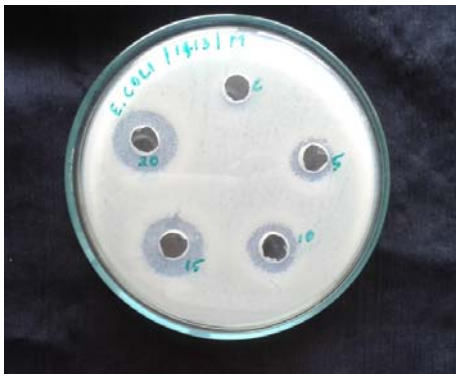
Table 3: Antibacterial activity

Zone of inhibition (mm)				
Bacterial sps.	**5(µg/ml)**	**10(µg/ml)**	**15(µg/ml)**	**20(µg/ml)**
Bacillus sps.	4	8	11	21
E. coli	9	12	16	19
Klebsiella sps.	0	5	9	14
Proteus sps.	0	0	5	10
Pseudomonas aeruginosa	0	4	8	11
Staphylococcus aureus	6	8	12	18

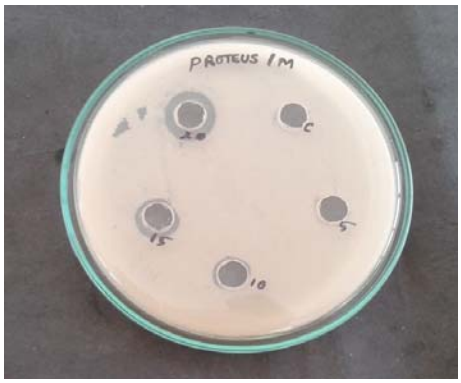
The highest zone of inhibition (21mm) was recorded against *Bacillus sps*. With 20µl concentration followed by *E.coli* (19mm) and *Staphylococcus aureus* (18mm) respectively. The cell free supernatant doesn't shows antagonistic activity against *Klebsiella sps., Proteus sps.,* and *Pseudomonas sps.* in the lowest concentration (5µl). Moderate levels of zone of inhibitions were observed against these pathogens as concentration increases.



a) *Bacillus sps.*



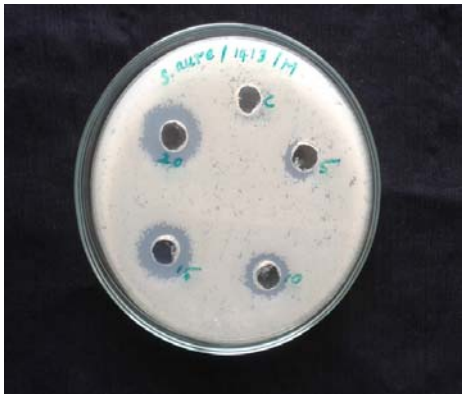
b) *E. coli*



c) *Proteussps.*

d) *Klebseilla sps.*

e) *Pseudomonas sps.*



f) *Staphylococcus aureus*

Fig 4: (a-f) antibacterial activity

3.7. Analysis of antioxidant activity

3.7.1. Total antioxidant activity

The total antioxidant activity of acetic acid produced was evaluated by phosho-molybdate method. The phosho-molybdate method has been routinely used to evaluate the total antioxidant capacity. In the presence of antioxidant compound the MO(VI) is reduced to MO(V) and forms a green coloured phosho-molybdenum complex which shows maximum absorbance at 695nm. The total antioxidant activity of the produced acetic acid was found to be 11.2µg/ml.

3.7.2DPPH radical scavenging activity

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The DPPH radical scavenging activity has been widely used as model system to investigate the scavenging activity of natural components (Yokozawa *et al*,. 1998) [14]. The reduction capability of DPPH radical is determined by decrease in its absorbance at 517nm as a discoloration from purple to yellow.

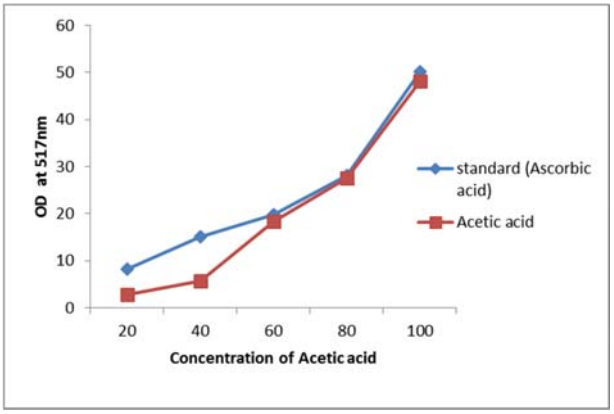


Fig 5: DPPH radical scavenging activity

4. Conclusion

In conclusion, eco-friendly methods of waste management could be effectively tuned for the production of commercially important bio products at reduced cost for the benefits of society.

5. Acknowledgement

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