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Research Article

GREEN CONVERSION OF WASTE FRUIT RINDS IN TO BIOETHANOL

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ABSTRACT

Biomass is a major energy source accounting for 10-14% of the world's energy supply. The fruit rinds are major biomass contains carbohydrates that are the ideal raw material for conversion into bio fuels majorly ethanol. The hydrolysed monosaccharide's after physical and enzymatic methods were converted to alcohol by the process of fermentation.

The bacteria which were used for saccharification were enumerated by physical methods involving the study of morphology, colony characteristics and staining techniques followed by the biological methods such as biochemical analysis and cellulose degrading activity and also molecular methods involving the sequencing of the 16srRNA of the bacteria. The bacteria which were used for saccharification i.e. H1 isolated from horse dung was identified as *Bacillus circulans* and C3 isolated from cow dung was identified as *Bacillus subtilis*, the yeast isolated from musk melon (G7) was identified as *Saccharomyces cerevisiae* by 18s rRNA sequencing, where G1 was the standard culture *Cellulomonas fimi*.

The saccharification process thus aided the fermentation process to occur more efficiently and thus yield more ethanol. Since the amount of utilizable sugars was high in the fruit rinds of *Carica papaya*yielded more amount of ethanol with*Bacillus subtilis* (60±2.04), followed by *Psidium guajava*with *Bacillus subtilis* (53.75±1.02) and then *Solanum lycopersicum* with *Cellulomonas fimi* (43.75±1.02).

The FTIR analysis of the *Carica papaya*sample saccharified with *Bacillus subtilis*showed the most characteristic peak in bio ethanol spectrum is peak one at 3429.49 cm^{-1} that lies between 3200-3550cm^{-1} which relates to alcohol (O-H) vibrations.

Keywords: Fruit rinds, Pre-treatment, Fermentation, Bioethanol

1. INTRODUCTION

Fossil fuels immensely contribute to environmental pollution and enhance greenhouse gas emission leading to depletion of ozone layer [1], hence biofuels are high priority alternative energy source because of rapid depletion of fossil fuel which instigated an attention to produce bio fuels from renewable resources [2] and increasing concern for the security of the oil supply has been evidenced by increasing in the oil price [3] therefore there is a great interest in exploring alternative energy source [4], where biofuels can derived from industrial and municipal waste and forestry / agricultural residues [5].

In recent years, the increased demand on food crops for fuel applications has resulted in concern about food scarcity, one possible alternative that has been a focus of extensive research is the use of terrestrial lignocellulosic biomass as raw materials for biofuel production. India alone generates over 400 million tons biomass every year [6]. These biomass sources are advantageous because of low cost, minimal land use change and avoidance of the competition between food and fuel which are the good source of raw materials in bio ethanol production [7, 8]. Recent reviews stated that different bioreactors used for the conversion of different lignocellulosic biomass including fruit wastes to obtain bio ethanol.

The production of ethanol from cheaper source of raw materials using efficient fermentative microorganism is the only possible way to meet the great demand for ethanol in the present situation of energy crisis [9]. Bio ethanol is being considered as a potential liquid fuel due to limited amount of natural resources [10] and recognize worldwide as an alternative to petroleum-based transport fuels, used mainly in blends with gasoline, where [11] reported that fruit wastes could be exploited as a potential source of bio ethanol.

Fruits are used in large scale by pulp and jam industries, in urban areas, the fruit waste generated by fruit juice vendors and restaurants is a considerable portion of solid waste where they usually discard the inedible parts of the fruits called rinds and in most cases unhygienic conditions developed because these rinds are dumped in landfills.

However, utilization of these in production of bio ethanol would be of great environmental and economic benefit as it could reduce the burden on conventional sources of energy and also get rid of the wastes [12, 13] where hydrolysing enzymes ferment the complex sugars to reducing sugars and then to ethanol. Vaitheki S and co-workers [14] reported that water adsorption with cellulose-based adsorbent could be an economical technique to produce anhydrous ethanol in country with large area of banana plantation which contain 5.40% of ethanol and Gosavi et al. [15] reported that after fermentation, pineapple waste produced 0.090%, sweet potato waste produced 0.079%, Indian water chestnut waste produced 0.045% and jackfruit waste produced 0.045% ethanol.

Karnataka is one of the most progressive states with great potential for horticultural crops; the geographical area is 190.50 lakh hectares of which an area of 104.89 lakh hectare comes under the cultivable area. Out of the total cultivable area 15.84 lakh hectares is covered under horticulture and out of this 2.57 lakh hectare come under fruits. The total fruits production was 40.29 lakh tonnes per year and the major fruits are banana, chikoo, custard apple, grapes, guava, jackfruit, mango, mosambi, orange, papaya, pineapple, pomegranate, water melon, etc., this would help to understand urgently the need of using the rinds generated by these fruit to produce bio ethanol.

The microorganisms of primary interest in fermentation of ethanol include *Saccharomyces cerevisiae* (ferment hexoses), *Pichia stipites* (ferment xylose), *Schwanniomyces alluvius* (hydrolyse starch) and *Kluyueromyces* yeast species (ferment lactose) [7].

The herbivorous grow with lignocellulose as main energy source, where only 10-35% crude fiber can be used; other was carried out with feces as undigested cellulose which was still high and fermentable. The microbes found in cecum and colon of herbivorous due to their colonized with undigested crude fiber in rumen lead to conduct isolation for collecting lignocellulolytic bacteria that has high ability to improve crude fiber degradation [16]. Cellulase is the enzyme that hydrolyzes the β 1, 4-glycosidic bonds in the polymer to release glucose units which is multi-enzyme system composed numerous isozymes act as synergy [17].

Before fermentation it is needed to soften the biomass and break down its structure to make it more susceptible to an enzymatic attack [18] which produces a complex mixture of ethanol and by products, from which the ethanol is isolated by distillation and the purity and international standards limit decides the performance of the ethanol as a fuel and specify the test methods to be used i.e. FT-IR [19] and during bio ethanol production culture conditions play significant role [20].

In consonance with the above raised issues and concerns, in the present study an attempt is made to produce bio-ethanol from different fruit rinds with isolated microorganism. This would also help in the fruit waste management and this would ultimately pave the way towards the establishment of bio economy.

2. MATERIAL AND METHODS

2.1. Collection and processing of substrate

Fruit rinds of *Solanum lycopersicum,Carica papaya*and *Psidium guajava*were collected in sterile container from Mother Dairy, Khajisonenahalli village, Whitefield-Hosakotehighway, Bengaluru, Karnataka, India. The samples were dried in hot oven at 65°C for 48 hours, then mechanically converted into powdered form and sieved to obtain average particle sizes of 5 micron. To 100g of each sample 100ml of distilled water was added to remove extractives and residues left was washed with distilled water.

2.2. Recovery of microorganism

Pure cultures of the organisms were obtained by quadrant streaking method on YEPD and LB agar medium and grown on YEPD and LB broth. Glycerol stocks were also prepared and maintained according to the methods described by Homer et al., [21]. The yeast species (G7) isolated from musk melon for the efficient ethanol production was collected from Prof. Harinikumar, University of Agricultural Sciences, Bengaluru.

2.3. Gram staining

Gram staining was performed according to the method described by Hans Christian et al. [22].

2.4. Scanning electron microscopic study

The SEM analysis of isolates was done according to the method described by Petra et al. [23].

2.5. Cellulose degrading activity (cmc)

The cellulose degrading assay was performed according to the method of Vashisthemraj [24] to know the zone of clearance and cellulolytic index of the isolates.

2.6. Biochemical tests

The biochemical tests like indole test, MR-VP test, citrate utilization test and catalase test were carried out [24].

2.7. Pre-treatment methods

The fruit rind samples were subjected to physical [25] and enzymatic pre-treatment [26] methods for hydrolysis of cellulose.

a. Physical method: 100g of powdered samples were soaked in 100ml of distilled water and autoclaved.

b. Enzymatic hydrolysis: After the physical pre-treatment the samples were further subjected to enzymatic hydrolysis. The powdered (5 micron particle size) substrates were inoculated with *Cellulomonas fimi* (G1), bacterial isolates of cow dung (C3) and horse dung (H1) were inoculated and incubated at 37°C for 3 days.

2.8. Estimation of reducing sugar, total sugar and cellulose

Reducing sugar estimation was done according to method of Miller at al., [27], total sugar estimation was done by methods as described by Dubois and Updegroff [28, 29] protocol was used for cellulose estimation.

2.9. Fermentative production of bio ethanol

Fermentation experiments were carried out in 250 mL conical flask. Flasks of each cultivar were inoculated with the yeast isolated from Muskmelon (G-7) and allowed to ferment for 3 days at 30°C.

2.10. Estimation of saccharified samples

DNS method was carried out to estimate the reducing sugar [27]. The total sugar content were determined as described by Dubois [28], cellulose content in biomass was determined by Anthrone method [29] and alcohol estimation was carried out by potassium dichromate method [30].

2.11. Molecular identification of isolated micro-organisms

The organism isolated from cow dung (C3), horse dung (H1) and muskmelon (G7) were sequenced according to Williams [30] protocol.

2.12. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of triplicate experiments.

3. RESULTS

3.1. Revival of organisms

The pure cultures of the rejuvenated cells *Cellulomonas fimi* (Figure 1A), cow dung isolates (Figure 1B), horse dung isolates (Figure 1C) and musk melon isolate (Figure 1D).

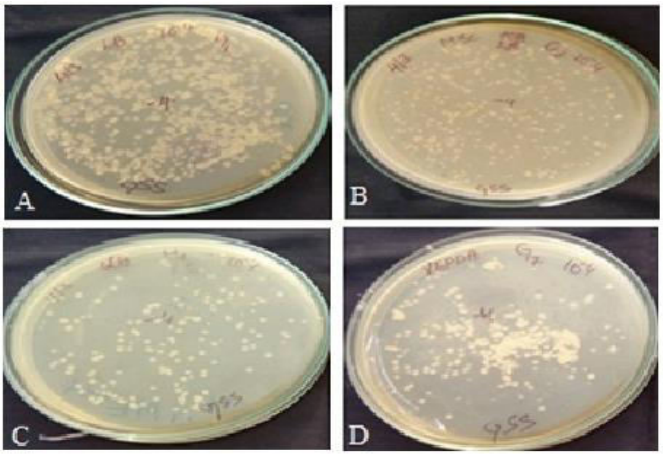

Fig. 1: The pure cultures of isolates A) Cellulomonas fimi B) cow dung isolate C) horse dung isolate D) musk melon isolate

3.2. Gram staining

The isolated bacteria were observed as gram positive rods G1 (Figure 2A), C3 (Figure 2B), H1 (Figure 2C) and G-7 (Figure 2D).

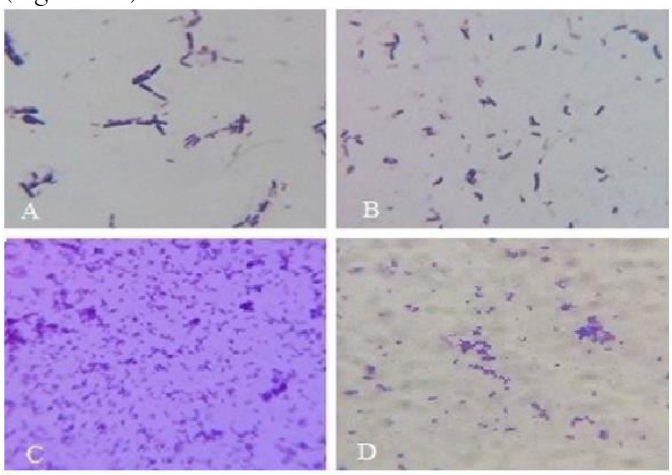

Fig. 2: Gram stained culture of A) *Cellulomonas fimi* B) cow dung isolates C) horse dung isolate D) musk melon isolate.

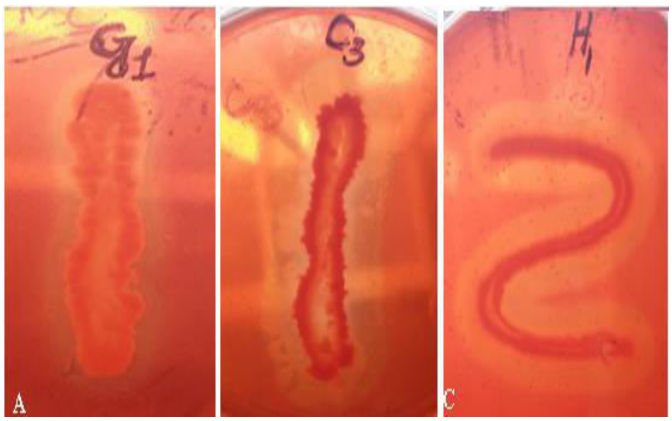

Fig. 3: Cellulose degrading activity of A) *Cellulomonas fimi* B) cow dung isolate C) horse dung isolate

3.3. Screening for cellulose degrading bacteria

The plates were checked for zone of clearance. G1 plate of *C. fimi* (Figure 3A), C3 plate of cow dung isolate (Figure 3B) and H1 plate of Horse dung isolate (Figure 3C) showed maximum clearing zone and the cellulolytic index was 13, 24 and 14mm and the hydrolytic capacity of organisms was 0.86, 3.00and 3.66 mm respectively (Table 1).

Table 1. Hydrolytic capacity (HC) and cellulolytic index of bacterial isolates.

Organism No	Colony diameter (mm)	Maximum clearing zone (mm)	Maximum HC value	Cellulolytic index
G1	7	13	6	0.86
C3	6	24	18	3.00
H1	3	14	11	3.66

3.4. Scanning electron microscopy (SEM)

The SEM of G7 showed chain like cells without clear boundary between each cell (Figure 4A). The Isolate of C3 had uniformly smooth spore coats with gently flowing ridges and did not exhibit an exosporium or appendages (Figure 4B). The H1 isolate was found to be slightly convex, with irregular margins (Figure 4C).

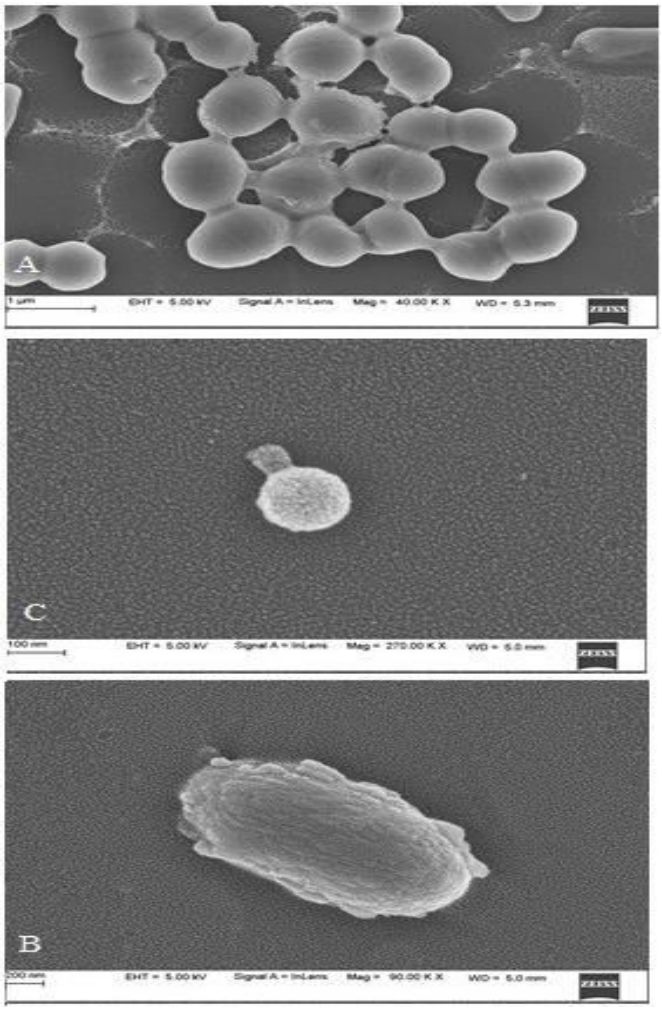

Fig. 4: SEM images of the organism isolated from A) musk melon B) cow dung and C) horse dung

3.5. Biochemical tests

The G1 and C3 organisms were showed positive results for indole, methyl red, citrate and catalase test, whereas H1 isolate showed the positive results only for voges proskauer and catalase test (Table 2).

Table 2. Biochemical tests for cellulase positive cultures

Isolates	Indole	Methyl red	Vogesproskauer	Citrate	Catalase
G1	+	+	-	+	+
C3	+	+	-	+	+
H1	-	-	+	-	+

3.6. Physicochemical analysis of samples before saccharification

The amount of reducing sugar was highest in *C. papaya* (203.70±18.46), followed by *P.Guajava* (193.84±9.23) and *S.lycopersicum* (43.07±10.65), total sugar content is maximum in *C. papaya* (110.56±14.89) followed by *P.Guajava* (94.30±5.63) and *S.lycopersicum* (22.76±5.62) and the amount of cellulose is highest in *C. papaya* (6.11±0.04) followed by *S. lycopersicum* (3.70±0.11) and *P.guajava* (3.50±0.06) (Table 3).

Table 3. Physicochemical analysis of samples

Substrate name	Reducing sugar (µl/ml)	Total sugar (µl/ml)	Cellulose (mg/ml)
Solanum lycopersicum	43.07±10.65	22.76±5.62	3.70±0.11
Carica papaya	203.70±18.46	110.56±14.89	6.11±0.04
Psidium guajava	193.84±9.23	94.30±5.63	3.50±0.06

Estimation of reducing sugar and total sugar: All samples showed the increase in the content of reducing sugar and total sugar upon number of days.

In *S. lycopersicum* sample, after Day 3 the amount of reducing sugar was reduced in C3 (107.68±3.08) and H1 (79.99±6.16). In *C. papaya* sample, after Day 2 the amount reducing sugar reduced in G1 inoculated sample (270.15±5.83). C3 inoculated sample showed maximum amount of reducing sugar (787.68±8.13). In *P. guajava* sample, H1 inoculated flask had maximum amount of reducing sugar (799.99±3.08) in Day 3(Figure 5).

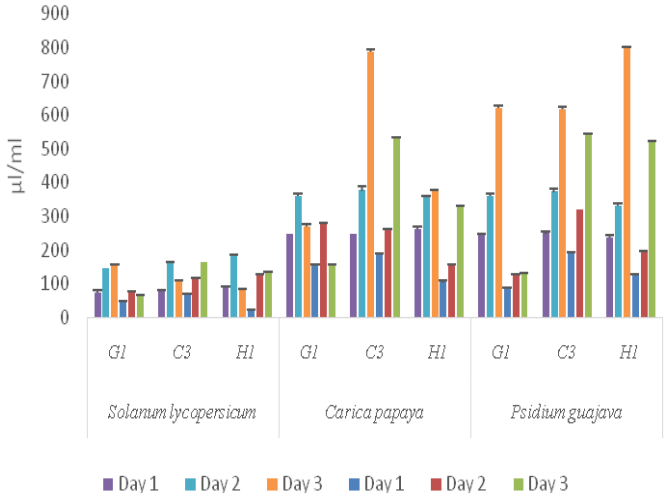

Fig. 5: Reducing and total sugar in different sample during saccharification.

The amount of total sugar has been increased in all the samples inoculated with C3 and H1organism and G1 inoculated flask in all the samples has shown less increase in total sugar as shown in figure 5.

Estimation of cellulose: All the samples inoculated with G1, C3 and H1 were estimated for cellulose content consecutively for 3 days. The amount of cellulose was decreasing as shown in figure 6.

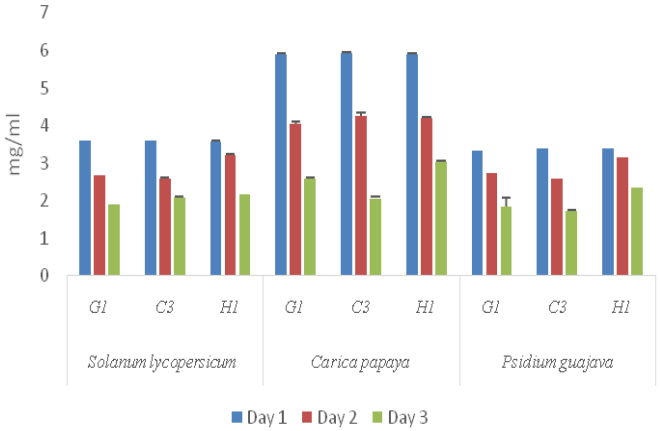

Fig. 6: Cellulose in different sample during saccharification

3.7. Percentage of alcohol production

The alcohol production was increased day by day with G7. In *S. lycopersicum* highest alcohol production was noted in G1 saccharified sample (43.75±1.02). The highest alcohol production was noted in *C. papaya* with C3 saccharified sample (60±2.04). In *P. guajava* the alcohol production is highest with C3 saccharified sample (53.75±1.02) (Figure 7).

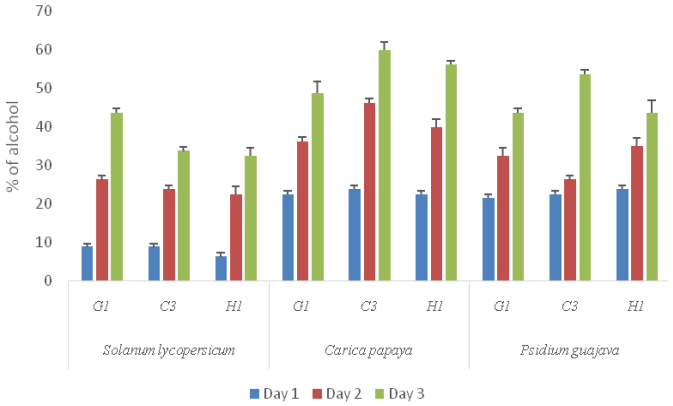

Fig. 7: Percentage of alcohol in different samples during fermentation

3.8. FTIR studies

The high yield of alcohol was obtained in the *C. papaya* sample, which was saccharified with C3. The FTIR analysis of the sample showed the most characteristic peak in bio ethanol spectrum is peak one at 3429.49 cm^{-1} that lies between 3200-3550 which relates to alcohol (O-H) vibrations and the figure 8 confirms the presence of alcohol.

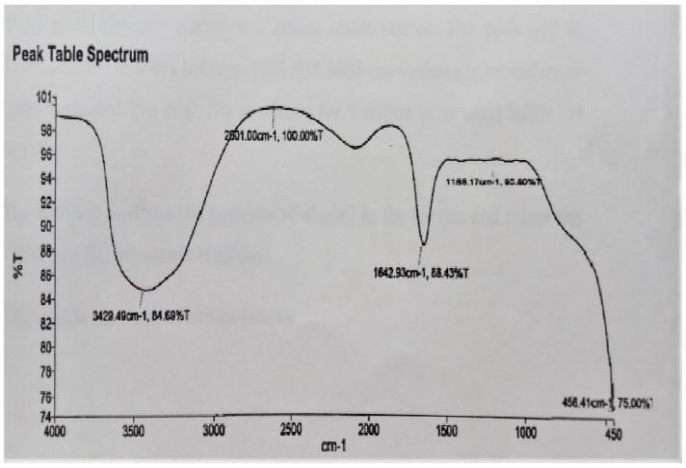

Fig. 8: Typical FTIR of *Carica papaya* fermented with C3 isolate.

3.9. Molecular identification of organisms

The bacteria isolated from cow dung (C3) and horse dung (H1) showed positive results for cellulose degradation and some biochemical test. Hence, it was

molecular characterized using 16s rRNA sequencing revealed that the isolates were *B.circulans*(H1) and *B.subtilis*(C3). Yeast isolated from musk melon (G7) was also sequenced for 18s rRNA sequencing revealed that the isolate was*S.cerevisiae*.The sequences of the isolates were compared with the reference strains obtained from NCBI Gene bank to arrive at the above results (Figure 9&10).

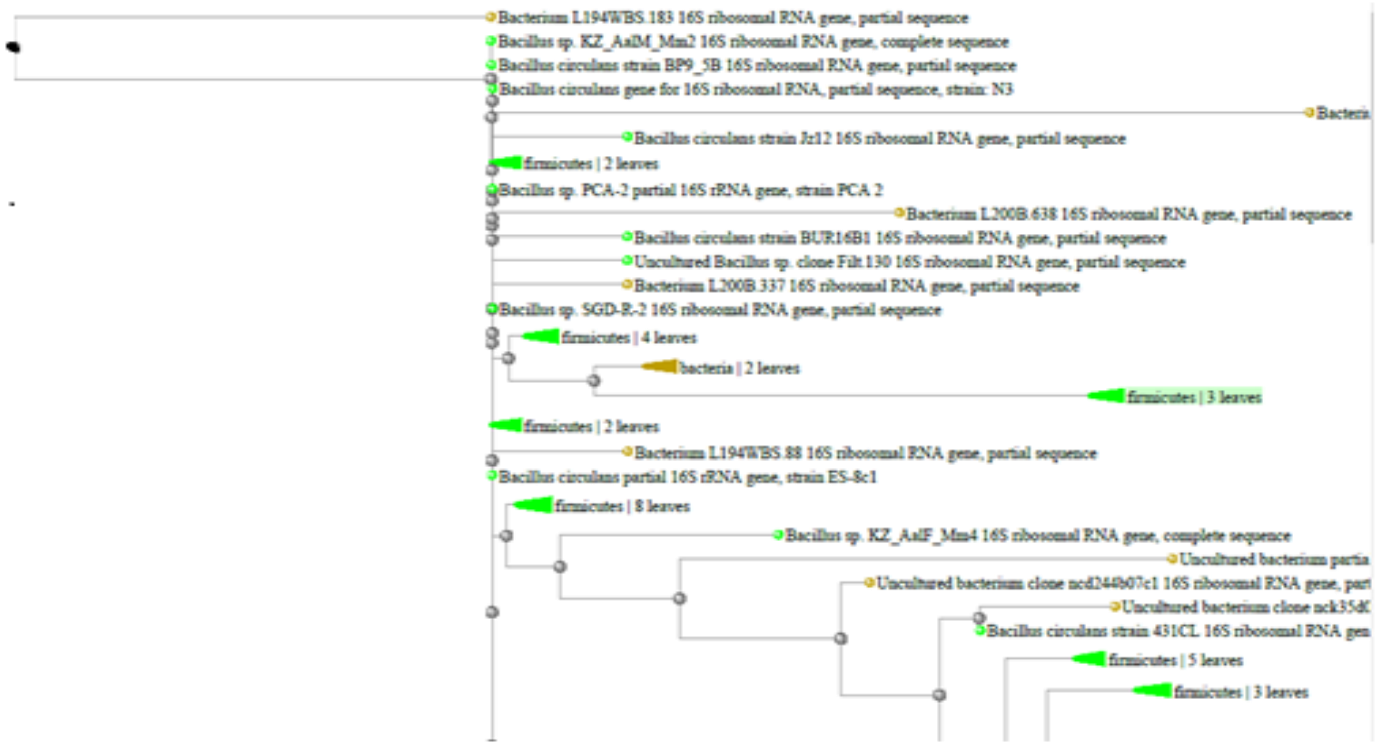

Fig. 9: Phylogenetic tree of *Bacillus circulans* (H1)

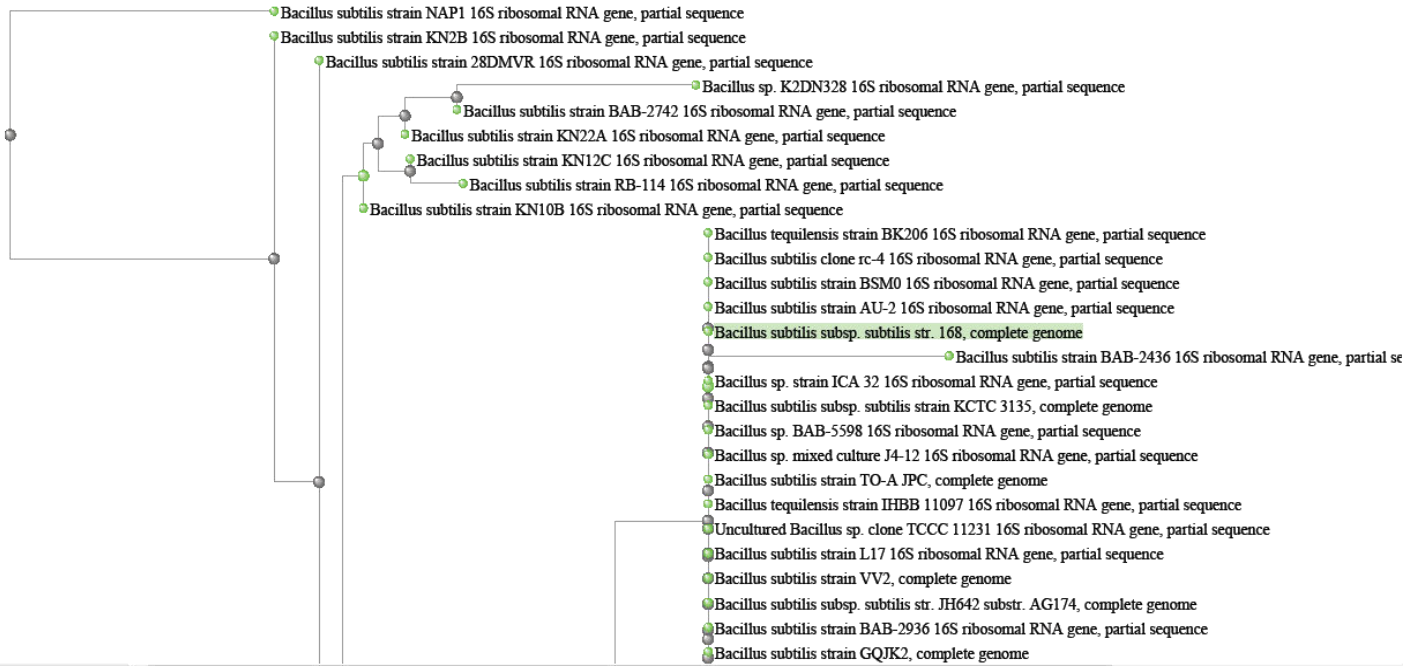

Fig. 10. Phylogenetic tree of *Bacillussubtilis* (C3)

4. CONCLUSION

The results of this investigation indicate that fruit rinds of *S. lycopersicum*, *C. papaya* and *P. guajava* are the source of readily fermentable sugars represents an untapped feedstock for bio ethanol production. From the results of this study the conclusions can be drawn that, the fruit rinds are a potential substrate which can be exploited in industries for the production of bio-ethanol and the isolates from dung samples *B. circulans* (from horse dung) and *B. subtilis* (from cow dung)suits best for saccharification. The fruit rinds served as the cheapest biomass for the production of bio ethanol which can be used as alternate fuel. It is much cleaner as it releases no toxic gases into the environment thus reducing pollution and it is not harmful for human health. The utilization of fruit rinds also reduces the accumulation of garbage thus keeping much disease at bay.

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