

Scanning Electron Microscope and Fourier Transform Infrared Analysis of Fruit Waste Samples Digested by Autothermal Thermophilic Aerobic Digestion Process

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Abstract - Scanning electron microscopy and Fourier Transform infrared spectroscopy are important techniques used to study the morphology, color, variation and different functional groups of both solid and liquid samples. The current work is collection of decayed fruit samples during different seasons and subjecting it to Autothermal Thermophilic Aerobic Digestion and SEM and FTIR – ATR analysis of sludge samples for the presence or absence of bacteria, their deformities. The variation of functional groups in raw and treated samples were studied using FTIR. In the present study, the control sample, 0th hour of sample and ATAD treated samples collected at various intervals were taken for study. Indicator organisms along with the indigenous strains were present in the 0th hour sample, which was evident from the SEM images. The deformed bacterial strains in the samples collected further were also observed. In case of FTIR analysis, the samples were subjected to ATR mode. In control samples, the intensity of peaks, corresponding functional groups were observed. In the successive samples, even though, the peaks obtained were in the same range, the intensity of peaks were not as sharp as obtained in the control samples and corresponded to different functional groups. This variation may be attributed to the thermophilic degradation of the samples in the ATAD reactor.

Key words: Scanning Electron Microscopy, Attenuated Total Reflection, deformed bacterial strains, peak intensity, functional groups

INTRODUCTION

Scanning electron microscopy (SEM) is one of the techniques suitable to show bacteria in great detail (Kalab *et al.*, 2008). SEM has been used to analyze the changes in microbial morphophytes in samples of vegetable waste substrates that were subjected to anaerobic digestion of swine manure (Molinuevo-Salces *et al.*, 2012). It has been in use to analyze samples synthetic wastes (Yang *et al.*, 2008) and in bacterial cellulose production from industrial waste and by-product streams (Tsouko *et al.*, 2015). SEM is the predominant choice for investigation to study the ultrastructure of the biofilm and its environment (Priester *et al.*, 2007, Sangeetha *et al.*, 2009, Alhede *et al.*, 2011). Analysis of effluent samples such as palm oil mill effluent, empty fruit bunch using eSEM has been reported (Baharuddin *et al.*, 2010, Kala *et al.*, 2009, Kananam *et al.*, 2011, Zainudin *et al.*, 2014). SEM images has been used to interpret the bioconversion of empty fruit bunches using *E. eugeniae* (Lim *et al.*, 2015).

Fourier Transform Infrared spectroscopy (FTIR) is used to obtain infrared spectrum of the compound taken for study. Volatile organic pollutants discharged into water, indoor air (Mo *et al.*, 2009), emission characteristics of VOCs in sludge drying process (Deng *et al.*, 2009) has been determined using FTIR. Volatile compounds in fruits, processed fruits has been determined by FTIR (Wei *et al.*, 2011). Proximate and ultimate analysis of raw fuel biomass of agro-industrial wastes for feasibility of solid bio-fuel production has been determined using FTIR (Veeresh and Narayana, 2012). In a study, removal of heavy metals in peels of orange, lemon and banana in aqueous solution was carried out using FTIR for the analysis of variation in functional groups upon modification (Thirumavalavan, *et al.*, 2011); also in study of structure binding capacity, affinity and sites of metal ions in melanin, produced by soil microbial isolate on fruit waste extract (Tarangini and Mishra, 2014) and functional groups in citrus peel extracts (Gnanasaraswathi *et al.*, 2014). Pre-treatment study of anaerobic digestability of oil palm empty fruit bunches revealed the growth of syntrophic acetate-oxidizing bacteria and hydrogenotrophic methanogens (Saelor *et al.*, 2024). Apart from volatile functional groups, fruit peels waste is a potential feed stock for production of secondary metabolites (Muigano *et al.*, 2024). In our study, the homogenized fruit waste samples were analyzed for SEM and FTIR before, during and at the end of ATAD digestion process to study the morphological variations of bacteria in the samples and the different types of functional groups observed during various time intervals.

MATERIALS AND METHODS

Sample Collection and preparation

Decayed fruit samples were collected from 3 different sites in 3 different seasons. Sweet lime, papaya, orange, banana were predominantly present. Collected samples were immediately brought to lab and homogenized to a paste like consistency.

SEM Analysis

The sample and control were studied for the bacterial morphological studies in SEM to identify the size before and after subjecting to ATAD. The SEM analysis was carried out using the instrument ICON analytical, FEI QUANTA 200. Dried samples were examined under SEM at 12-15kV with a tilt angle of 45° at a magnification range from 10000X-13000X.

FTIR Analysis

Both control and samples collected at different intervals were subjected to FTIR analysis. FTIR spectrum of samples were recorded using the BRUKER TENSOR 27 FTIR spectrophotometer. The spectrum was recorded in the Attenuated Total Reflectance (ATR) mode in the range from 600 – 4000 cm⁻¹.

STATISTICAL ANALYSIS

Statistical analyses were done to confirm whether the parameters were correlated and the significance of correlation (two tailed) was significant at the 0.05 level (moderate) or at the 0.01 (perfect). The parameters analyzed at various seasons formed a triplicate data for the analysis in the SPSS (Version 13) software for which the ANOVA was determined with the mean and S.D. values. The experiments performed at three different seasons were expressed as mean± standard deviation (SD). The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with a probability of $P \leq 0.05$.

RESULTS

Scanning electron microscopic analysis of the samples was carried out to analyze the morphological characters / presence of indigenous, pathogenic, indicator bacteria in the samples before and after ATAD process. The biosolids of the samples collected from Tiruchirappalli were taken for the SEM analysis.

Control Samples:

In the control samples (mixture of samples with indigenous culture of bacteria), there were very few colonies of bacteria. At the initiation of the ATAD process, the 0th hour sample was inoculated with indicator organisms which were subsequently reactivated in nutrient broth and incubated at 37 °C for 24 hours. The SEM images indicated that the 0th hour sample had a diverse culture of bacteria ranging from the indigenous culture already present in the decayed fruits mixture and the indicator organisms that was added into the digester. Figures 1 and 2 show the SEM image of the control and 0th hour's samples respectively. In fig.1, the encircled portion shows the indigenous bacterial species present before the ATAD process. Fig. 2 shows the diverse species of organisms (along with the indicator organisms) in the sample.

Fig. 1. SEM analysis of the control sample

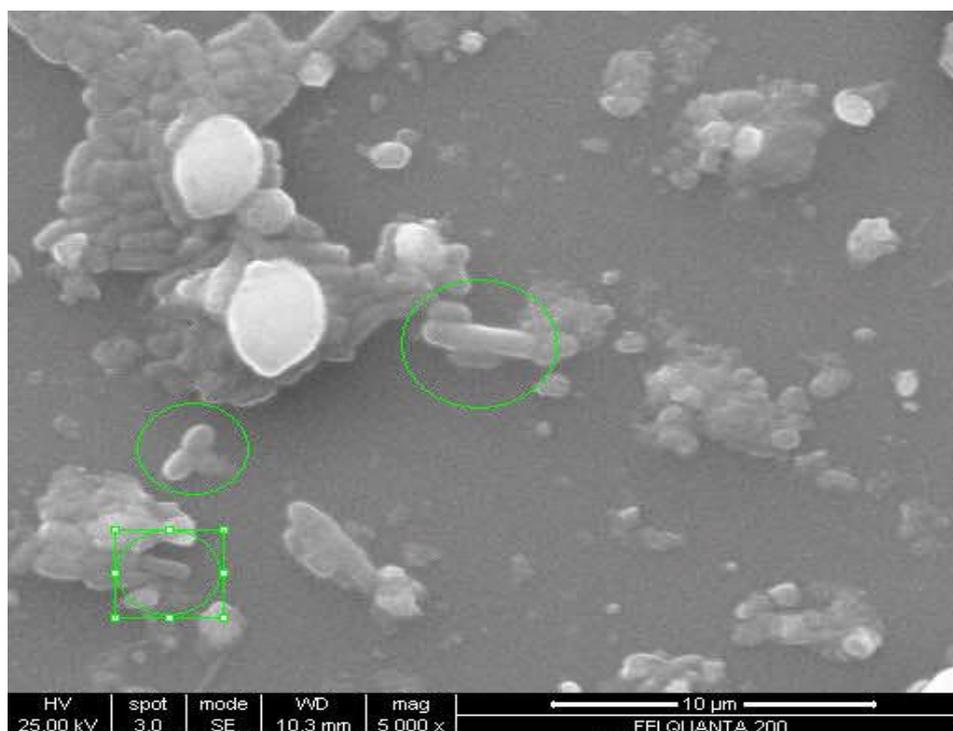
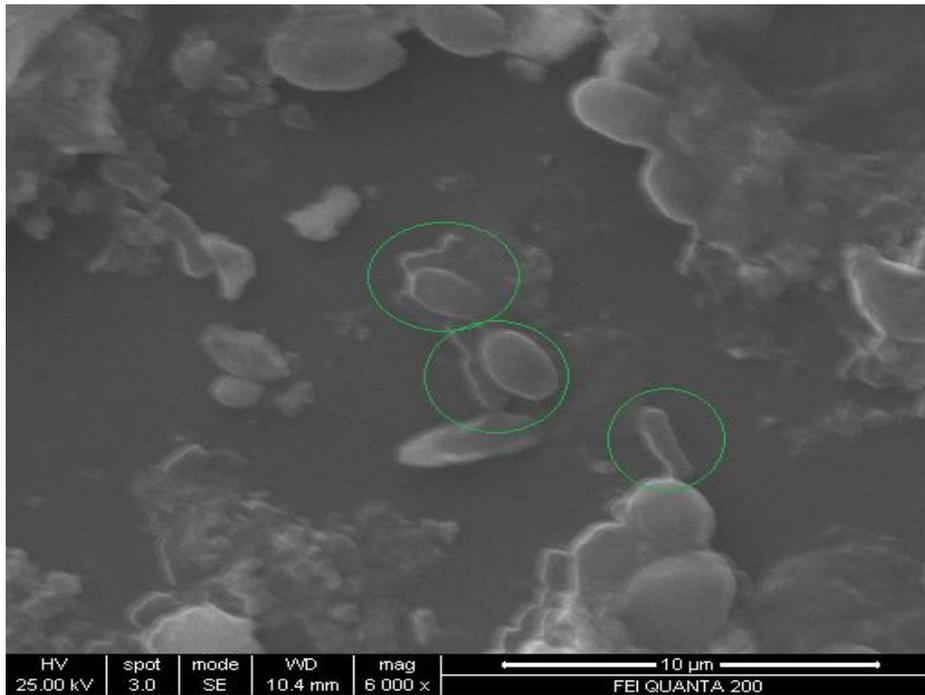


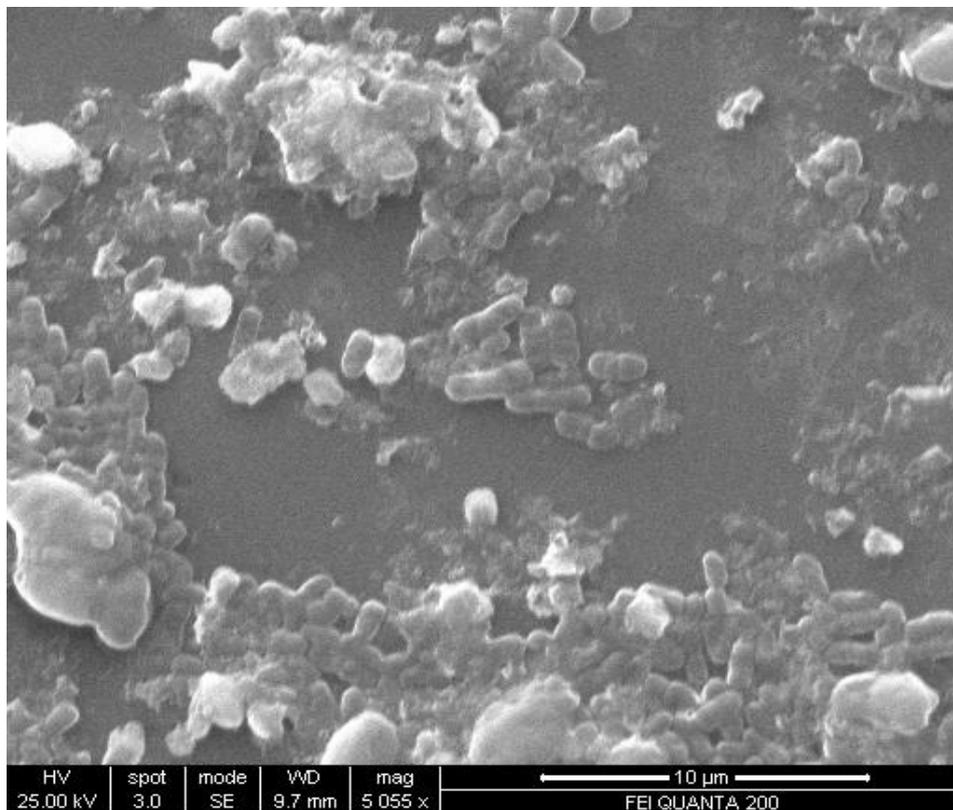
Fig. 2. SEM analysis of the sample collected at 0th hour (before the start of digestion)



Sample collected during 7 – 35 hours of digestion

During the digestion process, population of the indicator organisms and the indigenous species started reducing slowly since they cannot sustain the heat generated by the thermophilic organisms inside the reactor. Fig. 3 shows the SEM image of the sample taken after a digestion period of 7 – 35 hours.

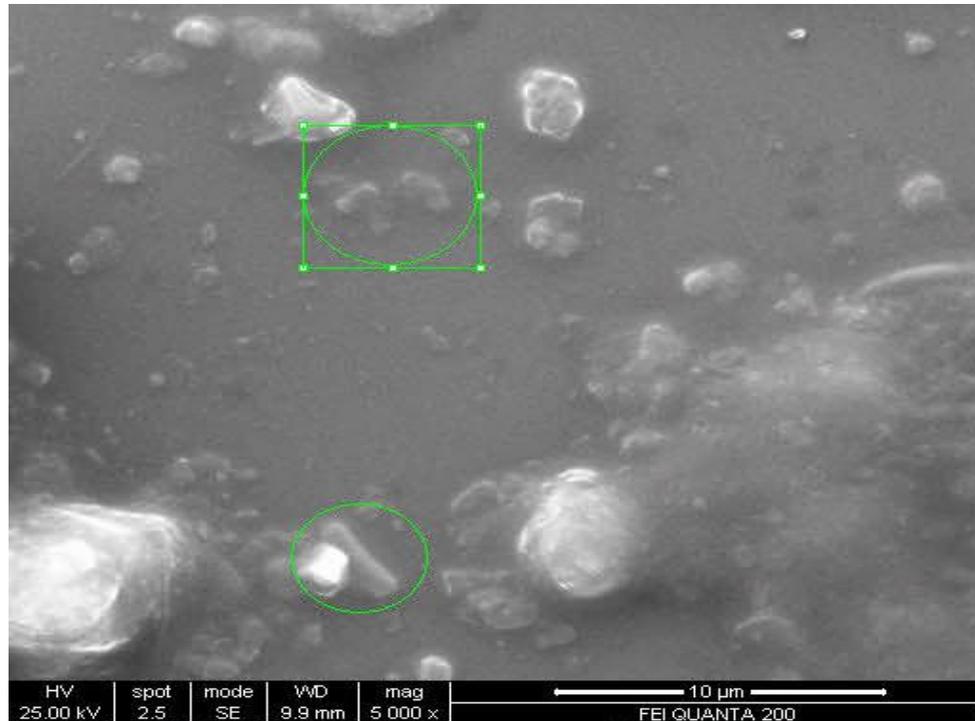
Fig. 3. SEM analysis of the sample collected after 35 hours of digestion



Sample collected during 36 – 56 hours of digestion

After 56 hours of digestion, the bacterial cultures started reducing and the thermophilic organisms alone sustained. This is evident from the SEM image in which the deformed bacterial species could be seen. This may be attributed to the heat generated by the thermophilic bacteria.

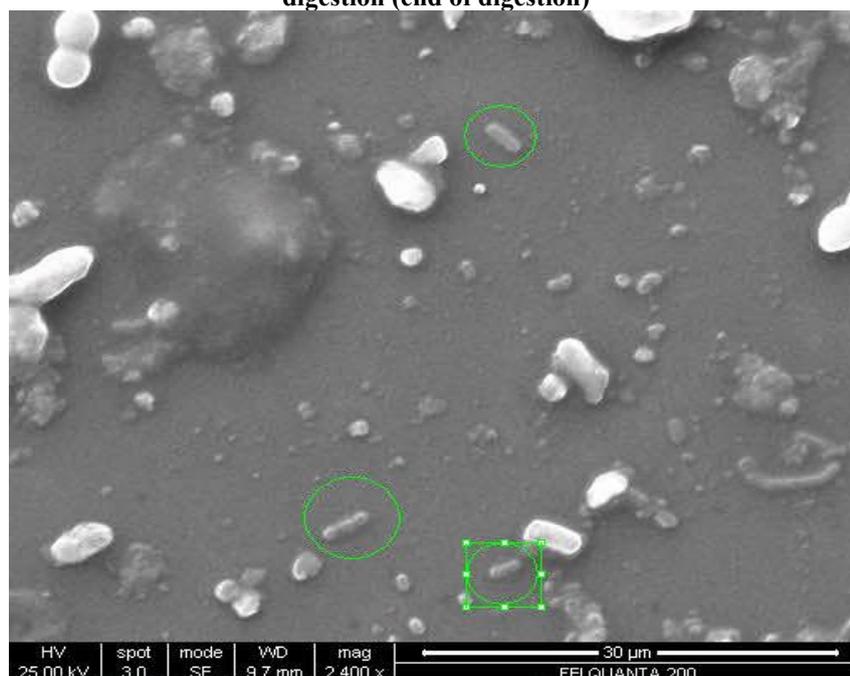
Fig. 4. SEM analysis of the sample collected after 56 hours of digestion



Sample collected after 70 hours of digestion

After 70 hours of digestion, except the thermophilic bacteria, indigenous and the inoculated species reduced completely which is characteristic of the ATAD treated biosolids. From Fig. 5, it is evident that the sample has been completely degraded and due to the heat generated, only a very few bacterial cells are visible.

Fig. 5. SEM analysis of the sample collected after 70 hours of digestion (end of digestion)



The color of the sludge remained same before and after the ATAD treatment. During each time of sample collection, 10 – 15 mL of the biosolid sample was removed from the reactor and tested. Sample was stored air-tight and stored at 4°C for analysis of biosolids.

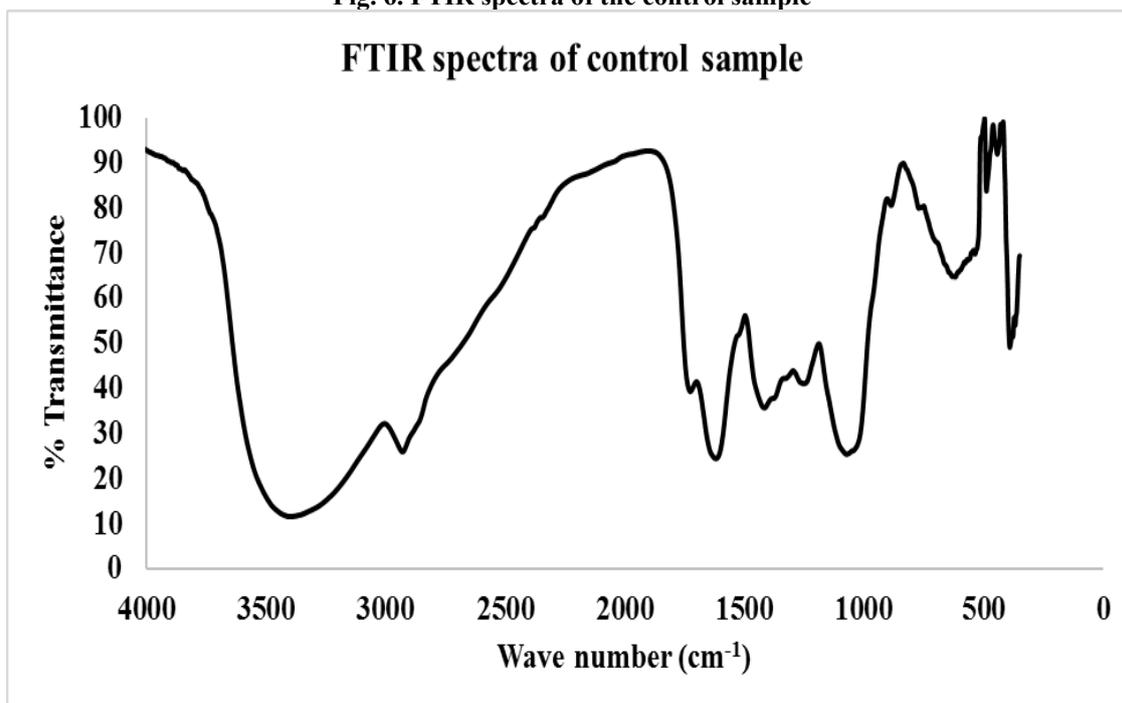
FTIR analysis

Analysis of the FTIR spectra was performed to identify the major functional groups and to compare the spectral differences between the samples collected (from Tiruchirappalli) at the beginning and after ATAD digestion. The infrared spectra of the samples within the wavenumber range 4000 to 500 cm^{-1} are in the figures.

Control

Control sample and the samples collected at various time intervals were analyzed. An FTIR spectrum of the powdered form of control samples is shown in Fig. In the control sample, broad absorption band in the region 3570-3200 cm^{-1} corresponds to the characteristic O-H stretching vibration and hydrogen bond of the hydroxyl groups. The broad peak is centered at 3395.07 cm^{-1} which shows strong absorbency for N-H stretch for aliphatic primary amines. An O-CH₂ stretching band at 2929.34 cm^{-1} was observed in control sample. A strong peak of aromatic combination bands was observed at 1724.05 cm^{-1} . Peaks at 1623.77 cm^{-1} which corresponds to Alkenyl C=C stretch, aryl substitution of C=C, primary and secondary amine, NH bend and carboxylic acid were observed. Absorption band in the region of 1300 – 700 cm^{-1} corresponds to skeletal C-C vibrations. The broad peak is centered at 1241.93 cm^{-1} along with another peak of 1040.41 cm^{-1} , which corresponds to cyclohexane ring vibrations and silicate ion and aromatic phosphates group. A strong peak of 773.315 cm^{-1} observed corresponds to Skeletal C-C vibrations. Other peaks including 540.935 cm^{-1} (corresponding to aliphatic iodo compounds) and 476.331 and 416.692 cm^{-1} were also observed.

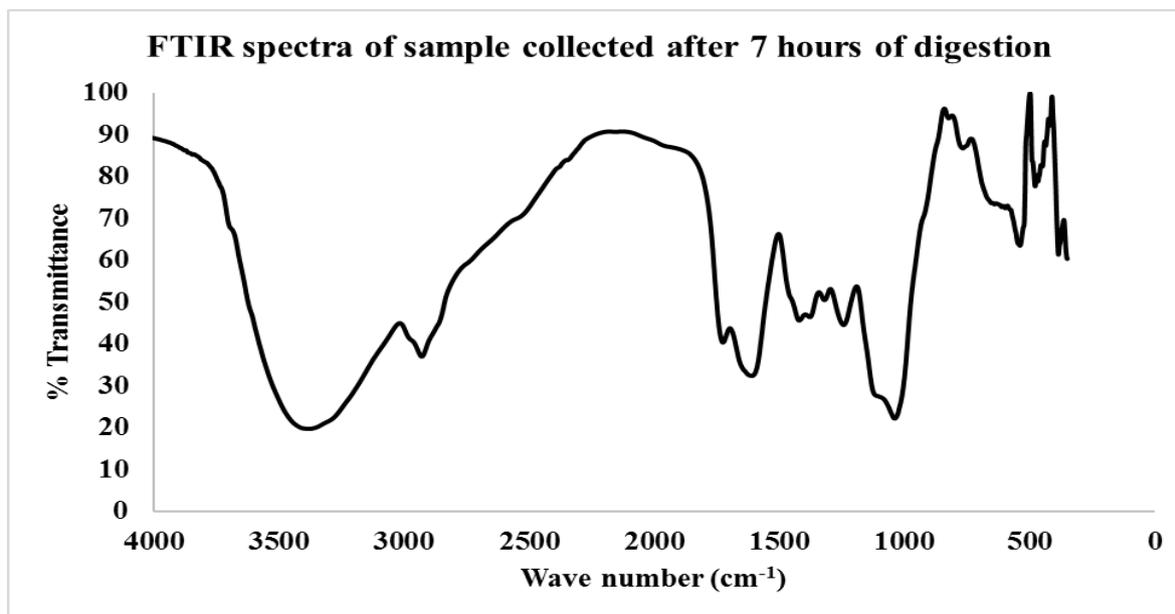
Fig. 6. FTIR spectra of the control sample



Sample collected after 7 hours of digestion

FTIR spectrum of the powdered form of the sample collected after 7 hours of digestion is shown in the figure. A broad absorption band in the region 3600 – 3100 cm^{-1} corresponds to the characteristic O-H stretching vibration and hydrogen bond of the hydroxyl groups. Peak observed at 1725.98 cm^{-1} corresponds to aromatic combination bands, aldehyde, ester, carboxylic acid and ketone. Peak at 1609.31 cm^{-1} corresponds to Alkenyl C=C stretch, aromatic ring C=C-C, primary and secondary amine, carboxylic acid, conjugated ketone, open chain imino and azo groups. Peaks – 1421.28 and 1243.86 cm^{-1} are obtained in the absorption band region 1470-1430 and 1270 – 1230 cm^{-1} respectively, Methyl C-H asym./sym. Band, vinyl C-H in-plane bend, Carboxylate, Aromatic ethers, epoxy and oxirane rings, organic and aromatic phosphates. The peaks 1243.86 cm^{-1} and 1039.44 cm^{-1} corresponds to the absorption band 1300 – 700 cm^{-1} which corresponds to skeletal C-C vibrations.

Fig. 7. FTIR spectra of the sample collected after 7 hours of digestion

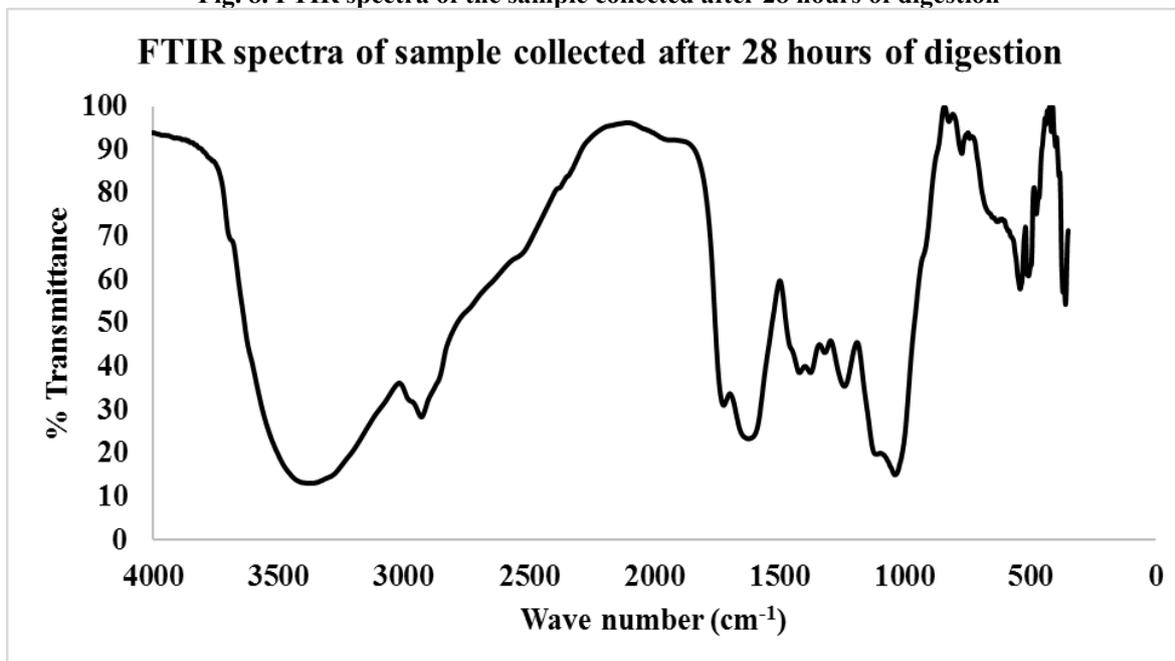


Sample collected after 28 hours of digestion

With increasing time of digestion, the first significant change observed was the decrease in the intensity of peaks in the successive samples, which may be attributed to the biodegradation of hydrocarbon compounds such as phenol and aliphatic groups.

In the sample collected after 28 hours of digestion absorption band in the region 3570-3200 was observed. The peak obtained was 3373.85, for which the intensity is lesser than that of the peak in control sample. A peak of 1623.77 cm^{-1} corresponding to Alkenyl C=C stretch, primary and secondary amine, conjugated ketone was obtained. There were shorter peaks – 540.935, 507.187 and 476.331 cm^{-1} , which were not sharp as that obtained in the control.

Fig. 8. FTIR spectra of the sample collected after 28 hours of digestion

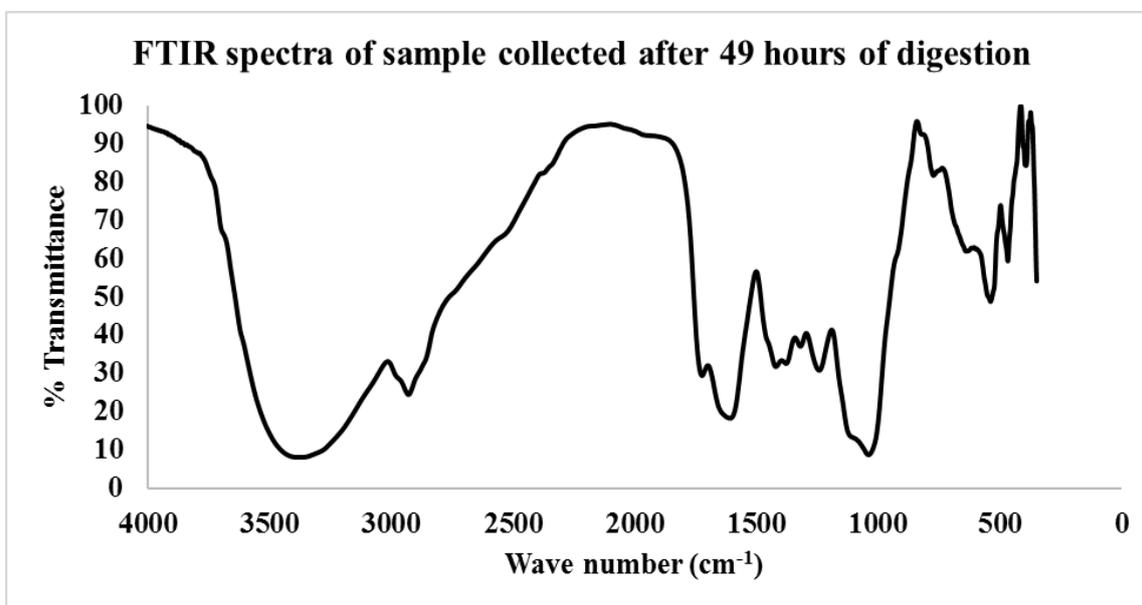


Sample collected after 49 hours of digestion

The peak obtained for the sample collected after 49 hours of digestion was 3367.1 cm^{-1} , which is lower than that of the control. This corresponds to the Hydroxy group,

H-bonded, normal polymeric OH stretch, aliphatic and aromatic primary amine, aliphatic secondary amine. A sharp peak of 1724.05 cm^{-1} corresponding to aromatic combination bands, aldehyde, ketone, ester and six-membered ring lactone was obtained. This peak was same as the one obtained in the control sample. The peak 1320 cm^{-1} corresponds to methylene C-H bend, primary or secondary alcohol, phenol or tertiary alcohol, aromatic amines, carboxylates, organic phosphates and dialkyl sulfones. The peak 1241.93 cm^{-1} corresponds to skeletal C-C vibrations, epoxy and oxirane rings, organic and aromatic phosphates.

Fig. 9. FTIR spectra of the sample collected after 49 hours of digestion

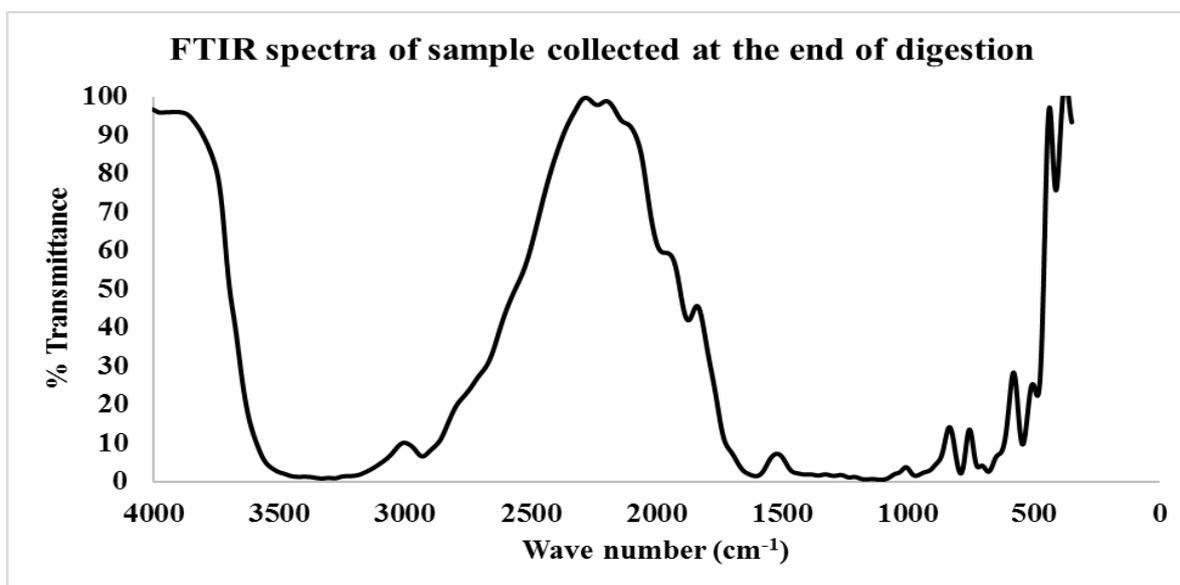


Sample collected after 70 hours of digestion

The samples collected at the end of ATAD process showed appreciable decrease in the intensity of bands between the range of 1873.51 and 1605.45 cm^{-1} , which was a significant change. The peak obtained in the absorption band region $3400 - 3200$ was 3330.46 cm^{-1} , which is much lower than the peak obtained in the control sample. The peak obtained is much lower than the peak obtained in the sample collected after digestion of 49 hours. The peak 1108.87 cm^{-1} corresponds to $1300-700$ skeletal vibrations and several aromatic C-H in-plane bends. Also the peak corresponds to secondary alcohol,

C-O stretch, whereas in control samples, the functional group corresponding to the same range of band was organic siloxane, cyanate, sulfate, phosphate and silicate ions.

Fig. 10. FTIR spectra of the sample collected after 70 hours of digestion



DISCUSSION

Structural damage and viable microbes in the untreated waste activated sludge, whereas, after alkali pretreatment, damage to the cells, swelling and softening were detected (Cho *et al.*, 2013). In the present study, the control samples (before ATAD digestion) indicated the presence of diverse culture of bacteria. After 70 hours of digestion, the cells were damaged and swollen due to the heat produced inside the ATAD digester. Deng *et al.*, (2014) isolated and characterized a novel hydrocarbon-degrading bacterium from crude oil contaminated seawater. The isolated strain *Achromobacter* was Gram negative, rod shaped bacteria with creamy white, opaque convex and smooth surfaces. In the present study, in the control samples the cells isolated were *Bacillus*, *Corynebacterium*, *Arthrobacter*, which are Gram positive and *Serratia*, which is Gram negative. *Bacillus* and *Corynebacterium* were rod shaped (*Serratia* was in short rods form). Some strains of *Arthrobacter* were rods and some were cocci. Circular colonies were observed in *Serratia* and *Corynebacterium*. Irregular colonies were observed in *Bacillus* sp. Whitish and slightly convex colonies were observed in *Arthrobacter*.

Fourier transform infrared is an important technique for characterizing chemical composition of complex probes and has been successfully used in fungal identification (Santos *et al.*, 2010), fruit bunch composites (Sefadi, 2010), oil palm empty fruit bunch (Isroi *et al.*, 2012) and cell wall analysis of weeds (Dunn *et al.*, 2007). Dong *et al.*, (2013) studied spoilage of strawberry via its volatile compounds using longpath FTIR. Alcohol absorption bands: 2830 – 3040 cm^{-1} , 1000 – 1120 cm^{-1} and 855 – 916 cm^{-1} were observed. The spectra was analyzed and found that the concentration of esters, alcohols, ethylene and similar compounds changed with deterioration. In the present study, the spectral bands of the samples analyzed after 49 hours of digestion showed the presence of primary or secondary alcohol, phenol, aromatic amines and carboxylates. The peak 1241.93 cm^{-1} corresponds to skeletal C-C vibrations, epoxy and oxirane rings, organic and aromatic phosphates. Viegas Jr *et al.*, (2007) studied the lipoperoxidation and cyclooxygenase enzyme inhibitory piperidine alkaloids from *Cassia spectabilis* green fruits. The IR spectrum implied the presence of secondary amine, ketone and olefinic moieties. In the present study, secondary amine, aromatic amine and secondary alcohol were observed in the sample collected after 49 hours of digestion. A peak 1320 cm^{-1} corresponding to methylene C-H bend was obtained. Several amines and conjugated ketones were obtained in the samples collected after 7 hours of digestion.

CONCLUSION

ATAD treatment of fruit wastes has direct effect on the deterioration of indicator organisms that were added to the slurry at the 0th hour of digestion. Deformation of bacterial strains was first observed in samples collected after 35 – 56 hours of digestion, which may be attributed to the heat generated in the reactor. Presence of indigenous strains alone in the sample obtained after 70th hour of digestion is evident from the SEM images. FTIR results showed the variation of functional groups in the raw and treated sample. When compared with the control samples, even though peaks were obtained in the same range, functional groups varied in the samples collected at various intervals. Also, the peak intensity was also not much sharp as obtained in the control samples. This shows the variation in volatile fatty acids and phenolics in the fruit wastes as time and temperature increased. The present study gives an idea about the morphological changes of bacterial strains in the raw and treated samples and different functional groups of the volatile organic compounds at various time intervals.

AUTHORS CONTRIBUTION STATEMENT

G Srinivasan carried out the whole study that includes collection of samples, setting up the ATAD digester, isolation of bacteria and phenotypic characterization of the strains. A Abirami carried out the SEM and FTIR analysis and statistical analysis of the work. Both the authors prepared the manuscript, read and approved the final version.

CONFLICT OF INTEREST

Conflict of interest declared none.

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