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Regular Article

Isolation, Characterization and Comparative Study of *Lactobacillus Sp.* using FTIR Based Statistical Analysis

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ABSTRACT: There are different types of Lactobacilli found in milk. Lactobacilli have antimicrobial activity. In this experiment we try to characterize the lactobacilli found in different sources. For characterization we have used various lactobacilli sources such as Benny Buttermilk, Aavin curd and Ranipet curd, all of which are locally available. Lactobacillus strain Lactobacillus MTCC 2997 and probiotic drinks Bifilac and Darolac were also used as lactobacilli sources. Gram staining and other biochemical tests like Voges Proskauer Test, Methyl Red Test, Citrate Utilization Test, Catalase Test, as per their standard procedures were performed. Fourier-transformation infrared spectroscopy (FTIR) was applied to characterize Lactobacillus from different sources at the genus and species level. Similarity matrices, cluster analysis and correlation plots were made to compare how similar 2 strains of isolated Lactobacillus sp. are over the entire FTIR range of wavenumbers are. The scatter plot and similarity matrix shows strong positive correlation of 0.966 between Lactobacillus sp. isolated from Benny buttermilk and that of Ranipet curd hence these two strains have a very similar lactic acid production rate. ANOVA tables and curve estimation was done to compare each isolated strain with MTCC 2997. During this analysis it was obtained that, Lactobacillus MTCC 2997 and Lactobacillus sp. isolated from Bifilac were 94.6% similar. When Hierarchical Cluster Analysis was performed, the dendogram also depicted that Lactobacillus MTCC 2997 and Lactobacillus sp. isolated from Bifilac were closely related .The importance of this method is that it states the amount of similarity between the organisms using statistical analysis. Based on this similarity percentage, measured in terms of Pearson correlation, we can predict the strain type, differentiate up to the sub species level, which was not possible with biochemical characterization.

Key words: Lactobacilli, milk

Introduction

The aim of study was to isolate, characterize and do a comparative study of *Lactobacillus sp.*, from commercial products and locally available curd. Members of LAB share the property of being Gram-positive bacteria that ferment carbohydrates into energy and lactic acid. A comparative study was carried out between isolates from various sources, to determine the best strain. The antimicrobial effect of *Lactobacillus* is mainly due to their lactic and organic acid production, causing the pH of the growth environment to decrease. Molecular techniques, especially polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE), FTIR (Fourier Transform Infrared Spectroscopy) are regarded important for specific characterization and detection of LAB strains. Fourier-transform infrared spectroscopy (FTIR) was applied to characterize *Lactobacillus* from different sources at the genus level and at the species level. The organism spectra were analyzed by Hierarchical clustering analysis (HCA) using Grouped Linkage method and the similarity was done using Pearson's coefficient .Spectral windows for homofermentative bacteria. Isolates were found to be homofermentative and were used as "starters" and inoculated into pasteurized milk and curdling was allowed to happen for a period of six hours and then analyzed using FTIR to find out the spectral changes.

Materials and Methods

Microbial growth is dependent on intrinsic factors, such as water activity and acidity, and extrinsic factors, such as temperature and oxygen availability. The basic objective was to inoculate six different *Lactobacillus* strains isolated from six sources into milk and compare the curdling process based on several parameters such as growth kinetics, pH, lactose conversion to lactic acid by FT-IR and do a comparative analysis.

Materials, media and reagents used

Sources of *Lactobacillus*

Lactobacillus MTCC 2997, Aavin Curd, Ranipet Curd, Benny Buttermilk, Bifilac Sachet, Darolac sachet.

Media used

- *Lactobacillus* MRS Agar (HIMEDIA)
- Agar Powder (HIMEDIA) – Bacteriological
- Buffered Glucose Broth (MR VP Medium) – (HIMEDIA)
- Simmons Citrate Agar (HIMEDIA)
- *Lactobacillus* MRS Broth (HIMEDIA)
- Safranin

Collection of *Lactobacillus* sources

Lactobacillus MTCC 2997 standard reference strain was ordered from MTCC at Institute of Microbial Technology, Chandigarh. Aavin Curd and Benny Buttermilk were bought from retail outlets in Vellore. Home-made curd from a household in Ranipet was collected. Bifilac and Darolac probiotic sachets were bought from Kayes Pharmacy in Vellore.

Isolation of *Lactobacillus* from Aavin and Ranipet Curd and Benny buttermilk

conical flask of 100 ml MRS agar was prepared and were autoclaved 25 eppendorfs were taken, 10 each for Aavin and Ranipet Curd and 5 for Benny buttermilk for serial dilution till dilution factor of 10^{-10} is reached.

50 µl each was spread plated with an L-rod onto autoclaved MRS agar plates.and were left overnight in the incubator at 37°C.The plates were observed for presence of mixed colonies and were tested by Gram staining and Biochemical tests

Once identified, the *Lactobacillus* colony was subcultured and streaked onto an autoclaved plate of MRS agar to obtain pure colonies.

Gram Staining

Each morphologically varying colony was tested for identification of *Lactobacillus* by Gram Staining which after Gram staining appear as purple rods.

Biochemical Tests

The following Biochemical tests were carried: Voges Proskauer Test;Methyl Red Test; Citrate Utilization Test; Catalase Test as per the standard procedures.

Study of effect of various parameters on the process of curdling of milk

Seven flasks were taken and autoclaved. one flask with 100 ml of milk was used as a control.To the other 6 flasks 100 ml milk was added and they were inoculated with a single colony of *Lactobacillus* MTCC 2997 and *Lactobacillus.*The flasks were kept on a shaker for half an hour and left at room temperature for a period of six hours.

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Study of variation of absorbance,pH,colony count;amount of lactic acid.Titration was also done.

FTIR analysis

Samples for FT-IR were prepared and were lyophillised.Processing of samples was done by powdering the samples and mixing hem with KBr to form pellets. The pelletized sample was placed in the sample chamber of the FT-IR instrument.
Spectra were obtained.

Statistical analysis from FTIR spectra data

The FTIR data obtained was used for statistical analysis using SPSS version 17.0 and Microsoft Excel 2007. Spectral changes were observed. Changes in peak area and height for characteristic lactic acid groups were done using Microsoft Excel 2007.

Using the software SPSS version 17.0, a number of analysis were done. Similarity matrices cluster analysis and correlation plots were made to compare how similar 2 strains of isolated *Lactobacillus sp.* are over the entire FTIR range of wavenumbers are. ANOVA tables and curve estimation was done to compare each isolated strain with MTCC 2997. Distance correlation was also done to confirm results obtained by curve estimation. Hierarchical cluster analysis was also done to determine the most closely related strains.

Results and Discussion

Colony morphology

The colonies seen in the MRS agar plates for 6 *Lactobacillus* sources were observed to have a size of >0.1 mm and were circular and milky white in appearance.

Results of Gram staining

Source	Length	Thickness
1.Aavin milk.	5.18	1.14
2.Benny buttermilk.		
3.Bifilac.	2.91	0.758
4.Darolac.	2.27	0.505
5.Ranipet curd.	3.03	0.632
	5.18	1.14

Biochemical test results

	MTCC 2997	AAVIN	RANIPET	BENNY	BIFILAC	DAROLAC
VP TEST	Positive	Positive	Positive	Positive	Positive	Positive
MR TEST	Positive	Positive	Positive	Positive	Positive	Positive
CITRATE UTILIZATION TEST	Negative	Negative	Negative	Negative	Negative	Negative
CATALASE TEST	Negative	Negative	Negative	Negative	Negative	Negative
GROWTH AT 15°C	Negative	Negative	Negative	Negative	Negative	Negative
GROWTH AT 45°C	Positive	Positive	Positive	Positive	Positive	Positive

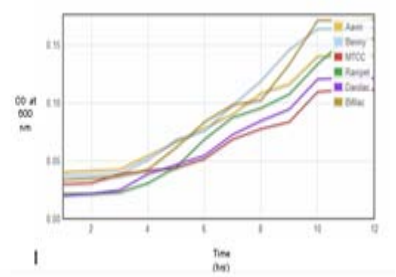
Variation of absorbance at 600 nm with time

At every hourly interval, for 12 hours, the absorbance at 600 nm of the milk inoculated with *Lactobacillus* MTCC 2997 and *Lactobacillus* isolated from Aavin and Ranipet curd, Benny buttermilk, Bifilac and Darolac was checked and the growth curve plotted.

	MTCC 2997	AAVIN	DAROLAC	BIFILAC	RANIPET	BENNY
1 hour	0.030	0.041	0.020	0.034	0.022	0.038
2 hours	0.031	0.042	0.022	0.035	0.022	0.038
3 hours	0.039	0.043	0.025	0.037	0.023	0.039
4 hours	0.042	0.055	0.039	0.043	0.031	0.052
5 hours	0.044	0.067	0.047	0.062	0.045	0.069
6 hours	0.052	0.079	0.055	0.084	0.069	0.077
7 hours	0.069	0.091	0.073	0.099	0.088	0.099
8 hours	0.078	0.109	0.065	0.103	0.096	0.120
9 hours	0.084	0.117	0.042	0.134	0.108	0.147
10 hours	0.110	0.141	0.121	0.171	0.134	0.165
11 hours	0.112	0.141	0.122	0.172	0.156	0.165
12 hours	0.112	0.142	0.121	0.173	0.156	0.166

Below are the graphs of OD vs Time for each *Lactobacillus sp.* source:

Fig. 1: Comparative graph of *Lactobacillus sp.* From all 6 sources



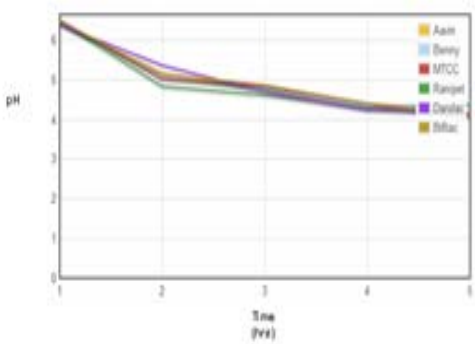
Variation of pH with Time

At every hourly interval, the pH of the milk inoculated with *Lactobacillus* MTCC 2997 and *Lactobacillus* isolated from Aavin and Ranipet curd, Benny buttermilk, Bifilac and Darolac was seen to decrease indicating the production of lactic acid. Following is a table showing the change in pH with time.

Table 4: Values of pH obtained

SOURCE/ TIME	MTCC 2997	AAVIN	RANIPET	BENNY	BIFILAC	DAROLAC
After 1st hour	6.53	6.43	6.48	6.50	6.50	6.40
After 2nd hour	5.03	5.19	4.85	5.07	5.16	5.38
After 3rd hour	4.86	4.75	4.63	4.81	4.88	4.71
After 4th hour	4.42	4.32	4.37	4.46	4.41	4.24
After 5th hour	4.18	4.10	4.30	4.33	4.13	4.16

Fig. 2: Comparative graph of *Lactobacillus sp.* From all 6 sources



Bifilac shows the most lactic acid production since its pH drops to the lowest value of 4.13 after 5 hours.

Study of the amount of free lactic acid produced in milk

Table 5: Values of free lactic acid produced per 100 cc of milk

	Bifilac	Darolac	Aavin	Benny	Ranipet	MTCC 2997
1 hour	0.65 cc.	1.96 cc.	1.31 cc.	1.31 cc.	1.31 cc.	2.62 cc.
2 hours	1.96 cc.	2.23 cc.	1.57 cc.	1.83 cc.	1.83 cc.	3.93 cc.
3 hours	2.1 cc.	2.36 cc.	2.10 cc.	2.1 cc.	2.1 cc.	8.13 cc.
4 hours	21.0 cc.	7.87 cc.	3.93 cc.	8.4 cc.	3.61 cc.	16.8 cc.
5 hours	26.2 cc	13.9 cc.	13.12 cc.	18.9 cc.	23.6 cc.	34.12 cc.

Hence, it was seen that the most amount of lactic acid of 34.12 cc for 100 cc. of milk after 5 hours, was produced by *Lactobacillus* MTCC 2997 and nearest to it is *Lactobacillus sp.* from Bifilac that produces 26.2 cc for 100 cc of milk.

Variation in Colony Count with Time

Colonies were counted using the colony counter and the following results obtained.

Table 6: Colony count

	After 2 hours	After 4 hours	After 6 hours	After 8 hours	After 10 hours	After 12 hours
Bifilac	54	137	201	369	422	430
MTCC 2997	61	141	250	400	439	444
Aavin	72	169	279	391	470	480
Ranipet	65	150	220	303	412	420
Benny	40	123	200	319	405	410
Darolac	47	130	222	336	400	403

FTIR Spectra Analysis

The prominent groups of lactic acid are the carboxylic group (OH-C=O) which lies in the range of wavenumbers 1760 – 1670 cm^{-1} and the alkyl group ($-CH_3$) which lies in the range of wavenumbers 2960 – 2850 cm^{-1}. The detection and increase of these peaks in the spectra obtained by FTIR indicate the positive production of lactic acid. An increase in peak height is directly proportional to an increase in quantity.

Comparison of spectra on 1st hour and 7th hour

Fig. 3: MTCC samples at 1st hour and 7th hour

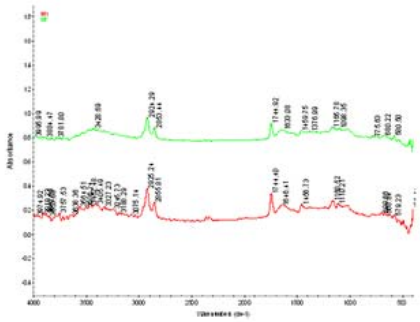


Fig. 4: Comparison of Darolac samples at 1st hour and 7th hour

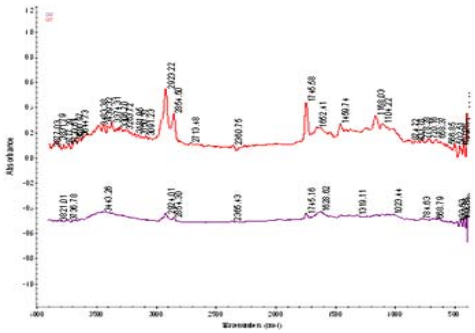


Fig. 5: Comparison of Ranipet samples at 1st hour and 7th hour

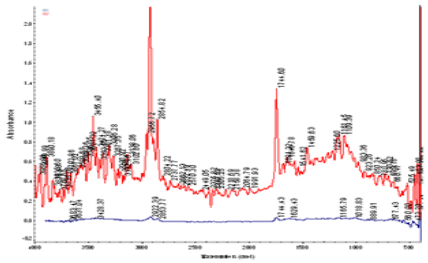


Fig. 6: Comparison of Aavin samples at 1st hour and 7th hour

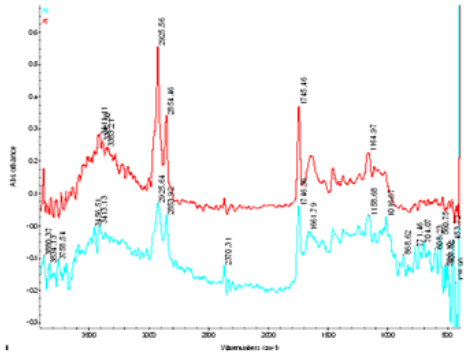


Fig. 7: Comparison of Bifilac samples at 1st hour and 7th hour

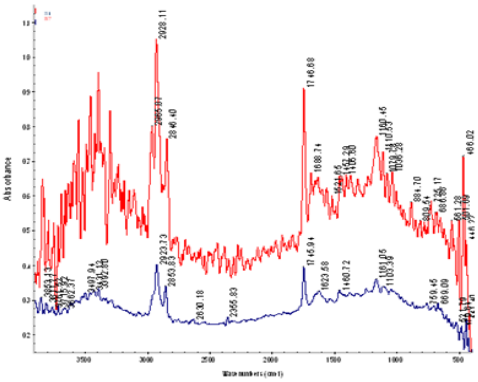
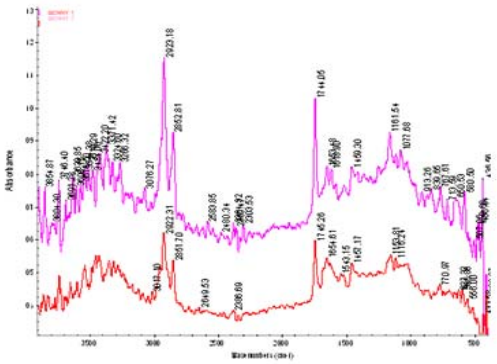


Fig. 8: Comparison of Benny samples at 1st hour and 7th hour



Statistical Analysis Using SPSS Statistics Data Editor

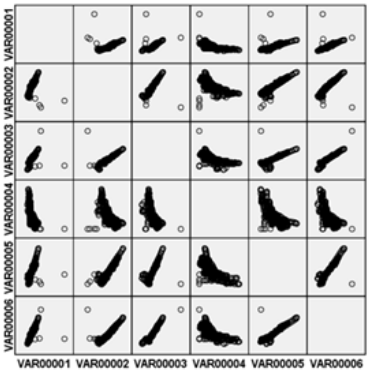
The spectral data obtained from FTIR was converted into excel files giving individual wave numbers and peak heights. Statistical analysis was used to obtain confirmatory results of the theoretical analysis obtained from the spectra of FT-IR. Scatter plots, similarity matrices, curve analysis, distance correlations and hierarchical analysis was done.The excel file peak height data was divided into three sets- the first set corresponding to wavenumbers 1800-399 cm^{-1} **,** the second set corresponding to 2700-1801 cm^{-1} and the third set corresponding to 3900-2710 cm^{-1}. The average of the peak heights of each source was taken. *Example:* Average of peak heights of Set1 for A_0 to A_7 is taken for Aavin.

Similarity Matrices and Correlation

Table 7: Similarity matrix

	Correlation between Vectors of Values					
	Aavin	Bifilac	Benny	Darolac	MTCC 2997	Ranipet
Aavin	1.000	.645	.730	-.652	.753	.787
Bifilac	.645	1.000	.851	-.707	.943	.899
Benny	.730	.851	1.000	-.695	.860	.966
Darolac	-.652	-.707	-.695	1.000	-.726	-.723
MTCC 2997	.753	.943	.860	-.726	1.000	.937
Ranipet	.787	.899	.966	-.723	.937	1.000

The scatter plot for all possible combinations of sources is shown below:



VAR00001 – Aavin; VAR00002 – Bifilac; VAR00003 – Benny; VAR00004 – Darolac; VAR00005 – MTCC 2997; VAR00006 – Ranipet

For the region between wavenumbers 1800 – 399 cm^{-1}, the scatter plot and similarity matrix shows the highest strong positive correlation of 0.966 between *Lactobacillus sp.* isolated from Benny buttermilk and that of Ranipet curd hence these two strains have a very similar lactic acid production rate. Some other strong positive correlations of 0.943 and 0.937 also occur between *Lactobacillus* MTCC 2997 and *Lactobacillus sp.* isolated from Bifilac and between *Lactobacillus* MTCC 2997 and *Lactobacillus sp.* isolated from Ranipet curd respectively.

Similarly the same correlation is carried for different sets.(set2 and set3).

Table 8: Comparing results for all 3 sets

	1st highest correlation	2nd highest correlation	3rd highest correlation
1800-399 cm^{-1}	Benny and Ranipet	MTCC and Bifilac	MTCC and Ranipet
2700-1801 cm^{-1}	MTCC and Bifilac	Bifilac and Benny	MTCC and Benny
3900-2710 cm^{-1}	Bifilac and Benny	MTCC and Benny	MTCC and Bifilac

Curve Fit and Estimation

In this method, *Lactobacillus* MTCC 2997 was used as a reference to compare *Lactobacillus sp*. isolated from the other sources and their similarity to *Lactobacillus* MTCC 2997. *Lactobacillus* MTCC 2997 was used as the independent variable. The Curve fit and ANOVA table was obtained.

Aavin and MTCC

Fig. 9: Curve fit for Aavin and MTCC

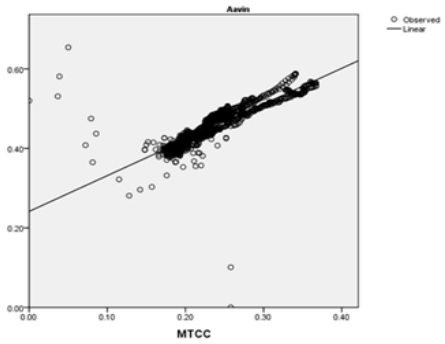
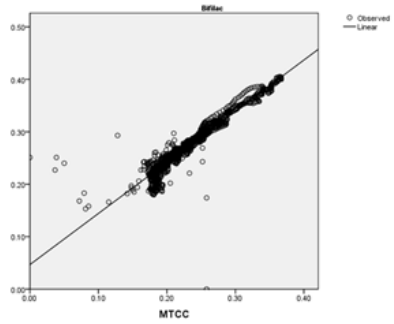


Fig 53: Curve Fit for Aavin vs MTCC

Lactobacillus MTCC 2997 and *Lactobacillus sp.* isolated from Aavin milk were 85.7% similar.

Bifilac and MTCC

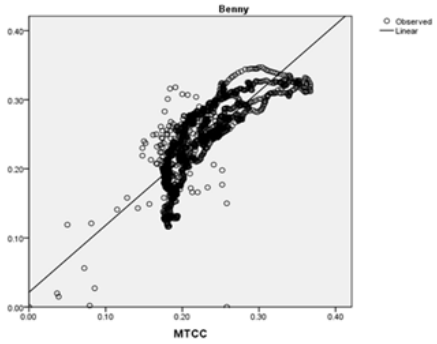
Fig. 10: Curve fit for Bifilac and MTCC



Lactobacillus MTCC 2997 and *Lactobacillus sp.* isolated from Bifilac were 94.6% similar.

Benny and MTCC

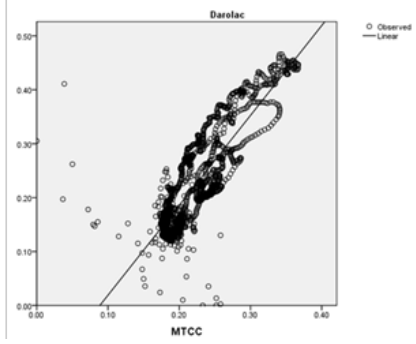
Fig. 11: Curve fit for Benny and MTCC



Lactobacillus MTCC 2997 and *Lactobacillus sp.* isolated from Benny buttermilk were 80.8% similar.

Darolac and MTCC

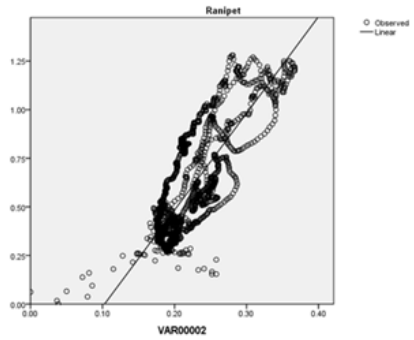
Fig. 12: Curve fit for Darolac and MTCC



Lactobacillus MTCC 2997 and *Lactobacillus sp.* isolated from Darolac were 84.8% similar.

Ranipet and MTCC

Fig. 13: Curve fit for Ranipet and MTCC

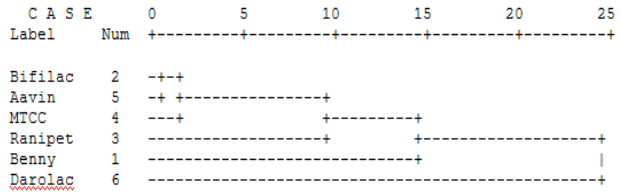


Lactobacillus MTCC 2997 and *Lactobacillus sp.* isolated from Darolac were 82.7% similar.

Hierarchical cluster analysis

Fig 14: Dendrogram using Average Linkage (Within Group)

Rescaled Distance Cluster Combine



From the above dendrogram, it is seen that Bifilac and MTCC 2997 are closely related. This is generated by applying group linkage method.

Conclusion

Lactobacillus is a widely used probiotic organism which produces lactic acid and causes curdling of milk. It is used commercially for curing lactose intolerance and to maintain the intestinal pH. The sample processing in our project was done using lyophiliser. Characterization is done by using conventional biochemical tests which have shown lacto bacillus positive.The method of FTIR for characterization of organisms is adopted . The importance of this method is that it states the amount of similarity between the organisms using statistical analysis. Based on this similarity percentage, measured in terms of Pearson correlation, we can predict the strain type, differentiate up to the sub species level, which was not possible with biochemical characterization. Hence Fourier Transform Infrared Spectroscopy was found to show spectral changes as an organic reaction proceeds, here in case of fermentation, which can also be used for industrial purposes with further improvement.

Future work

Though FTIR is a rapid technique to characterize and analyze microorganisms sample processing time can be reduced by handling other techniques. These techniques are already available, but simple and cheaper techniques should be devised which will make this technique an inevitable one in future generation. Product quality can be assessed in quality assurance departments and in research laboratories it can be used to study metabolic changes in a microorganism.

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