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Production of Fruity Aroma by Non Lactose Fermenting *Lacto bacilli* Species Isolated from South India Hills

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Abstract

The study describes about the discovery of fruity aroma producing micro organisms and determination of their chemical structure. Fresh flowers, pollen, over-ripe fruits, water stream and leave samples were isolated from pollution free low temperature regions of Nilgris hills of Tamil Nadu state by adopting selective isolation media. The sensory evaluation of fruity aroma odor was evaluated by primary and secondary screening methods. Non lactose fermenting lactobacilli was found to produce volatile fruity aroma. The volatile compound was extracted in diethyl ether and GC-MS analysis was done. As a result of GC-MS analysis the compound was identified as Benzoic acid, 4 ethoxy ethyl ester.

Keywords: Fruity aroma, Volatile organic compounds

Introduction

Flavors and fragrances are important to the food, cosmetic, pharmaceutical and chemical industries. Although chemical synthesis remains important, there has been a trend in consumer preference toward natural flavors motivating the biotechnological development of aromas. Natural aroma production can be obtained from enzymes, plant cell cultures and microorganisms[1].Few strains of microbial species produce volatile fruity aromas during their growth in culture media (Schindler and Schmid1982).The existing technology for the production of flavors is mainly based on natural plant materials where seasonal and other fluctuations are major disadvantage.

On the other hand, synthetic chemical flavor products lack stereo selectivity. For these reasons, the microorganisms are potential sources of natural fruit essences.[8]

Microorganisms can synthesize flavours as secondary metabolites during fermentation on nutrients such as sugars and amino acids. Amino acid catabolism by *Lactococcus lactis* and mesophilic lactobacilli has been studied recently (S. Gummalla and J. R. Broadbent, 1996). In these lactic acid bacteria (LAB), amino acid catabolism is mainly initiated by a transamination reaction, which requires the presence of a keto acid as the amino group acceptor.[6]The key aroma compounds identified in Swiss-type cheese results mainly from the catabolism of branched-chain amino acids (3-methylbutanal, is butyric acid, isovaleric acid, and their derived esters), methionine (methional, methanethiol, and Dimethyl trisulfide), and phenylalanine (phenyl acetic acid and phenyl acetaldehyde). Most of these compounds are considered beneficial to the cheese flavor. Isobutyric acid and isovaleric acid participate in the sweaty and strong cheese notes, respectively, while 3-methylbutanal has a malty aroma.[3]

All the other non-conventional sources such as free-living microbial cells, including food-grade micro-flavored volatile organic compounds are recognized as alcohols and esters are presently increasing in considerable interest from an industrial viewpoint demand. Because they were widely used in the food, beverage, pharmaceutical and cosmetic industry (Scholler et al. Welsh et al. Vandamme; Lomascolo et al.) Consequently, in the past key ingredients, year most industries have carried out extensive in the size of the flavor market being worldwide estimating the screening programmes on naturally occurring micromated at 9.7 billion US$ in 1994 (Cheetham 1997).

The VOC's (Volatile Organic Compound) were chemically synthesized by the food processing industries. But the significant trend in consumer preferences was towards the natural flavours extracted from plant sources. However, compounds extracted from plant sources (e.g. fruits) flavored VOC producers face the problems of seasonal and regional variation (Comi et al. 2001; Rojas et al. 2001).[4]

Hence GRAS microorganism based technologies for the production of flavored VOC are important to limit the agriculture limitations. The present study describes the discovery of fruity aroma producing micro organism by miniaturized screening by sensory evaluations and determination of their chemical structure and GCMS analysis of the compound.

Materials and Methods

The samples were collected from top hills at Nilgris of Tamil Nadu state. Around 30 samples of fresh flower pollen, over-ripe tropical fruits and plants, water, twig, bark, leaf and soil samples were collected in sterile polythene bags. The samples were carefully transferred to Microcore R&D lab under frozen packing. Samples were shaken for 15-30min on sterile reverse osmosis water and processed by axenic culture techniques on a spread of autoclaved media.

Primary screening

5g/L of Soluble Starch (Sdfine), 5g/L of Glucose (Sdfine), 3g/L of Soya flour d/t (Sdfine), 5g/L of Yeast Extract (Hi-media), 7g/L of Peptone (Hi-media) , 0.05 g/L of MgSO4,0.05g/L of NaCL(Merck), 27g/L of Agar agar (Hi-media) with 0.0001g/L Chloramphenicol (Sigma) and 0.0001g/L Azithromycin (Sigma) prepared at pH 6 and inoculated with serially diluted samples are plated and incubated at 30°C for 2 days. Totally 389 colonies are observed in 500 plates and examined for the sensory evaluations.

Secondary screening

Primary screening media with increased peptone concentration 10 g/L of Peptone (Hi-media) was plated aseptically. The templates of the 7 slimy colonies suspected for the fruity odor was transferred to secondary screening media and also secondary screening broth prepared in a shake flask of 100 ml capacity. Among the isolated the single isolate IS39 was found to produce fruity odor, observed by direct sensory evaluation. The isolate is preserved at liquid vial preparation and stored at -80 and named as MRLICC-05 (Microcore Research Laboratories Culture Collection).

Morphology

The fruity aroma producer MRLICC-05 was observed under compound microscope (Automated Magnus MIPS-USB) it is a white to creamy colonies. The strain was identified as Gram positive *Lacto bacilli, non- spore forming, non-shiny white colonies.*

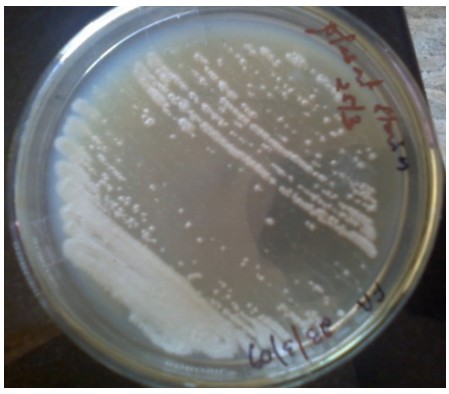

Image 1: MRLICC-05 Fungal Agar Media

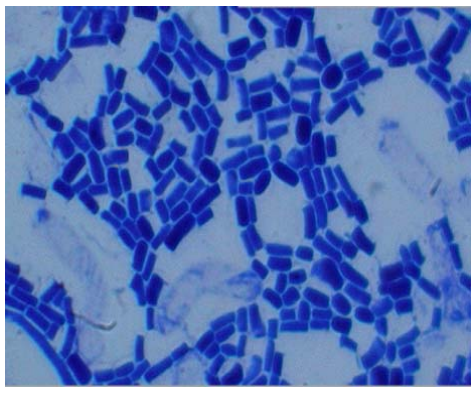

Image 2: MRLICC-05 Gram Stain

Biochemical Characterization of MRLICC-05

The fruity aroma producing strain MRLICC-05 was grown in media containing various carbohydrate sources and tested for the fermentation and also sensory aroma production and the results are tabulated.

Table No 1: Carbohydrate Fermentation of MRLICC-05

S.No	Carbohydrates	24 hrs	48 hrs
1	Glucose	(++)	(++)
2	Sucrose	(+)	(+)
3	Lactose	(-)	(-)
4	Glycerol	(+)	(++)
4	Maltose	(+)	+
5	Fructose	(+)	(++)
6	Soluble starch	(+)	(+)
7	Cellulose	(-)	(-)
8	Carboxyl methyl cellulose	(+)	(+)
9	Trehalose	(-)	(-)
10	Arabinose	(-)	(-)
11	Mannitol	(+)	(+)
12	Xylose	(++)	(++)

The table No-1 indicates that the MRLICC-05 ferments glucose and Xylose at 48 log hrs for high odor production and it do not ferments Arabinose, Trehalose, Cellulose and Lactose.

Physiological test

The MRLICC-05 was inoculated in following media that includes Nutrient Agar, DYMA Agar, PDA Agar, Fungal Agar, Oat meal Agar, Skim Milk Agar, Muller Hinton Agar, Yeast extarct Agar, Czapeck Dox Agar. The organism has growth in all the above mentioned media but fruity aroma production was noted in few media only.

Table 2: Sensory Evaluation on Media

S. No	Media	Aroma Odor
1	Nutrient Agar	-
2	DYM Agar	-
3	PDA Agar	+
4	Fungal Agar	+
5	Oat meal Agar	+
6	Skim Milk Agar	-
7	Muller Hinton Agar	-
8	Yeast extract Agar	+
9	Czapeck Dox Agar	-

The results illustrates that MRLICC-05 produced strong aroma in Potato dextrose agar media (PDA), fungal agar media (FA), Oat meal agar media and Yeast Extract Agar Media. It is evident that the peptone and oligo-peptides increased the enhanced production of fruity aroma. The best odor production was noted in fungal agar (FA) media.

Media Optimization

The strain MRLICC-05 is tested with various concentration of the organic protein (peptone) and tested by sensory evaluation of the Fruity odor is deducted and tabulated below. The organism produces aroma at the case where 10g/L of peptone (Hi-media) and 10g/L of CSP (Sdfine) the organism produces strong aroma odor and tabulated below

Table 3: Peptone Concentration optimization

S. No	Peptone Concentration (g/L)	pH	PCV (%)	Log Hrs	Aroma
1	Nil	3.93	10	48	Very less
2	10	4.2	13	48	Normal
3	10	7.21	12	66	No odor
4	20	7.64	3.89	48	Strong
5	20	7.64	7.1	66	No odor
6	10g/L Peptone+10g/L Corn steep Powder	4.1	13	48	Strong flavor

Results of the Table No 2 indicates that the peptone plays a critical roll in increasing the aromas production and hence the peptone concentration is optimized in the following The media optimization process evident that the organism produces strong odor where the media contains 10g/L of peptone (Hi-media) and 10g/L of CSP (Sdfine).

Shake flask production

MRLICC-05 is grown in the optimized media for the mass production of the aroma compound by submerged fermentation in shake flask. The flask was inoculated and incubated in (Orbitek Shaker) 200RPM at 30˚C for 48hrs.On the 48^{th} log hr the extreme aromatic odor was produced and followed by extraction.

Compound Extraction & GCMS Analysis

The broth was extracted at 48^{th} log hr in diethyl ether and concentrated in rotatory evaporator (Roteva) and analyzed for GCMS Gas chromatograph (Model: Agilent Technologies 6890N Network GC SystemTM) equipped with a mass spectrometer (Model: Agilent 5973 Network TM) with nonpolar capillary column (HP5- MS; 30 m by 0.25 mm; 0.25-µm film thickness) at a helium flow rate of 8 psi. The oven temperature needs to be held at 35ºC for 5 min programmed to rise from 35 to 240ºC

at 7ºC/min and the final temperature maintained for 5 to15 minutes. Data Analyzed by comparing the mass spectrum of the compounds with the Wiley 275 L mass spectral data base and NIST MS Search Program (2002) or the latest Ref 2008.

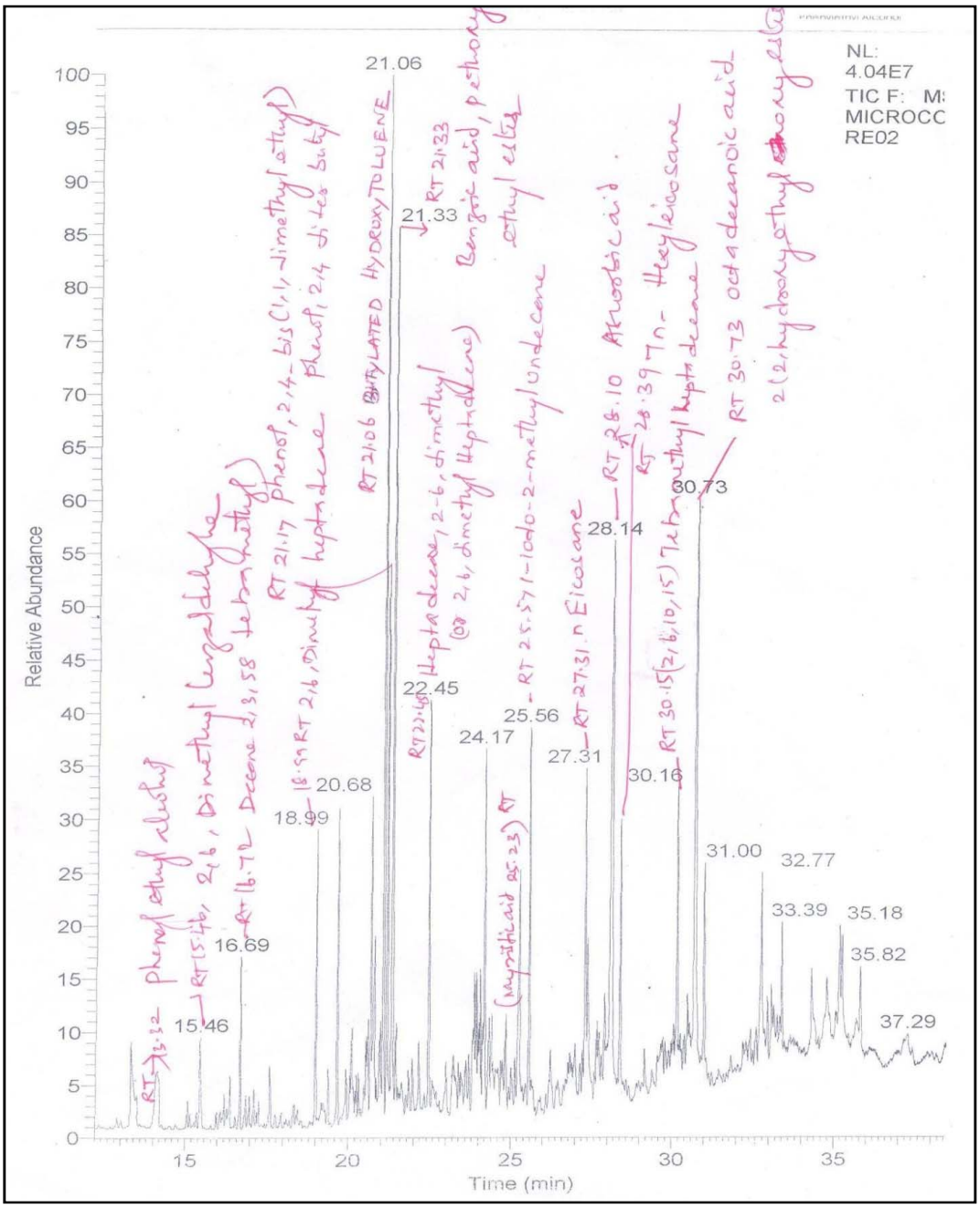

Image 3: GCMS Chromatogram

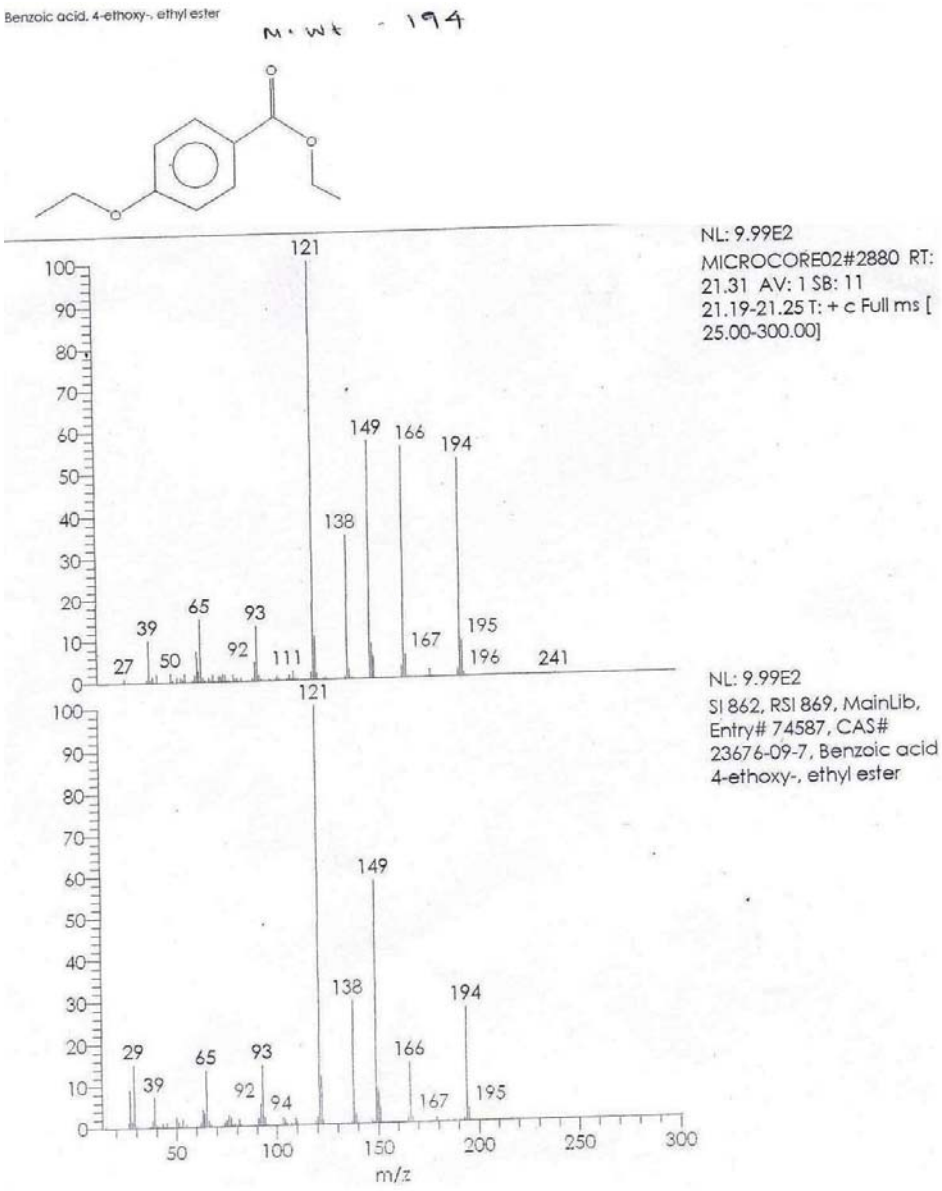

Image 4: Fragmentation pattern Benzoic acid, 4-ethoxy ethyl ester

Results and Discussion

Lactobacilli strain isolate of fruity aroma producing microorganism was extracted in diethyl ether and the GC-MS Chromatogram was obtained hence compounds in volatile samples are chemically identified as Benzoic acid, 4-ethoxy ethyl ester (RT 21.31).

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