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ISOLATION, SCREENING AND CHARACTERIZATION OF RIBOFLAVIN PRODUCING LACTIC ACID BACTERIA FROM KATPADI, VELLORE DISTRICT

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Abstract
Riboflavin is a basic component of the cellular metabolism since it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Riboflavin producing lactic acid bacteria are of potential importance to the fermented food industry since the chemical processes is being replaced by the fermentative biotechnological methods. The present study was carried out to identify and characterize the best riboflavin producing lactic acid bacteria from the yoghurt samples of the Vellore district. The strains isolated belong to the genus *Lactobacillus, streptococcus, Pediococcus*, and *Leuconostoc*. *Lactobacillus* was the predominant strain. The best riboflavin producing strain was characterized to be *Lactobacillus fermentum* by both biochemical as well as molecular methods from the Institute of microbial technology (IMTECH), Chandigarh, India and deposited with the accession number MTCC 8711. The strain could be a better starter replacing the conventional lactic acid bacteria where in the fermented foods could be naturally fortified with the riboflavin.

Key Words: Lactic acid bacteria – Riboflavin – *Lactobacillus sp.*

Introduction
Lactic acid bacteria (LAB) are a class of industrially prominent microorganisms used in food and dairy industry owing to their fermentative properties. LAB are considered as generally regarded as safe (GRAS) organisms and their application as probiotics is immense as they play major role in maintaining health [1]. They produce a range of metabolites that are collectively termed as 'nutraceuticals', which include B vitamins like riboflavin (B2), folate (B11) and cobalamine (B12), low calorie sugars like mannitol and sorbitol, exopolysaccharides, diacetyl and L-alanine. One of the vital nutraceutical produced by LAB, namely the riboflavin (Vitamin B_2) is an indispensable component in the human diet, which is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The recommended daily allowance of riboflavin is 1.6 mg and the deficiency of riboflavin leads to loss of hair, inflammation of the skin, sore throat, hyperemia, edema of oral and mucous membranes, cheilosis and glossitis [2].

Although riboflavin is found in a wide variety of foods, riboflavin deficiency is common in many parts of the world, particularly in developing countries [3]. The deficiency can be treated with dietary supplement of riboflavin or by consuming fermented foods such as cheese, yogurt fortified with riboflavin in the daily diet. Riboflavin has traditionally been produced by chemical processes, but in recent times this has been replaced by the microbial fermentation processes [4]. This study was aimed to isolate riboflavin producing LAB strains from yoghurt samples.

Materials and Methods

Sample collection and maintenance
Thirty seven fermented milk samples were collected from the individual house holds in rural parts of Katpadi, in the Vellore district, Tamilnadu, India. Samples were obtained in duplicates in sterile glass vials and stored at 4ºC until they were used in the experiments.

Isolation of Lactic acid bacteria
The fermented milk samples were homogenized in a homogenizer (Polytron). The homogenized samples were serially diluted using physiological saline (0.9%) and plated in *Lactobacillus* MRS [5] and M17 [6] agar plates. The plates were incubated at 37ºC for 48 hrs.

Forty eight colonies were randomly selected from all the MRS and M17 plates of lower dilutions. Selected colonies were grown in MRS and M17 broth and the purity was checked by streaking on the respective agar plates. Glycerol stocks of the isolates were prepared with 50% glycerol and maintained at -20°C. A set of MRS stabs were also made and stored at 4°C for use as working culture. These forty eight strains were subjected to the genus level identification of LAB and further screening of riboflavin producing ability in the modified chemically defined medium [7].

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Genus level identification of LAB

LAB were identified based on the differentiation scheme up to genus level as described by Schillinger and Lucke [8] which included Gram's staining, catalase test, gas production test, arginine test, growth studies on 6.5% sodium chloride, growth at 10°C and growth at 15°C.

Riboflavin assay

Riboflavin producing ability of the isolates was estimated as described by Sauer et al. [9]. In brief, 0.8 ml of culture broth was mixed with 0.2 ml of 1 M NaOH. A 0.4-ml vol of the resulting solution was neutralized with 1 ml of 0.1 M potassium phosphate buffer (pH 6.0), and the absorbance at 444 nm was measured. The riboflavin concentration was calculated using an extinction coefficient of 1.04×10^{-2} $M^{-1}cm^{-1}$.

Screening of riboflavin production by the isolates

The capability of LAB to synthesize riboflavin varies, depending on the species or even the strain. This was best determined by growth on chemically defined medium in the presence or absence of the vitamin i.e. riboflavin. The chemically defined medium was prepared according to Otto et al [7] by omitting riboflavin. About 1% of the overnight inoculums of the respective cultures were inoculated into the sterile medium and incubated at 37ºC under static conditions. Culture samples were analyzed for the presence of riboflavin after 24 hrs of growth.

Characterization of the best riboflavin producer

The biochemical as well as the molecular characterization of the best riboflavin producing strain was carried out at the Institute of Microbial Technology (IMTECH) Chandigarh, India. The 16S rRNA sequence has been submitted to the GenBank with the accession number GU21340.

Results and Discussion

Isolation of Lactic acid bacteria

Forty eight LAB strains were subjected for the genus level identification using the differentiation scheme for genus level identification (Fig.1) of LAB. Based on the results for the physiochemical test for the genera level identification, the strains were identified up to their genus level which is presented in Table 1. Among the forty eight strains, fifteen were found to be *Enterococcus sp.* which was omitted for further studies. Seven strains were *Lactobacillus* (hetero fermentative), *Leuconostoc* were two in numbers. Fifteen strains were *Lactobacillus* (homo fermentative) and three strains belonged to *Streptococcus sp.* and five were *Pediococcus sp.* Among the group, *Lactobacillus sp.* was predominant.

Table 1. Identification of genus for the strains isolated from fermented milk

Genus	Isolates
Lactobacillus (heterofermentative)	2,3,11,19,35,44,45,48
Leuconostoc	7,36
Lactobacillus (homofermentative)	5,6,13,14,15,18,22,23,24,25,27,28,30,31,32
Streptococcus	16,29,43
Enterococcus	1,8,9,12,20,21,26,33,34,37,38,39,40,41,46
Pediococcus	10, 4,17,42,47

Screening of riboflavin producing LAB

The results of the screening are presented in Table 2. Excluding the fifteen *Enterococcus sp.* the remaining thirty three strains were subjected to screening for their ability to produce riboflavin by growing them in the chemically defined medium short of riboflavin [7]. Nine strains were not able to survive in the chemically defined medium short of riboflavin exhibiting their inability to synthesize the vitamin. The levels of riboflavin in the remaining twenty four strains were assayed after 24 hours and the best riboflavin producing strain was identified as the strain number forty eight belonging to the genus *Lactobacillus sp.* The strain forty eight produced 2.29 mg/L of riboflavin after 24 hrs of growth (A_{600} - 0.847) in the modified chemically defined medium.

Table 2. Screening of riboflavin production by LAB

Isolates	A_{600} nm at 24h	Concentration of riboflavin in mg/L at 24h
2	0.311	0.64
3	0.26	0.60
4	0.31	0.64
5	0.26	0.60
6	0.21	0.55
7	0.23	0.57
10	0.25	0.89
11	0.32	0.68
13	0.20	0.54
14	0.28	0.61
15	0.29	0.62
16	0.25	0.89
17	0.50	0.76
18	0.50	0.76
19	0.20	0.54
22	0.53	0.76
23	0.51	0.76
24	0.28	0.61
25	0.30	0.64
31	0.49	0.68
32	0.10	0.45
44	0.49	0.68
45	0.10	0.45
48	**0.847**	**2.29**

Table 3. Morphological and biochemical characteristics of the strain MTCC 8711.

Characteristics	Response
Gram reaction	+
Shape	Rod
Endospore	–
Motility	–
Indole test	–
Methyl red test	–
Voges Proskauer test	–
Citrate utilization	–
Casein hydrolysis	–
Esculin hydrolysis	–
Nitrate reduction	+
Catalase test	–
Oxidase test	–
Arginine dihydrolase test	–
Ornithine decarboxylase test	–
Acid from Glucose	–
Fructose	–
Lactose	+
Sucrose	+
Cellobiose	+
Maltose	+
Mannitol	–
Salicin	–
Rhamnose	–
Galactose	–
Arabinose	–
Melebiose	–

Characterization of the Riboflavin producing strain

The strain was characterized based on the biochemical and physiological characterization at the Institute of microbial technology (IMTECH, Chandigarh, India) up to the species level. The morphological and physiological characteristics of the strain are listed in Table 3. Microbial Type Culture Collection (MTCC) centre at the Institute of Microbial Technology, Chandigarh, India, had confirmed it as *Lactobacillus fermentum* and the culture has been deposited with the accession number MTCC 8711.

Molecular characterization of the strain was also carried out at the Institute of microbial technology (IMTECH, Chandigarh, India) using the universal bacterial 16S rDNA primers. Genomic DNA of the strain MTCC8711 was extracted and the gene coding for 16S rRNA was PCR amplified using the universal bacterial 16S rDNA primers.

The amplified fragment was sequenced. The sequence was compared with previously published bacterial 16S rDNA sequences at seqmatch server (www.rdp.cme.msu.edu/seqmatch) of the Ribosomal Database Project (RDP). The 16S rDNA sequence of the MTCC 8711 exhibited highest similarity with a strain of *Lb fermentum* NRIC 0135 (99.9%) and also with the 16s rRNA of the other *Lb. fermentum* strains. The results of 16S rDNA based phylogenetic analysis are presented as the similarity matrix in Table 4 and as the phylogenetic tree in Fig. 2. In phylogenetic analysis, the strain MTCC8711 formed a separate cluster along with other reported strains of *Lb. fermentum*. Therefore, the riboflavin producing bacterium was designated as *Lb. fermentum* MTCC 8711 and its 16S rRNA gene sequence has been submitted in GenBank (Accession No. GU21340).

Table 4. Sequence similarity matrix of 16S rDNA of *Lb. fermentum* MTCC 8771 with the related bacterial strains*

Organisms	1	2	3	4	5	6	7	8	9	10	11
1. *Lb. fermentum* MTCC 8711	-										
2. *Lb. fermentum* NRIC 0135 (AB362616)	99.99	-									
3. *Lb. fermentum* K3 (EU621850)	99.84	99.84	-								
4. *Lb. brevis* T10-3 (AB368912)	92.05	92.05	91.18	-							
5. *Lb. plantarum* T14-8 (AB368910)	90.99	90.99	90.92	95.03	-						
6. *Lb. paracasei* T2-2 (AB368899)	90.71	90.71	90.57	92.20	92.61	-					
7. *Lb. johnsonii* FI9785 (NC_013504)	90.54	90.54	90.47	89.93	90.62	89.01	-				
8. *Lb. pentosus* 124-2 (NR_029133)	91.22	91.22	91.15	95.03	99.77	92.76	90.70	-			
9. *Lb. delbrueckii* NCC725 (NR_029106)	90.03	90.03	89.96	89.55	88.87	89.62	92.51	89.10	-		
10. *Lb. harbinensis* T12-11 (AB368916)	90.38	90.38	90.23	92.20	92.23	93.15	89.10	92.44	89.32	-	
11. *Lb. parabuchneri* T9-11 (AB368914)	91.38	91.38	91.31	95.33	93.82	93.36	90.50	93.97	89.42	92.74	-

*GenBank accession numbers are given in parentheses. The values are given in percentage sequence identity.

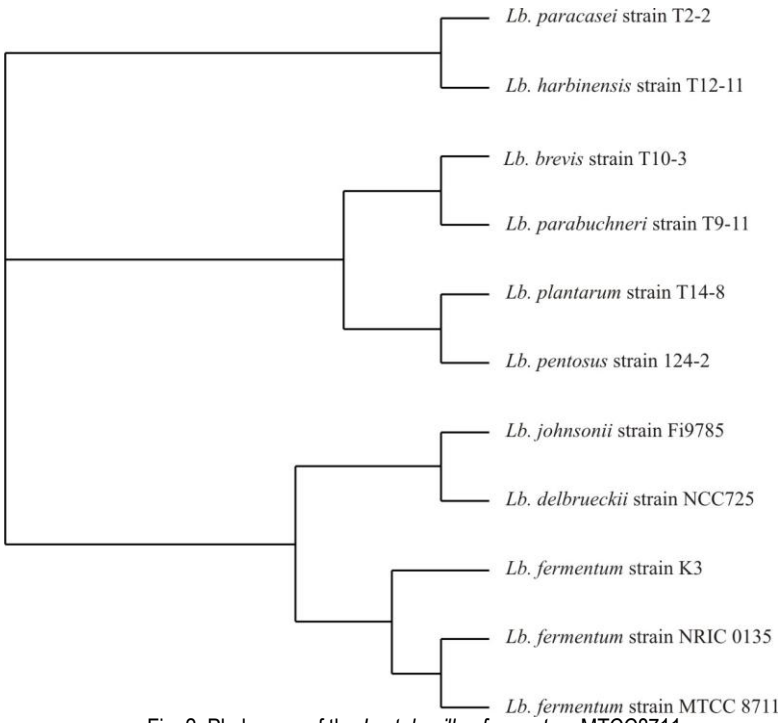

Fig. 2. Phylogram of the *Lactobacillus fermentum* MTCC8711

Growth pattern and riboflavin production of *Lb. fermentum* MTCC 8711

Lactobacillus MRS broth [5] is an optimized and selective media known for the cultivation of *Lactobacillus sp*. Fermentative production of riboflavin by the strain *L. fermentum* MTCC 8711 was carried out in 250-ml Erlenmeyer flask containing 50 ml of MRS broth. The MRS broth consisted (g L^{-1}) peptone, 10; beef extract, 10; yeast extract, 5; tween 80, 1; glucose, 20; sodium acetate, 5; ammonium citrate, 2; K_2HPO_4, 2; magnesium sulphate, 0.2; manganese sulphate, 0.05. Riboflavin was estimated in the culture supernatant, and the samples were collected at every four hours interval.

The fermentation pH was maintained at 6.5 and temperature at 37° C. The strain produced maximum riboflavin (2.8 mg/L) with A_{600} of 1.75 at 16 hours of fermentation in the *Lactobacillus* MRS broth (Fig. 3).

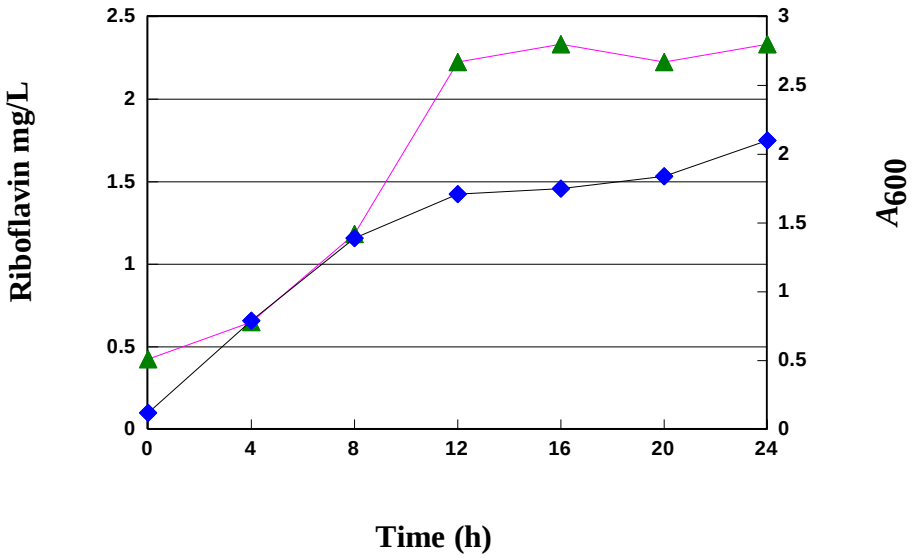

Fig. 3. Kinetics of growth and riboflavin production by *Lb. fermentum* MTCC 8711. The fermentation was carried out under static conditions with the *Lactobacillus* MRS broth at pH 6.5 and 37°C. Cells were harvested at 4 h intervals and the growth (♦); riboflavin production (▲) was estimated.

Thus the LAB with riboflavin synthesizing ability was isolated from the yoghurt samples of the Vellore district. The organism could be further exploited for the enhanced production of riboflavin using various strain improvements strategies that could result in the development of a better starter stain in the fermented food industry. LAB with riboflavin synthesizing ability could better replace the conventional strains that are being employed in the LAB based fermented products.

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