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BIO PROSPECTING OF RIBOFLAVIN PRODUCING BACTERIA FROM DIFFERENT RIBOFLAVIN ENRICHED FOOD SOURCES

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Abstract

Riboflavin is an essential, water-soluble vitamin (B_2) and a component of basic cellular metabolism. The aim of the present study is to isolate and characterize riboflavin producing bacteria from different food sources. Ten different riboflavin enriched food sources were collected from Vellore district. Totally 72 bacterial strains were isolated and cultured on nutrient agar plates. Out of these, 43 strains were identified as riboflavin producers. Isolated bacterial strains HDS27, HDS07, HDS14, HDS18, HDS38 and HDS54 isolated from milk, mushroom, spinach, lamb kidney, beef liver and mackerel fish were found to be potent riboflavin producers. Based on morphological, biochemical and molecular characterization, the potent strains were identified as *Lactobacillus plantarum* (HDS27), *Bacillus cereus* (HDS07), *Delftia tsuruhatensis* (HDS14), *Citrobacter freundii* (HDS18), *Enterobacter cloacae* (HDS38) and *Bacillus cereus* (HDS54). The selected potent isolates HDS27 from milk and HDS07 from mushroom showed a maximum riboflavin production of 3.69 mg/L and 2.9mg/L respectively. The present study explores the riboflavin producing novel bacteria from different food sources. This is the first report that the *Enterobacter cloacae* isolated from beef liver, *Delftia tsuruhatensis* from spinach and *Citrobacter freundii* from lamb kidney has the ability to produce riboflavin. These potent strains could be a better starter for substituting the conventional bacteria for large scale production of riboflavin in industry.

Keywords: *Bacillus cereus. Citrobacter freundii. Lactobacillus plantarum.* Riboflavin. Vitamin B_2.

1. Introduction

Riboflavin-a water soluble vitamin is necessary for the development and reproduction, in both humans and animals. Riboflavin is a co-enzyme of flavin adenine dinucleotide (FAD) and flavin mononucleotide FMN (Sybesma et al. 2003). Deficiency of riboflavin causes dermatitis, glossitis and cheilosis (Krymchantowski et al. 2002). The normal need of riboflavin for children, men, women, pregnant women and during lactation was found to be 0.4, 1.3, 1.1, 1.6 and 1.8 mg/day (Thakur et al. 2016). Generally humans and animals are unable to produce riboflavin by their own. Either they can intake through dietary supplements like meat and dairy products or in the form of medicine (Burgess et al. 2004). Riboflavin and its various forms are used in different sectors like pharmaceutical, feed and food industries (Liu et al. 2020). As a global demand, nearly 2500 tons of riboflavin is required to treat vitamin deficiency in humans and animals. (Kato et al. 2006). To meet the demand, industries have to increase the production of riboflavin. For the enhanced production, more efficient fungal and bacterial strains like *Eremothecium gossypii* (*Ashbya gossypii)* and recombinant *Bacillus* sp. have been used for the synthesis of riboflavin (Stahmann et al. 1994;

Kalingan et al. 1997; Lin et al. 2001). The genes involved in biosynthetic pathway of riboflavin production are RIB G, RIB A, RIB H, and RIB A. The RIB gene has been mutated in different microbial strains to enhance the production of riboflavin (Yunxia et al. 2010). The large scale riboflavin manufactures BASF and DSM were using *Eremothecium gossypii* (*Ashbya gossypii)*, a fungal strain for the production of riboflavin (Kato et al. 2006). Industrialists are probing for the efficient riboflavin producing strain for enhanced production of riboflavin. Moreover, after fermentative production, recovery of riboflavin from fungal strain was more challenging than bacterial strains. After several rounds of replication, the potency of both wild and recombinant strain was reduced. It also affects the yield of riboflavin. The present study was intended to isolate riboflavin producing potent bacterial strains from riboflavin enriched food sources.

2. Material and Methods

Sample collection and isolation of riboflavin producing bacteria from different food sources

Beef liver, lamb kidney, spinach, almond, *mackerel fish, egg, sundried tomato, milk, curd and mushroom are the major sources of riboflavin. These food sources were collected from Vellore district, Tamil Nadu, India.* Samples were washed with distilled water and homogenized. A Gram of homogenized sample was serially diluted with 0.85% of saline. The diluted food samples were plated on nutrient agar and MRS agar medium. The plates were incubated at 37℃ for 24 h (Stahmann et al. 2000).

Screening for riboflavin producing bacterial strains

The selected colonies were screened for the production of riboflavin by using chemically defined medium (CDM). CDM was prepared without riboflavin according to Otto et al (1983). All the selected strains were inoculated in 100 mL of CDM and incubated at 37℃. To select the potent riboflavin producers, growth in the culture broth was analyzed at 600nm using UV- spectrophotometer (Otto et al. 1983).

Riboflavin assay

The selected culture broth was centrifuged at 6000 g at 4℃ for 15 min. The crude supernatants were collected for riboflavin assay. The crude supernatant (0.8 mL) was mixed with 1M sodium hydroxide (0.2 mL) and 0.4 mL of resulting solution was mixed with potassium phosphate buffer (pH 6.0). The presence of riboflavin in crude supernatant was analyzed at 444nm using UV-spectrophotometer. Based on the extinct coefficient (1.04×10^{-2} $M^{-1}cm^{-1}$), the concentration of riboflavin was calculated and the standard graph was plotted using standard riboflavin (Sigma Aldrich) (Sauer et al. 1996).

Characterization of potent strains

The selected potent strains were identified by morphological and biochemical characterization. (Thakur et al. 2015).

Molecular characterization of potent strains

The potent six riboflavin producing strains were characterized at species level by 16s rDNA sequencing. The selected pure potent strains of DNA were extracted by phenol chloroform method (Edwards et al. 1989). The 16s rDNA of selected potent strains were amplified by polymerase chain reaction. The amplified 16s rDNA sequences were run in BLAST using National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990). Multiple sequence alignment was evaluated using the program MUSCLE 3.7 and the aligned sequence was corrected using Gblocks 0.9 1b (Edgar et al. 2004). The program MEGA 7.0 software was used for the analysis of phylogeny (Kumar et al. 2016). The 16s rDNA sequences were submitted to GenBank and the accession numbers were generated.

3. Results

Isolation and screening for riboflavin producing bacterial strains

From ten different food sources, 72 isolates were obtained and the isolated strains were named as HDS01 to HDS72. Out of 72 isolates, 43 isolates were able to grow in chemically defined medium (CDM). The other 29 strains were not able to grow in CDM that showed the lack of riboflavin production (Figure 1).

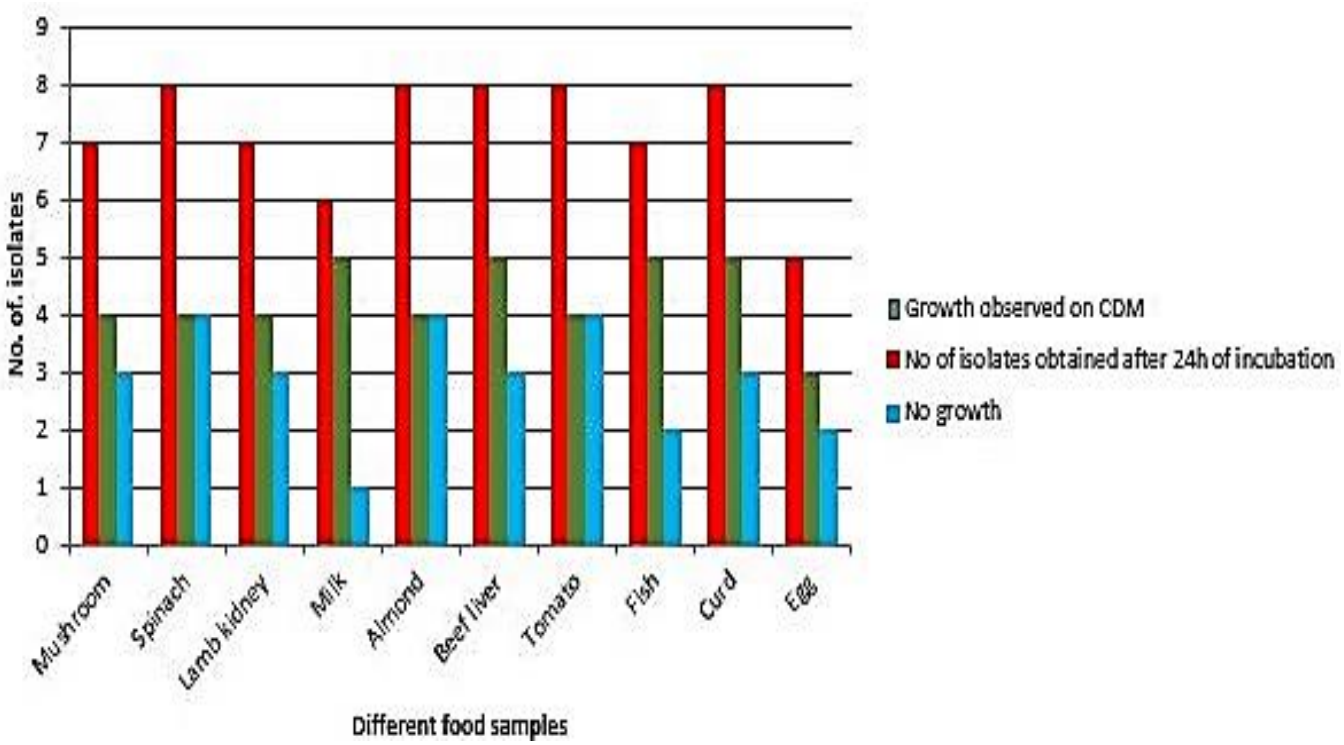

Figure 1. Number of isolates and the growth on chemically defined medium.

More number of isolates was obtained from beef liver, spinach, almond, tomato and curd (8 strains from each sample). From egg sample, only 5 isolates were obtained, out of 5 only 3 showed growth on CDM. From mushroom, lamb kidney and mackerel fish sources, 7 isolates were obtained and only 4 from each food sources showed good growth in CDM (Figure 2A) and (2B). Six potent riboflavin producing strains were selected based on the absorbance value at 600 nm (Figure 3). The absorbance of selected six potent strains HDS07, HDS14, HDS18, HDS27, HDS38 and HDS54 was found to be 1.26, 0.79, 0.94, 1.83, 1.09 and 0.73 respectively. The growth of six potent riboflavin producing strains on MRS plate was shown in Figure 4.

Riboflavin assay

The selected crude supernatants of potent bacterial strains were analysed to estimate the concentration of riboflavin. The absorbance of six potent strains at 444nm was found to be HDS07 - 0.083, HDS14 - 0.035, HDS18 - 0.063, HDS27 - 0.109, HDS38 - 0.069 and HDS54 - 0.031. The corresponding riboflavin concentration was 2.9, 1.45, 2.29, 3.69, 2.48 and 1.39 mg/L respectively.

The potent strain HDS07 isolated from mushroom produced 2.9 mg/L of riboflavin. The isolates HDS01, HDS02, HDS05 from mushroom sample produced 0.48, 0.75 and 0.86 mg/L of riboflavin respectively. The potent strain HDS14 isolated from spinach produced 1.45 mg/L of riboflavin. The other 3 strains produced, HDS08 - 0.64, HDS11- 0.78, HDS13 - 0.75 mg/L of riboflavin respectively. From lamb kidney, 4 riboflavin producing strains were isolated. Strain HDS18 isolated from lamb kidney was identified as one of the potent strain and produced 2.29 mg/L of riboflavin. The isolated strains HDS17 and HDS19 produced lesser amount of riboflavin 0.41 and 0.47 mg/L respectively. Moderate level (0.53mg/L) of riboflavin production was observed in strain HDS21. Milk is a major source for isolating riboflavin producing bacteria.

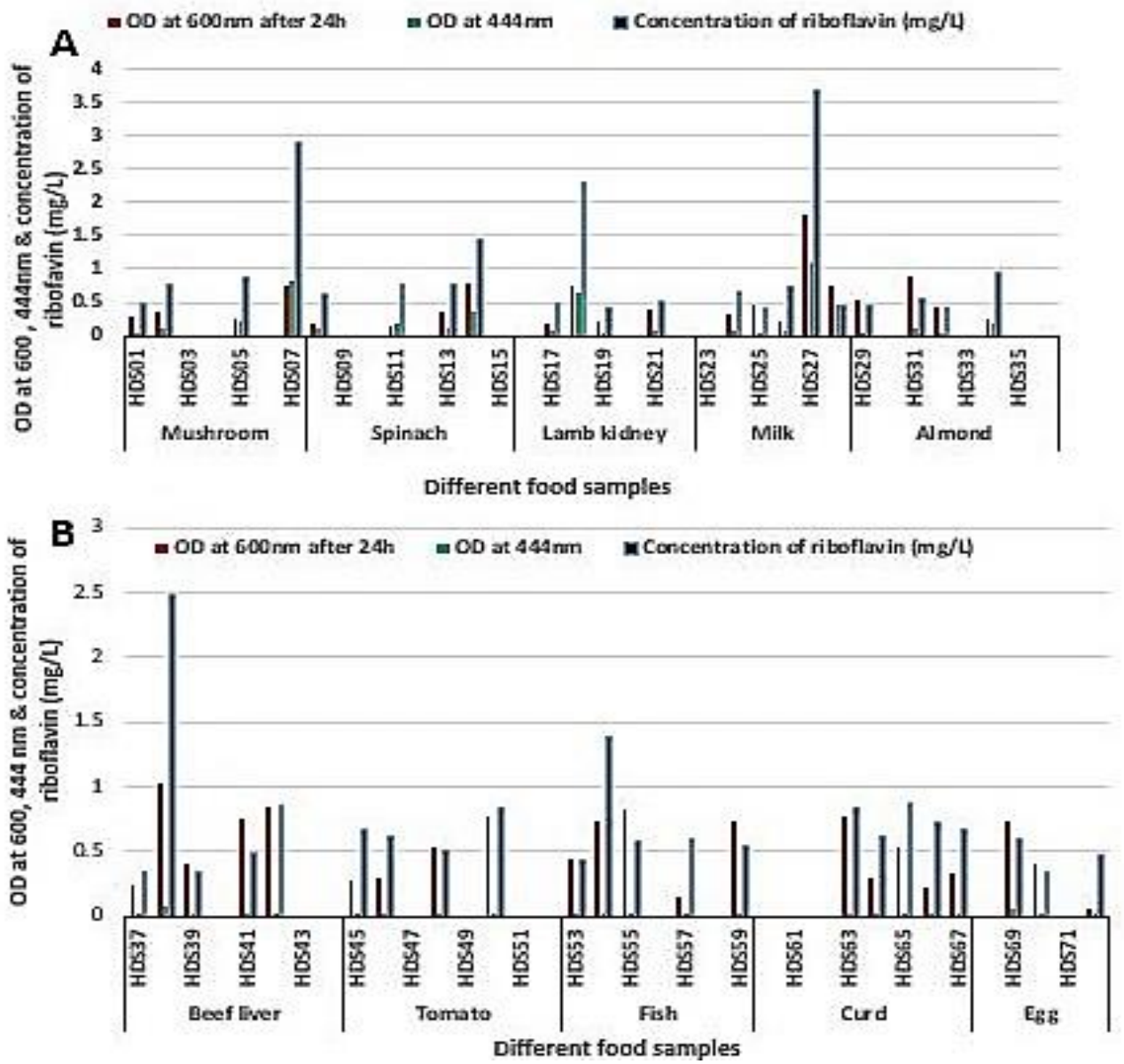

Figure 2. Riboflavin producing potency of bacterial strains isolated from different food sources (A) Strain HDS01- HDS35, (B) Strain HDS36- HDS72.

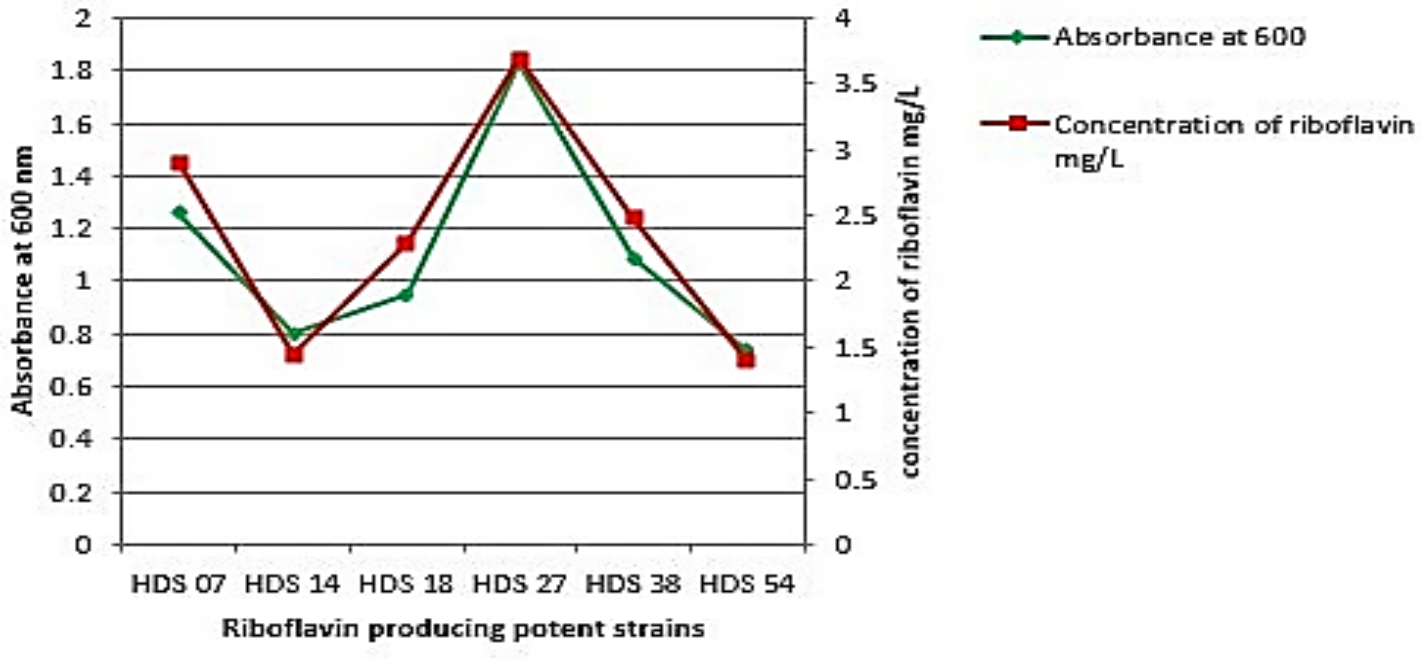

Figure 3. Growth and riboflavin production of potent bacterial strains.

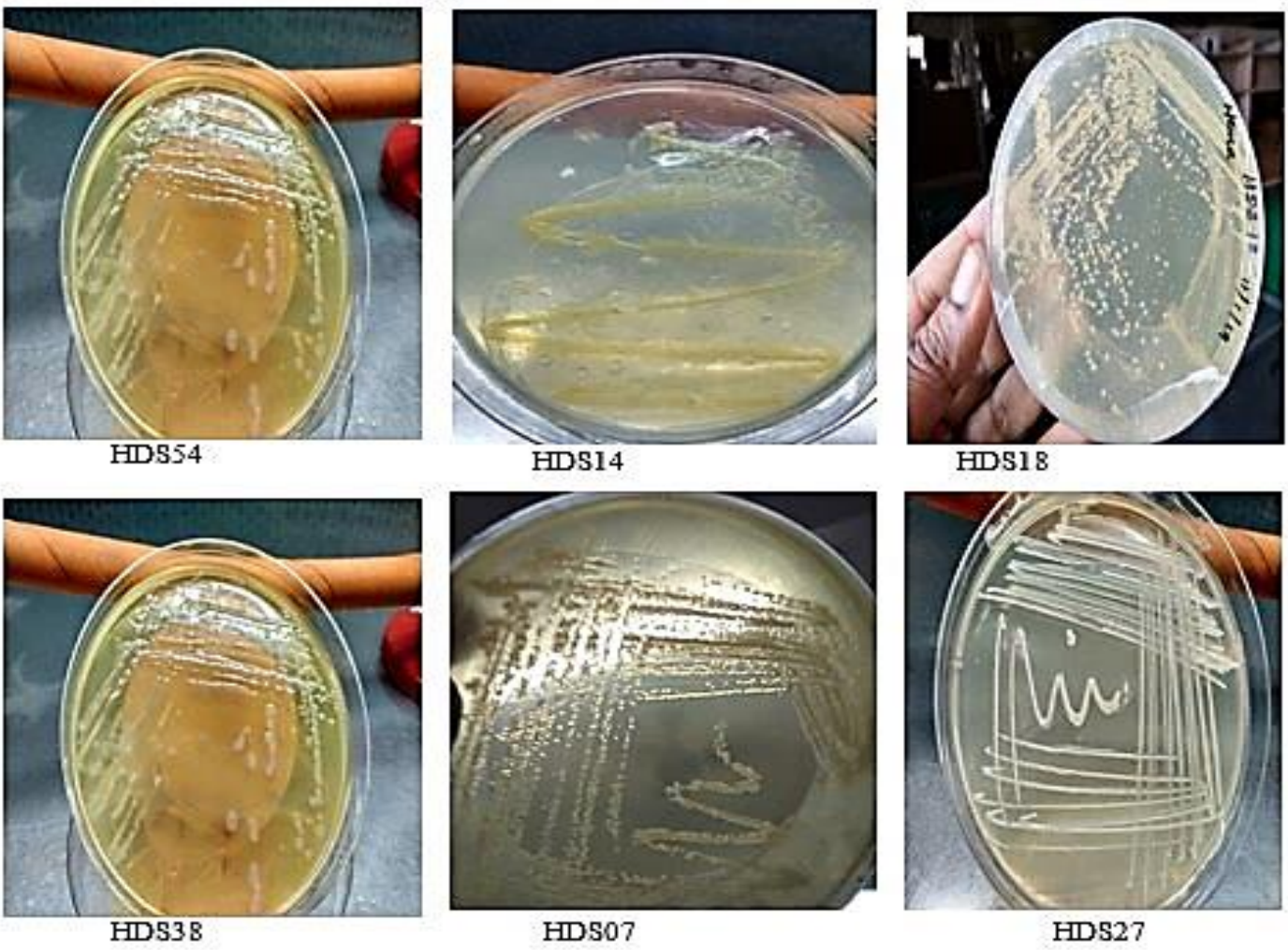

Figure 4. Different riboflavin producing potent bacterial isolates on chemically defined medium.

Based on the growth in CDM, 5 strains were selected. The isolate HDS27 was found to be the most potent strain and its riboflavin production was 3.69 mg/L. Other strains from milk source, HDS26, HDS28, HDS24 and HDS25 were also found to be the moderate producers of riboflavin. The riboflavin concentration was found to be 0.73, 0.6, 0.66 and 0.4 mg/L. From almond, only 4 strains were grown in CDM and minimum riboflavin production was observed. The amount of riboflavin was found to be HDS29- 0.43, HDS31 - 0.57, HDS32 - 0.4 and HDS34 - 0.93 mg/L. Generally, beef liver is a best source of riboflavin. The potent strain HDS38 isolated from beef liver produced 2.48 mg/L of riboflavin. From the strain HDS42 0.86mg/L. of riboflavin was obtained. The other strains HDS37, HDS39 and HDS41 produced very less amount of riboflavin 0.34, 0.35 and 0.48 mg/L respectively. Sun dried tomato was another one sample used for the isolation of riboflavin producing bacteria. Strains HDS45, HDS46 and HDS48 produced moderate level of riboflavin 0.67, 0.61, 0.5 and 0.84 mg/L. From mackerel fish, 5 strains showed very good growth on CDM. One of the potent strains HDS54 produced 1.39 mg/L of riboflavin in CDM. A very minimal concentration of riboflavin, 0.44, 0.57, 0.59 and 0.55 mg/L was obtained from the strains HDS53, HDS55, HDS57 and HDS59 isolated from mackerel fish. Curd is a best and suitable source for the selective isolation of lactic acid bacteria (LAB). A total of 6 different LAB was selected based on its growth in CDM. Most of the strains from curd HDS60, HDS63, HDS64, HDS65, HDS66 and HDS67 showed varied concentrations 0.58, 0.84, 0.61, 0.87, 0.73 and 0.66 mg/L of riboflavin. From egg source, only 3 strains exhibited good growth in CDM. The strains HDS68, HDS70, HDS72 produced only minimum concentration of riboflavin 0.6, 0.34 and 0.46 mg/L.

Characterization of potent strains

Based on morphological, biochemical and phylogenetic analysis, the six potent strains HDS07, HDS14, HDS18, HDS27, HDS38 and HDS 54 were identified as *Bacillus cereus*, *Delftia tsuruhatensis, Citrobacter freundii, Lactobacillus plantarum, Enterobacter cloacae* and *Bacillus cereus*. (Table 1). The generated

GenBank accession numbers of the potent bacterial strains are HDS07 – MK177597, HDS14 – MH397231, HDS18 – MH397228, HDS27 – MK314098, HDS38 – MH397226, HDS54 – MK185103 (Figure 5).

Table 1. Biochemical characterization of potent bacterial strains.

S:No	Characteristics	HDS07	HDS14	HDS18	HDS27	HDS38	HDS54
1	Gram's staining	+	_	_	+	_	+
2	Shape	Rod	Rod	Rod	Rod	Rod	Rod
3	Indole	_	_	_	_	_	_
4	Methyl red	_	_	+	_	_	_
5	Voges Proskauer	+	_	_	_	_	+
6	Nitrate reduction	+	+	+	_	+	+
7	Citrate	+	W +	+	_	+	+
8	Catalase	+	+	+	_	+	+
9	Motility	+	+	+	-	+	+
10	Glucose	+	_	+	+	+	+
11	Spore	+	_	_	_	_	+
12	Oxidase	_	+	_	_	_	_
13	Pigment	_	_	_	_	_	_

['+' (positive), '_' (negative), W+ (weakly positive)]

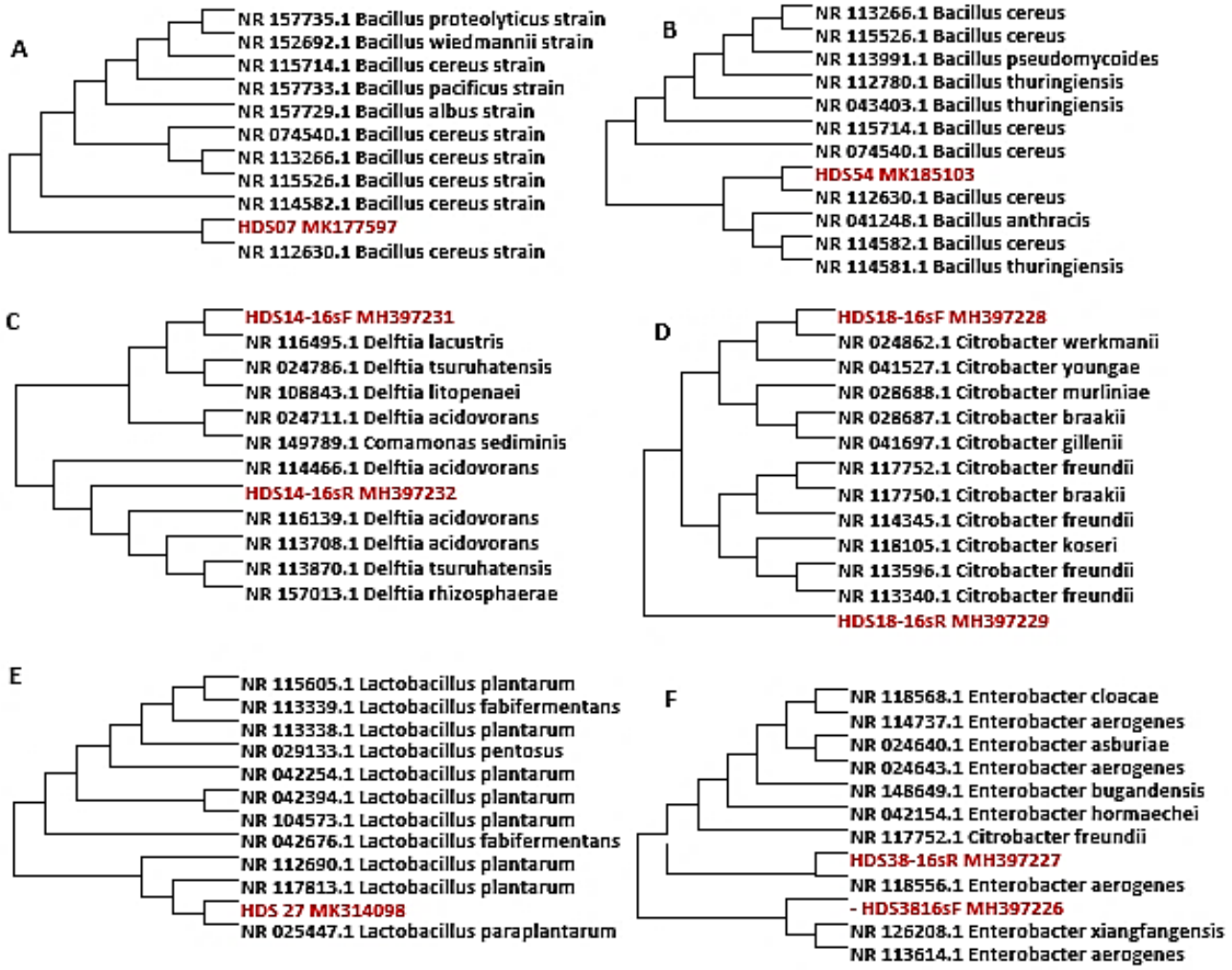

Figure 5. A - Phylogram of *Bacillus cereus* HDS07; B - *Bacillus cereus* HDS54; C - *Delftia tsuruhatensis* HDS14; D - *Citrobacter freundii* HDS18; E - *Lactobacillus plantarum* HDS27; F - *Enterobacter cloacae* HDS38.

4. Discussion

Riboflavin is produced from several microbes and its biosynthetic pathway has been studied extensively in *B. subtilis* and *E. coli*. Industry always endeavors to create novel starter strain for riboflavin

production with upgraded potentials. In the current study, potent riboflavin producing bacterial strains were isolated and identified from different food sources. In our study, from *Bacillus cereus* (HDS07 and HDS54) 2.9 and 1.39 mg/L of riboflavin was obtained. In the previous study, rib gene from *Bacillus cereus* ATCC14578 have been cloned in *B. subtilis* PY and the riboflavin production was 4.3g/L (Yunxia et al. 2010). Oraei et al. (2018) reported that *Bacillus subtilis* subsp. *subtilis* ATCC 6051 has been produced 12.08mg/mL of riboflavin in an optimized medium. In this study, the wild strain *Bacillus cereus* from mushroom and mackerel fish showed moderate production of riboflavin. Here, the wild strains HDS07 and HDS54 were produced considerable amount of riboflavin in CDM. Further optimization and strain improvement methods will enhance the production of riboflavin by *Bacillus cereus* (HDS07 and HDS54). To enhance the production of riboflavin, Wang et al (2011) cloned Zwf and gnd genes from *Corynebacterium* to *Bacillus subtilis* and it has been further improved by site- directed mutagenesis. The production rate of riboflavin from *Bacillus subtilis* mutant strain was found to be 33% increased than the wild strain (Wang et al. 2011). In the future study, rib genes from isolated *Bacillus cereus* (HDS07 and HDS54) will be cloned in potent *L. plantarum* (HDS27) to enhance the riboflavin production.

The bacterial strain HDS38 - *Enterobacter cloacae* has been produced 2.48mg/L of riboflavin. This is the first report that the *Enterobacter cloacae* isolated from beef liver has the ability to produce riboflavin. The strain HDS14 - *Delftia tsuruhatensis,* a Gram negative, rod shaped, and motile bacterium produced 1.45mg/L of riboflavin. Previously, it was reported that *Delftia tsuruhatensis*, a novel plant growth promoting bacteria has the ability to control plant pathogens (Han et al. 2005). In this study, a novel riboflavin producing bacterium, *Delftia tsuruhatensis* has been identified. From another one potent strain HDS18 - *Citrobacter freundii,* 2.29mg/L of riboflavin was obtained. This is the first report stating that *Citrobacter freundii,* a Gram negative facultative anaerobic bacterium isolated from lamb kidney has the ability to produce riboflavin for its growth. Lactic acid bacteria act as a cell factory for riboflavin production. The potent strain HDS27 - *Lactobacillus plantarum* isolated from milk produced 3.69 mg/L of riboflavin. Similarly, *Lactobacillus plantarum* KTP13 isolated from bamboo shoots has been produced 2.36 mg/L of riboflavin (Thakur et al. 2015). Moreover, in previous reports the presence of riboflavin (rib) genes RIB A, RIB G, RIB B, RIB H were investigated in *Lactobacillus plantarum* WCFS1, *L. plantarum* sub sp. *plantarum* ST – III, *L. plantarum* JDM1, *L. plantarum* CRL725 (Capozzi et al. 2012; Valle et al. 2014). Overexpression of rib genes (RIB A, RIB G, RIB B, RIB H) in *Lactococccus lactis* have been reported and produced 24mg/L of riboflavin (Burgess et al. 2004). A recent study reported that *Lactobacillus plantarum*- HY7715 isolated from Kimchi produced 34.5 mg/L of riboflavin (Kim et al. 2021). In the present study, the bacterial strain *Lactobacillus plantarum* HDS27 - MK314098 isolated from milk showed a maximum riboflavin production (3.69 mg/L). Further the study will be focused on strain improvement methods to enhance the production of riboflavin by *Lactobacillus plantarum* HDS27 and *Bacillus cereus* HDS07. Chu et al. (2021) stated the significance of microbial synthesis methods to increase the yield of riboflavin. Genetic engineering and analogue screening techniques have been used for the enhanced production of riboflavin by lactic acid bacteria (Liu et al. 2020). Future studies should include molecular cloning, inactivation of folE gene in *L. plantarum* HDS27 for the improvement of riboflavin production. Inactivation of folE gene may stimulate the increased availability of GTP for the production of riboflavin in *L. plantarum*.

5. Conclusions

The aim of the present study was to explore the riboflavin producing bacteria from different riboflavin enriched food sources. Several bacterial isolates from food samples were screened for riboflavin production. The riboflavin producing ability of the different bacterial isolates was compared. Based on the research findings, *Lactobacillus plantarum* from milk and *Bacillus cereus* from mushroom samples has been identified as the maximum riboflavin producers. In near future, these strains could be a superior starter culture in the pharmaceutical and food industries for the enhanced production of riboflavin.

Authors' Contributions: HEMALATHA, M.: acquisition of data, analysis and interpretation of data, drafting the article, and critical review of important intellectual content; DEVI, C.S.: conception and design, analysis and interpretation of data, drafting the article, and critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

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Ethics Approval: Not applicable.

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