Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Online International Journal Available at http://www.cibtech.org/jls.htm
2013 Vol. 3 (4) October-December, pp.169-176/Guru and Viswanathan
Research Article

RIBOFLAVIN PRODUCTION IN MILK WHEY USING PROBIOTIC BACTERIA- *LACTOBACILLUS ACIDOPHILUS* AND *LACTOCOCCUS LACTIS*

***Guru V. and Viswanathan K.**
Institute of Animal Nutrition, TANUVAS, CHENNAI-51
**Author for Correspondence*

ABSTRACT
A total number of six isolates of *L.acidophilus* were obtained from curd and chesse samples. The organisms were identified and confirmed through bio-chemical and sugar fermentation tests. They were inoculated in milk whey medium for production of riboflavin and the vitamin produced was estimated at periodical intervals using HPLC. It was observed that L. *acidophilus* yielded a higher riboflavin compared to *L. lactis*. Further *L. acidophilus* yielded a maximum riboflavin content of 2930 µg/ litre on 7th day in whey and declined on 9th day of fermentation. However, *L. lactis* yielded a maximum riboflavin content of 2610µg/litre till 5th day and declined thereafter. Among isolates of *Lactobacillus*, LB5 yielded maximum riboflavin (2930 µg/liter) on the 7th day of fermentation. Whey served as a better fermentation medium compared to skim milk

Key Words: *Probiotics- Lactobacillus-Dairy Products - Whey- Riboflavin – Production*

INTRODUCTION
Riboflavin is yellow green fluorescent water soluble pigment widely distributed in plants and animal cells. It is a component of co-factors FAD and FMN. It is required for metabolism of fats, carbohydrates and proteins. Large quantities of riboflavin are often included in multivitamins. Riboflavin is vital for normal reproduction, growth, repair and development of skin, eyes, connecting tissues, mucous membrane and immune and nervous system. Commercially synthesized vitamins are used in fortification of food product such as bread and breakfast cereals. It is also used in small amount as colouring agent in foods such as ice cream and sauces and also as a medical identification aid The recommended daily requirement of riboflavin is set as 1.3 mg (Food and Nutrition Board, 1999) and sufficient amount to be ingested regularly since the body is unable to store this vitamin. A large number of lactic acid bacteria such as *lactobacillus acidophilus, lactobacillus bulgaricus, lactobacillus plantarum, lactobacillus reuteuri, lactobacillus delbrueckii, lactobacillus rhamnosus, lactococcus lactis and lactococcus cremoris* and bifidobacterium species like *bifidobacteria infantis, bifidobacteria longum* have been reported to produce vitamins including riboflavin. Lactic acid bacteria are industrially important microbes that are used all over the world in a wide variety of industrial food fermentations. *Lactobacillus acidophilus* and *Lactococcus lactis* are far the most extensively studied lactic acid bacteria to produce riboflavin.
Whey is serum or watery part of milk that separates from curd in process of cheese or casein making. It is the largest by-product of dairy industry and becomes an environmental threat. Skim milk is the milk from which fats have been removed through cream separator Whey and skim milk contains about 4.5 percent lactose. We know that probiotic bacteria produce multivitamins. Since not much work had been done on lactic acid bacteria that produces riboflavin and also microbial production of riboflavin is more advantageous when compared to chemical production, a study to isolate and identify the lactic acid bacteria from fermented milk products such as curd and cheese samples from local market and to study riboflavin production by these bacteria in whey and rehydrated skim milk was taken.

MATERIALS AND METHODS
All the glassware used in the study were soaked overnight in the cleaning solution (10% potassium dichromate in 25%sulphuric acid for few hours and washed with detergent and rinsed in tap water, finally the glassware were rinsed with distilled water, dried in oven and sterilized when needed. Media were sterilized by moist heat at 121ºC at 15lbs for 15 min and glassware were sterilized by dry

heat at 160ºCfor 1hour.Sterile test tubes and sterile plastic containers were used for collection of all samples (curd, cheese).Sigma chemical company, USA was used as reference / standard riboflavin and to obtain standard curve (purity of riboflavin was 99.6%).Twenty curd samples and five cheese samples were collected aseptically from local markets and households and were brought to the laboratory in ice box and stored at less than 5ºC until further use. Appropriate dilution of curd and cheese samples were made in normal saline blanks and plated on MRS agar plates and Elliker agar plates respectively and incubated at 37ºC in candle jar for 24-48 hrs. Every 24 hours, colony growth was observed and checked with gram staining. At the end of 72 hrs of incubation, gram positive rods and gram positive cocci had become predominant. A total number of six isolates were grown and were further purified by repeated sub-culturing. A loopful of colonies from MRS agar plates and Elliker agar plates were taken and inoculated on Elliker broth flasks separately and incubated at 37ºC for 72 hrs for the growth of organisms. The growth is indicated by turbidity formation.

From each MRS agar plates and Elliker agar plates two loopful of colony growth was taken and inoculated into fermentation medium. (Skim milk and whey).The purified cultures were subjected to the following for identification: 1. Microscopic examination 2. Spore staining 3. Growth at 15ºC 4. Growth at 45ºC 5. Salt tolerance at 4% NaCl 6. Catalase test 7. Sugar fermentation tests

For all the above tests standard microbiological procedures were followed. All isolates were maintained in MRS broth and transferred at bi-weekly intervals. The purity of all cultures was regularly ascertained before use, by microscopic examination using gram staining technique. The cultures were activated before use by successive transfer at 18 hrs interval into appropriate MRS broth. All stock cultures were preserved in MRS glycerol broth and stored at -20ºC; glycerol was used at level of 15%.

Preparation of Fermentation Media

Whey: Whey was prepared from cow's milk by following procedure. First, the milk was warmed to 70ºC. Acidification of the milk was done by adding citric acid 2 %(w/v) solution drop by drop until coagulation occurred. Then it was filtered through a muslin cloth to remove coagulated particles. Whey was collected separately and pasteurised at 60ºC for 30 min. It was supplemented with chloramphenicol at 5µg/ml (Bhattacharya, 2001).

Skim Milk: Commercially dried, low fat, skim milk powder was rehydrated (13.5% w/v). The rehydrated milk was heated at 100ºC for 5 min and immediately cooled to 4ºC in iced bath. It was transferred into 500 ml flask and stored for 24 hrs before use. The rehydrated milk was supplemented with chloramphenicol at 5µg/ml (Leblanc *et al*., 2005).

Fermentation

Whey: About 100 ml of supernatant of sterile whey was placed separately in each of six numbers of 250 ml sterile volumetric flasks. They were inoculated with five isolates of *Lactobacillus* and one isolate of *Lactococcus* and allowed to grow in the dark, in the bench type water bath shaker at room temperature with a 55 rpm wrist shaking action. Samples were removed on completion of 3rd, 5th, 7th and 9th days under aseptic conditions and analyzed for riboflavin content. Similar inoculation conditions and procedures were carried out for rehydrated skim milk also.

Estimation of Riboflavin

Fluorimetric method (Rashid and Potts, 1980) The Riboflavin estimation was carried out at Madras Veterinary College (Tamil Nadu Veterinary and Animal Sciences University), Chennai using a Hitachi 650-10-S fluorescence spectrophotometer. The procedures for preparation of standard solutions, standard curves and estimation in samples were carried out as detailed below.

Riboflavin fluoresces in light of wave length of 440 -550 nm. Yellowish green fluorescence of riboflavin in UV light is dependent upon pH of the solution a well as its concentration. From values of optical density at 445nm, Quantity of riboflavin was determined from standard curve. The standard curve was prepared from quantity of pure riboflavin in known volume of distilled water.

Riboflavin Stock Solution-A (25µg riboflavin/ml)

25mg reference standard riboflavin which had previously been dried in vacuum desiccators was weighed and transferred quantitatively to a 1litre volumetric flask. About 750 ml of 0.02 N acetic acid was added and warmed to aid solution. It was cooled to room temperature and volume made to mark with distilled water. It was preserved under toluene, protected from light, in a refrigerator.

Riboflavin stock solution-B (10µg riboflavin/ml):
40ml of stock riboflavin solution A was taken in a 100ml volumetric flask and volume made to mark with distilled water.
Riboflavin working standard (0.1µg riboflavin/ml):
1 ml of riboflavin stock solution B was taken in a 100ml volumetric flask and volume made to mark with distilled water.

Calibration Curve

Six riboflavin sub-standard solutions were prepared as detailed below:

Vol. of working standard (0.1µg riboflavin/ml):(ml)	Vol. of distilled water (ml) Added	Conc. of riboflavin (µg/ml)
0.5	9.5	0.05
1.0	9.0	0.10
1.5	8.5	0.15
2.0	8.0	0.20
2.5	7.5	0.25
3.0	7.0	0.30

The fluorescence emissions of above concentrations of standard solutions were measured using photo-fluorimetry with an excitation filter of 440nm and emission filter of 565 nm and values were noted for plotting in a calibration curve.

Sample Preparation

20 ml of fermented medium was pipetted out into a 100 ml volumetric flask. 2 ml of 10 %(w/v) acidified lead acetate solution was added to it. Contents were mixed well and filtered with Whatman No. 42 filter paper The filtrate was centrifuged at 14,000 rpm for 20 minutes and supernatant was used for assay (1 ml was taken and diluted to 10 ml).

Estimation of riboflavin - HPLC method (Ashoor *et al.*, 1983).

The Riboflavin estimation was carried out at Veterinary College and research Institute, (Tamil Nadu Veterinary and Animal Sciences University), Namakkal using Shimadzu RF- 535 HPLC chromatographic system. The procedures for preparation of standard solutions, standard curves and estimation in samples were carried out as detailed below.

Riboflavin Stock Solution

Four riboflavin aqueous standard solutions were prepared by dissolving each of four weights (1.15mg, 2.4 mg, 4.56 mg, and 6, 48 mg) of standard riboflavin in 100 ml distilled water.

Riboflavin Working Standard

From each of above stock solution, 5 ml was taken separately and transferred into four separate volumetric flaks (100ml) and the volume made to mark, which served as working standard. The concentration working standards were determined as 23 ng, 48 ng, 91.2 ng and 129.6 ng per 20 µl

Sample Preparation

A volume of 10 ml of each fermented medium was acidified to pH 3 with aqueous glacial acetic acid (1:1v/v) added drop wise with constant stirring. The acidified fermented medium was stirred gently for additional 5 min, and then centrifuged at15, 000 pm for 15 min. supernatant was transferred to 25ml volumetric flask and the sediment was washed twice with 5 ml of 2% acetic acid solutions. The washings were combined and centrifuged at 15,000 rpm for 15 min .The second supernatant was added to the first volumetric flask and volume made to mark with 2% acetic acid solution.

Analysis: The Shimadzu RF- 535 HPLC chromatographic system consisted of a multi solvent pumping system (LC-10AS) an injector, Column- phenomenex C18 with computer software and printer. The detectors were photodiode array UV-VIS (SPD-10A detector Shimadzu) to monitor 270 nm at 0.02 sensitivity.

The chromatography was carried out on - phenomenex C18 reversed phase column. Mobile phase used was an isocratic system consisting of a mixture of water-methanol-acetic acid (68:32:0.1v/v). Flow rate was set at 1.0ml per min. all solvent used in the mobile phase were HPLC grade from Merck and degassed with helium gas for 10 min. For determination of riboflavin 20 µl of standards of known concentration were injected to obtain retention times for identification of peak. Calibration

curve were constructed, where the mass of riboflavin (ng) was plotted versus the average areas found for each peak.

The detection time limit of riboflavin was determined at 129.6 ng per ml.

Quantification of riboflavin in sample; An injection of 20 µl of each sample supernatant was carried out and the retention time, peak and area were noted. The riboflavin contents were obtained directly from calibration curve by extrapolation values.

RESULTS AND DISCUSSION

20 curd samples and five cheese samples were collected from house hold and organized dairy as per standard procedure and from them five curd isolates and one cheese isolate were taken for the study. A total number of six isolates were obtained.

On microscopic examination by gram staining, Gram positive rods with rounded ends occurring singly or in pairs and in short chains indicative of *Lactobacillus acidophilus* was observed. Further Gram-positive cocci occurring in pairs and in short chains spherical or ovoid in shape revealing *Lactococcus lactis* was observed. The results of biochemical and sugar fermentation tests are given in table no.1 & 2 respectively.

Table 1: Biochemical tests

Biochemical test	**Observation**	
Catalase	Negative	Negative
Growth at 15^{o}C	No	No
Growth at 45^{o}C	Yes	yes
Salt tolerance at 4% NaCl	Positive	Positive
Voges - Proskauer	Positive	Negative
Ammonia from arginine	Negative	positive
Esculin hydrolysis	Positive	positive
Inference	***L. acidophilus***	***L. lactis***

Table 2: Sugar fermentation tests for lactic acid bacteria

S.No.	**Name of sugar**	**Results**	
1	Arabinose	Negative	Positive
2	Cellobiose	Positive	Negative
3	Galactose	Positive	Positive
4	Lactose	Positive	Positive
5	Maltose	Positive	Positive
6	Mannitol	Negative	Positive
7	Melibiose	Positive	Negative
8	Raffinose	Positive	Negative
9	Salicin	Positive	Negative
10	Sorbitol	Negative	Negative
11	Sucrose	Positive	Positive
12	Trehalose	Positive	Positive
	Inference	***L. acidophilus***	***L. lactis***

Table 3: Normal riboflavin content

Medium	Quantity (µg per litre)
whey	1187
Skim milk	1250

Table 4: Riboflavin yield (µg per litre) on 3rd day fermentation

Isolate	Whey	Skim milk
LB1	2220	2050
LB2	2100	2000
LB3	2330	1970
LB4	2100	2000
LB5	2300	2100
LC	2400	2000

LB 1 to LB 5 –***Lactobacilllus acidophilus*** isolates
LC- ***Lactococcus lactis*** isolate

Table 5: Riboflavin yield (µg per litre) on 5th day fermentation

Isolate	Whey	Skim milk
LB1	2360	2300
LB2	2350	2300
LB3	2500	2200
LB4	2200	2100
LB5	2380	2350
LC	**2610**	2440

Table 6: Riboflavin yield (µg per litre) on 7th day fermentation

Isolate	Whey	Skim milk
LB1	2560	2440
LB2	2550	2500
LB3	2600	2390
LB4	2550	2500
LB5	**2930**	2600
LC	2220	2000

Table 7: Riboflavin yield (µg per litre) on 9th day fermentation

Isolate	Whey	Skim milk
LB1	2440	2330
LB2	2190	2390
LB3	2280	2220
LB4	2330	2330
LB5	2560	2400
LC	1830	1720

Table 8: Comparison of Riboflavin production (µg/litre) at different days of fermentation in whey and skim milk

Isolates	Fermentation period in Whey (days)				Fermentation period in Skim milk (days)			
	3rd	5th	7th	9th	3rd	5th	7th	9th
LB1	2220	2360	2560	2440	2050	2300	2440	2330
LB2	2100	2350	2550	2190	2000	2300	2500	2380
LB3	2330	2500	2600	2280	1970	2200	2390	2220
LB4	2100	2200	2550	2330	2000	2100	2500	2330
LB5	2300	2380	**2930**	2560	2100	2350	2600	2400
LC	2400	**2610**	2220	1830	2300	2440	2000	1720

Table 9: Riboflavin content (µg per litre) in whey – HPLC method

Normal whey	1187
5^{th} day fermentation – LC isolate	2610
7^{th} day fermentation –LB isolate	2931

Riboflavin Synthesis

It is biosynthesized in plants and many bacteria. Vegetables and milk are the main sources of vitamin in human nutrition. The daily recommended allowances for riboflavin are 1.3 mg. Fermentation processes are progressively replacing chemical manufacturing processes. Naturally occurring bacteria, yeast and fungi produce riboflavin in levels exceeding their requirement. Hence an attempt to study riboflavin production of flavogenic organisms was carried out.

Composition of Normal Whey and Skim Milk

The approximate composition of liquid whey was water- 93.35 %, total solids - 6.65%, comprising of fat- 0.5%, protein- 0.8%, lactose-4.85% and ash-0.5%

The approximate composition of skim milk is almost similar to whey excepting fat content, which varies 0.1 – 0.2%.

Riboflavin Content in Normal Whey and Skim Milk (Table 3)

In the present study, riboflavin content in normal whey and skim milk were found to be 1180 µg/ liter and 1250µ g / liter respectively Daniel and Norris (1944) noticed a value of 1240µg/ liter of riboflavin in whey and 1580µg/ litre in skim milk. A lower value of 850 µg/liter in whey (Alm, 1982) and 1020 µg/liter was reported (FAO, 1988) but higher riboflavin content of 1235 µg/ liter whey had been observed (Dierkson et *al.*, 1997).

The reconstituted skim milk used in the present experiment was found to contain 1250µg/litre, which is agreement with reported values of 1250 µg/litre (Rashid and Potts, 1980). The variation in the riboflavin content between the estimated value in the present study and the published values may be due to on farm practices such as breed of animals, diet, stage of lactation and seasonal changes which may exert influence on composition of milk or environmental factors like exposure of milk to sunlight/ artificial light resulting in reduction of riboflavin in content (Porter, 1978).Whey was selected as a nutrient in this study for its higher rate of production through out the world. The riboflavin production rate/ liter during the nine days of fermentation (at pH 6.6 and temperature 30ºC) in whey and skim milk were observed.

Riboflavin production increased most rapidly after three days of fermentation and continued to increase until 7^{th} days of fermentation in *L. acidophilus* isolates both in whey and skim milk media. The quantity of riboflavin produced by all isolates in whey medium except, LB5 isolates on the 7^{th} day of fermentation was between 2550 and 2600 µg/ liter, while LB5 yielded a maximum of 2930 µg/liter. Similarly LB isolates, exceptLB5, produced riboflavin yield of 2390- 2500 g/ liter in skim

milk medium on 7th day fermentation but highest at 2600µ g / litre for LB5 isolate. After the 7th fermentation there was a decline in riboflavin production in both media.

Riboflavin Production- L. lactis (Table 4 to table 9)

There was a time lag till lactose was fully utilized in riboflavin production consistent with literature (Prabakar *et al.*, 1993) Riboflavin production increased most rapidly after 2 days of fermentation and continued to increase until 5th day. It was observed that the riboflavin yield was higher (2400µ g/ liter) on third day in whey medium and attained its maximum level (2610 µg/ liter) on 5th day of fermentation. There was decline in the production rate noticed for *Lactococcus* isolate on the 7th day onwards. The same trend was observed for *Lactococcus* isolate in skim milk medium also. Similar pattern of increased riboflavin yield from 1st day of fermentation until 5th of fermentation and the highest yields after 5 days was observed in *Bacillus megaterium* and *Enterobacter aerogenes* on corn meal slurry (Chung and Fields, 1996).

A similar value of 2000 µg/liter by *Clostridium acetobutylicum* grown in corn meal was reported (Demain, 1972). A riboflavin yield of 6000 µg/litre liter of fermented whey supplemented with iron at 3ppm in *Clostridium aceto*butylicum was recorded (Meade and Pollard., 1942).An enhanced riboflavin was noticed when *Clostridium acetobutylicum* was fermented in whey and skim milk supplemented with iron and ammonium salts/ manganese/ zinc in pre-determined amount and ratio (Pollard *et al.*, 1948).

The present study demonstrated that *L. acidophilus* produced higher riboflavin yield (2930 µg/ liter) than *L. lactis* (2610 µg/liter).

The present study revealed that whey had served as a better medium than skim milk for riboflavin production, which coincided with earlier findings of (Rogosa, 1943) who had indicated that whey could be employed as a substrate for the production of ethanol, acetic acid and riboflavin.

Future Development

Riboflavin production is speculated to be 3000 tonnes per year and about 80% of these are produced through microbial fermentation. Production rate could be enhanced by

1. Employing genetically engineered micro-organisms encoding enzymes involved in bio-synthesis of riboflavin
2. Incorporating supplements such as iron, manganese, zinc and resin in the fermentation medium.

Conclusion

L. acidophilus yielded a higher riboflavin compared to L. lactis.

L. acidophilus yielded a maximum riboflavin content of 2930 µg/ litre on 7th day in whey and declined on 9th day of fermentation.

L. lactis yielded a maximum riboflavin content of 2610µg/litre till 5th day and declined thereafter.

Among isolates of *Lactobacillus*, LB5 yielded maximum riboflavin (2930 µg/liter) on the 7th day of fermentation. Whey served as a better fermentation medium compared to skim milk**.**

REFERENCES

Ashoor SH, Saperich GJ, Monte WC and Welty J (1983). HPLC determination of riboflavin in eggs and dairy products. *Journal of Food Science* **48** 92-94.

Bhattacharya DC (2001). Milk processing technology in dairy year book 150.

Chung HJ and Fields ML (1986). Production of riboflavin and vitamin B_{12} by *Bacillus megaterium* ATCC 13639 and *Enterobacter aerogenes* in corn meal. *Journal of Food Science* **51** 1514-1517.

Demain AL (1972). Riboflavin over-synthesis. *Annual Review of Microbiology* **26** 369-383.

Dierkson KP, Sandine WE and Trempy JE (1997). Expression of ropy and mucoid phenotypes in *L .lactis. Journal of Dairy Science* **80** 1528-1536.

FAO (1988). Food and Agricultural Organization of United Nations. Animal Production and Health Paper-113, Milk and milk products Rome, Italy.

Food and Nutrition Board, Institute of Medicine (1999). Riboflavin. In: Dietary reference intakes, Thiamine, Riboflavin, niacin, Vitamin. B_{12}, Biotin, Folate. National Academic Press, Washington DC 87-122.

Leblanc JG, Burgess C, Sybesma F, Savoy de Giori G and Van Sinderson D (2005). Ingestion of milk fermented by genetically modified *Lactococcus lactis* improves the riboflavin status of deficient rats. *Journal of Dairy Science* **88** 3535-3442.
Meade RE and Pollard HL (1942). Process for manufacturing a vitamin concentrate. *U.S. Patent* 2869680.
Pollard N, Rodgers E and Meade RE (1948). Method of enhancing the yield of vitamin in fermentation. *U.S. Patent* 2449140.
Porter JWG (1978). Milk as a source of lactose, vitamins and minerals. *Proceedings of the Nutrition Society* **37** 225-230.
Prabhakar G, Raju DC and Satyanarayana M (1993). Bioproduction of riboflavin from molasses and lentils. *Bioprocess and Biosystems Engineering* **8** 247-249.
Rashid I and Potts D (1980). Riboflavin determination in milk. *Journal of Food Science* **45** 748-749.
Rogosa M (1943). Synthesis of Riboflavin by Lactose-Fermenting Yeasts. *Journal of Bacteriology* **45** 459-460.