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# Soy-Fortified Herbal Curd Formulated Using Potential Probiotics Isolates *Lactococcus Lactis*" (Nrrl B-1232)

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## Abstract

Recently there has been an increased demand for functional foods to reduce the risk of cardiovascular diseases mainly related to hypercholesterolemia, because of undesirable side effects of traditional drugs. Hence, in the quest for natural and safer alternatives, this work is aimed to bring together the health-promoting properties of probiotics, soymilk, green tea and tulsi extract into an herbal product by using potential probiotic strain *Lactococcuslactis.* The study includes characterization of the probiotic strain, characterize the tolerance of *Lactococcuslactis* for pH and bile, production of soy milk, soy curd and soy fortified green tea curd and tulsi curd. Further estimation of protein, carbohydrate, amino acid, total solid and ash content. Finally, flavonoid, antioxidant and tannin content was measured by using chemical methods. This soy-fortified green tea curd will attract the health conscious consumers as it is a nutrition-packed functional food that could be equally helpful to healthy people of all age groups, as to people having certain health issues like lactose intolerance, hypertension, hypercholesterolemia, malnutrition, etc.

**Keywords**

Probiotic, Hypercholesterolemia, Soy Fortified, Malnutrition.

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## INTRODUCTION:

Recently the food industry has shown a growing interest in the so-called functional foods due to increase in consumers' desire for a healthier lifestyle and so for foods from the natural origin with enhanced nutritional and therapeutic values. Functional foods are more preferred over medications to reduce the risk of hypercholesterolemia and hypertension, which are the two major contributing factors in development of cardiovascular diseases (CVD) in order to avert various side effects from the traditional drugs.

Hypercholesterolemia and hypertension are projected to be the chief cause of fatality worldwide by 2020. However, frequent and prolonged administration of

the traditional drugs like statins induces undesirable secondary effects like myopathy and cognitive impairment. Hence, to develop more natural alternatives, several researchers have studied the in vitro and in vivo cholesterol reducing potencies of beneficial lactic acid bacteria (LAB) termed as probiotics. Thus, there is growing interest in the use of nutraceutical and pharmaceutical products formulated using probiotics and prebiotics alone or in combination (synbiotic) having cholesterol lowering and blood pressure reducing properties. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [(1)]. Probiotic bacteria are used in the food industry due to various beneficial properties including reduction in bowel irritation, immune-modulation, anti-hypertension and cholesterol reduction [(2)]. Pereira, McCartney and Gibson (2003) proved that the oral administration of probiotics and prebiotics significantly reduce the serum cholesterol levels in human subjects. Hypocholesterolaemia effects of probiotics can be accredited to bile salt hydrolase activity, bacterial cell wall attachment, and physiological role of the short-chain fatty acids (fermentation end products) [(2).]

Probiotics, defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" by the Food and Agricultural Organisation (FAO 2001), have become a major topic of lactic acid bacteria (LAB) research over the past 10 years [(3)]. These organisms can be the predominating members of the endogenous intestinal flora in humans, which are reported to exert beneficial effects including the activation of the immune system, reduction of serum cholesterol, and inhibition of the growth of potential pathogens that may cause infections in the host [(4)]. Therefore, incorporation of these probiotic bacteria into soymilk to increase its therapeutic value has become a popular trend [(5,6,7,8,9)]. Furthermore, probiotic bacteria generally do not grow rapidly in cows' milk, and therefore cannot attain a high enough viability as starter cultures in yoghurt manufacture [(10)]. However, many studies indicate that soy is a good substrate for probiotic bacteria, especially the probiotic Bifidobacterium [(11,12)]. These bioactive phytochemicals possess potent antioxidant, antimutagenic, anti-inflammatory and anti-microbial properties and the synergistic effect of tea and soy in lowering risks of several cancers and cardiovascular diseases has been established both in vitro and in vivo [(13,14,15,16)]. Thus, it is desirable to increase the daily intake of flavonoids, especially from natural sources. Yoghurt produced from soymilk are considered to have poor consumer acceptability and sensory attributes. Development in flavor and texture of soymilk yogurt needs a grouping of treatments. The present study employed some treatments including thermal, added flavor essence, sweetener, and animal's milk-based products to develop soymilk yoghurt

Despite the common association of *Lactococcuslactis* with dairy products, the bacterium was originally isolated from plants where it was believed to be dormant, and only became active and multiplied in the gastrointestinal tract after being consumed by ruminants [(17)]. Originating from the streptococcus genus and re-classified into the *Lactococcus* genus in 1985, *L.*lactis is divided into three subspecies namely *L. lactis* subsp. *lactis, L. Lactis*subsp. *cremoris*, and *L. lactis* subsp. *Hordniae* [(18)]. Phenotypically, it is classified as a gram-positive, spherical, homolactate, non-sporulating, and facultative anaerobic gut bacteria with hundreds of strains and bio variants published to date [(19,20)].

*Lactococcuslactis* has been used for centuries in the fermentation of food especially cheese, yoghurt, sauerkraut and the like, thereby rendering it's generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA). Apart from imparting flavour, *L. lactis* being a lactic acid bacteria (LAB) also produces acid which preserves food. Some strains further enhance this preservation property with the production of bacteriocins, thus reinforcing its role in the food industry. Other than its important function in food*, L. lactis* has become the model LAB when it comes to genetic engineering. Several factors including its small-sized fully sequenced genome (2.3 Mbp), and the development of successfully compatible genetic engineering tools such as cloning and expression systems with customizable options, have rendered it a desirable model. Over the past two decades,*L. lactis* has vastly extended its application from food to being a successful microbial cell factory, and on many occasions, acting as a gram-positive alternative to *Bacillus subtilis* and *Lactobacillus plantarum*, or its gram-negative counterpart, *Escherichia coli*[(21)].

**MATERIALS AND METHOD:**

**pH and Bile Tolerance:**

Tolerance to low pH and bile content was assessed with minor modifications the ability of the strains to grow at low pH was Evaluated in acidified M17 broth (finalpH2.5), containing 1000 unit per ml of pepsin (sigma USA). The tolerance of strains to bile (ox gall) was determined in M17 broth containing 0.3% ox gall the 10 l of each type of modified M17 was in occulated with a Bacterial suspension to find the cell concentration of approximately 1.0x10-7 cfu/ml. The pH tolerance was evaluated by measuring survival after 3hours of incubation at 37°C. Bile tolerance was evaluated by measuring after 24hrs of incubation at 37°C. In These experiments, 100ml was plated induplication to M17 agar.

**Milk fermentation ability test:**

Litmus milk test is a qualitative procedure used to determine the action of Individual isolates on milk (Mac Faddin, 2000). The ability to ferment milk is an indispensable characteristic for the selection of probiotic as the starter culture. Sterile litmus milk medium from Hi-Media (skim milk powder, 100g/L; litmus, 5g/L; pH6.8) was in occulated with overnight cultures of respective isolates followed by incubation at 37°C for 72h. Then changes in color and texture of the media were recorded.

**Colony Characterization:**

M17agar media was prepared and sterilized at 121°C for 30 minutes. After the sterilization was over organism was inoculated and incubated at 37˚C for 24 hrs. Grams staining, Catalase test was performed.

**Production of Soymilk:**

Commercial Soybean seed were socked in distilled water for 10-12 hours. After socking seeds were blanched by using 0.5% $NaHCo_3$. Cool the blanched soy seed. dehull the seeds. At last soybean cotyledon was blend as the result soymilk was produced.

**Production of Soy Curd:**

Prepared soymilk was pasteurized at 60-80˚C. Cool the pasteurized soymilk and add the starter culture to ferment the soymilk into soy curd.

**Preparation of Green Tea and Thulasi Extract:**

For 100ml of distilled water 2 gram of green tea and thulasi was added separately and boil for 10 minutes. After cooling the extract was filtered by vacuum filter.

**Preparation of Soy Fortified Green Tea and Thulasi Curd:**

To 5 ml of soy curd 1 ml of green tea and thulasi extract added separately in a sterile test tube and kept in room temperature.

**Estimation of Carbohydrate:**

Weighed 100mg of the sample into a boiling tube, hydrolysed by keeping it in a boiling water bath for three hours with 5.0 ml of 2.5 N HCl and cooled to room temperature. Neutralized it with solid sodium carbonate until the effervescence as made up the volume to 100 ml and centrifuged, collected the supernatant and take 0.2 to 1.0 ml for analysis. Prepared the standards by taking 0.2-1.0 ml of the working standards. 1.0 ml of water serves as a blank made up the volume to 1.0 ml in all the tubes with distilled water, then added 4.0 ml of anthrone reagent, heated for eight minutes in a boiling water bath, cooled rapidly and read the green to dark green colour at 630 nm.

**Estimation of Protein:**

To estimate protein content Pipette out 0.1 ml sample in test tube and make the volume to 1ml. A tube with 1ml of distilled water serves as blank. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. Make up the volume to 1.0 ml in all the test tubes. A tube with 1.0ml of water serves as the blank. Add 5.0 ml of reagent C to each tube including the blank. Mix well and allowed to be standing for 10mins. Then add 0.5 ml of reagent D, mix well and incubate at room temperature in the dark for 30min, blue colour is developed. Take the reading at 660nm.Draw a standard graph and calculate the amount of protein in the sample.

**Separation of Protein by Sds- Page Method:**

**Running the Gel:**

Separating gel and stacking gel were prepared and pour it in gel tray. Remove comb and assemble cast gel into Mini-Protean II apparatus. Add freshly prepared 1x running buffer (300 ml) to both chambers of the apparatus. Load the prepared samples into the wells of the gel. Run the gel at 100 V until the dye front migrates into the running gel (~15 min) and increase to 200 V until the dye front reaches the bottom of the gel (~45 min).

**Staining & Distaining the Gel:**

Remove the run gel from the apparatus and remove the spacers and glass plates. Place the gel into a small tray. Note: Never us a metal spatula to separate the

glass plates. Add ~20 ml staining solution and stain for > 30 min with gentle shaking. Pour off and save the stain. Add ~5 ml destain solution and destain for ~1 min with gentle shaking. Pour off and discard the destain solution. Add ~ 30 ml of destain solution. Destain with gentle shaking until the gel is visibly destained (> 2 hr). Pour off and discard the destain solution. Rinse with DDI H2O. Add ~30 ml DDI H2O and rinse for 5 min with gentle shaking. Dry the gel on the gel dryer at 60°C for 1 hr with a sheet of Whatman filter paper below the gel and a piece of Seran wrap over the gel.

**Estimation of Amino Acid:**

To 0.1 ml of extract, add 1ml of ninhydrin solution. Make up the volume to 2ml with distilled water. Heat the tube in a boiling water bath for 20min..Add 5ml of the diluents and mix the contents. After 15min reat the intensity of the purple colour against a reagent blank in a colorimeter at 570 nm. The colour is stable for 1h. Prepare the reagent blank as above by taking 0.1ml of 80% ethanol instead of the extract.

**Estimation of Total Flavonoid Content:**

**Aluminium Chloride Colorimetric Method:**

0.5 ml of different curd sampleswere taken. 0.1 ml of 10 % aluminium chloride was added and 0.1 ml of 1M potassium acetate was added. 4.3 ml of distilled water was added. It was incubated at room temperature for 30 min. Reading was noted at 415 nm in UV spectrophotometer.

**Determination of Total Phenolic Content:**

**FolinCioCalteau Method:**

400µL of different curd samples was taken.2.0 ml of folinciocalteau reagent (10 times pre diluted) was added. It was incubated for 5 min at room temperature.1.6 ml of 7.5 % of sodium carbonate solution was added. It was incubated for 60 min at room temperature. O.D value was noted at 765 nm in UV spectrophotometer.

**Determination of Total Tannin Content:**

**Vannilin HCl Method:**

1 ml of 4 different curd samples were treated with 5 ml of reagent mixture. 4 % Vannilin in methanol and 8% of concentrated HCL in methanol, 1:1 ratio. Colour was developed after 20 min of incubation at room temperature. O.D value was noted at 500 nm in UV spectrophotometer.

**Determination of Ash Content**:

Dry curd sample at 100˚c for 1 hr and cool it for 30 min. Take 1gm of dried sample heat it Bunsen flame and weight was measured.

$$Ash\ \% = \frac{weight\ of\ ash}{weight\ of\ sample} \times 100$$

**Determination of Fat Content:**

The curd sample was dried at 105˚c for 1hr and 0.3 gm of dried sample was taken and 25 ml of petroleum ether was added. The sample was kept in water bath for 3 hrs and evaporate the solvent and after the complete evaporation weight of sample was measured.

$$\text{Fat\%} = \frac{weight\ of\ fat}{weight\ of\ sample} \times 100$$

**Determination of pH:**

The pH of the GTC was measured before fridge ration (0day), then at 24h after storage (1$^{st}$ day) with an electronic pH meter. The curd samples were homogenized properly with alittled is tilled water before pH measurement.

**Antioxidant Activity:**

Antioxidant activity of soy fortified green tea curd and soy fortified tulsi were estimated. Different concentrations of fortified curd namely 20, 40, 60, 80 and 100 µg/ ml. These different concentrations of fortified curd samples were added to 3 ml of DPPH solution. The mixture was mixed thoroughly. It was incubated for 30 min at 20ºC. The readings were noted at 517 nm in UV Spectrophotometer.

$$= \frac{OD\ of\ blank - OD\ of\ sample}{OD\ of\ blank} \times 100$$

**Antimicrobial Activity:**

Sterile Nutrient broth tubes were prepared and 1ml of green tea and tulsi contain curd was added. *E.coli*, *Pseudomonas* sp, *Streptococcus mutans*culture was added to each test tubes and incubated at 37˚C for 24 hours. nutrient broth Without any extract was serves as control.

**RESULTS**

**pH tolerance:**

Organisms are observed in M17 agar plate which has pH 2.5. This will indicate that *Lactococcuslactis* have the ability to grow at low pH.

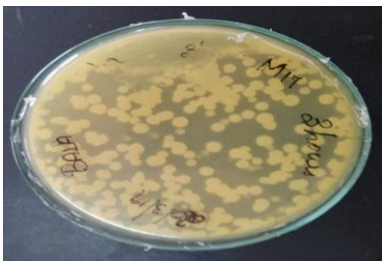


**Figure 1**
**pH tolerance**

**Bile Tolerance:**

Organisms are observed in M17 agar plate which contain bile. This indicate that *Lactococcuslactis*has bile tolerance.

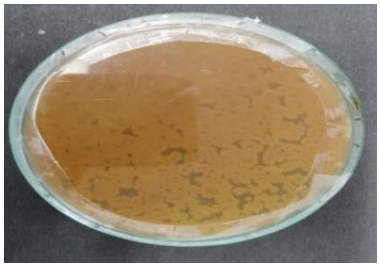


**Figure 2**
**Bile tolerance**

**Gram Staining**:

Gram positive cocci in chain appeared purple in color for *Lactococcuslactis*.

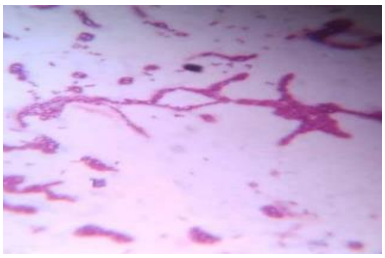


**Figure 3-**
**Gram staining**

**Catalase Test:**

For Catalase hydrogen per oxide was added to the culture *Lactococcuslactis* its shows positive results.

**Figure 4**
**Catalase test**

**Production of Soymilk:**

The Soymilk was produced using the soybean, using blended method.

**Figure 5**
**Soymilk**

**Production of Soy Curd:**

Soy curd was prepared by using probiotic stain *Lactococcuslactis*

**Figure-6 (a) Primary fermentation process**

**Figure- 6 (b) Secondary fermentation process**

**Production of Green Tea and Tulsi Extract:**

Green tea and tulsi extract were prepared and added to soymilk separately.

**Figure 7 (a)- Green tea extract**

**Figure 7 (b)- Tulsi extract**

**Milk Fermentation Ability Test:**

After one-day incubation milk was changes into pink color which indicate the presence of lactic acid.

**Figure 8- Milk Fermentation Ability test**

**Estimation of Carbohydrate, Protein, Amino acid, Total Solid Ash Content:**

Various parameters are measured for Soycurd, Green tea curd, Tulsi curd, and Mixed curd were measured.

**Table: 1**

| Parameter | Soycurd | Green Tea Curd | Tulsi Curd | Mixed Curd [Green Tea &Tulsi |
|---|---|---|---|---|
| Carbohydrate | 4.00 | 2.636 | 2.785 | 2.984 |
| Protein | 3.873 | 3.965 | 3.983 | 4.080 |
| Amino acid | 0.78 | 0.48 | 0.43 | 0.48 |
| Total Solid | 0.069 | 0.079 | 0.071 | 0.085 |
| Ash Content | 2.5 | 2.7 | 2.3 | 2.6 |

**Separation Of Protein by SDS Page Method:**

Protein in the Soy curd, Green tea curd and tulsi curd was separated by using SDS PAGE method according to the molecular weight of the protein molecule.

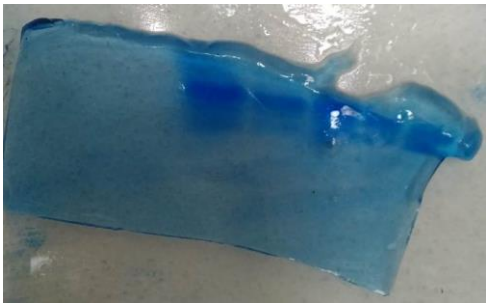


**Figure 9- SDS PAGE**

**Determination of pH:**

Shows the effect of refrigerated storage on pH, total acidity and total microbial count of the soy fortified GTC. There was a gradual decrease in pH from $0^{th}$ day to 1 day. The decrease in pH is showed to increase in lactic acid content due to fermentation.

***Antioxidant Content:***

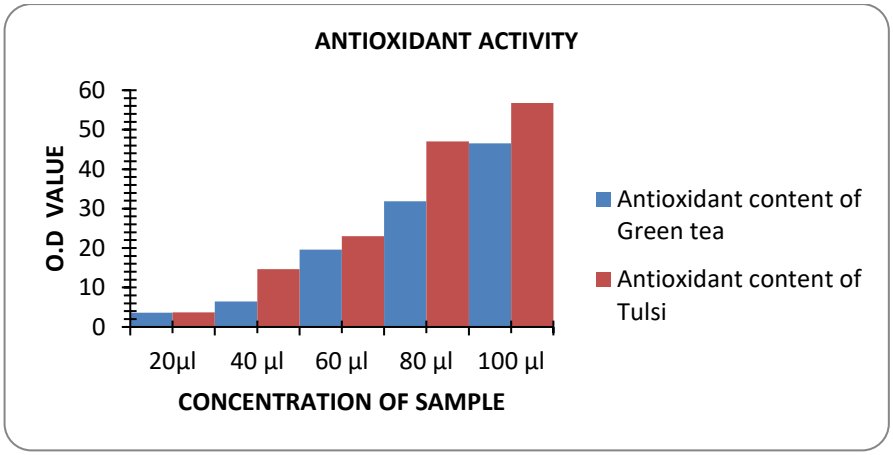
Antioxidant content was measured by DPPH method the reading is noted at 517 nm in UV spectrophotometer.

**TABLE: 2**

| S.NO | Concentration of sample (µl/ml) | Antioxidant content of Green tea (µl/ml) | Antioxidant content of Tulsi (µl/ml) |
|---|---|---|---|
| 1 | 20µl | 3.588 | 3.685 |
| 2 | 40 µl | 6.495 | 14.645 |
| 3 | 60 µl | 19.592 | 22.987 |
| 4 | 80 µl | 31.813 | 47.041 |
| 5 | 100 µl | 46.556 | 56.741 |

**Graph -1**

**Antioxidant activity**




**Estimation of Flavonoid, Tannin and Phenolic Content:**

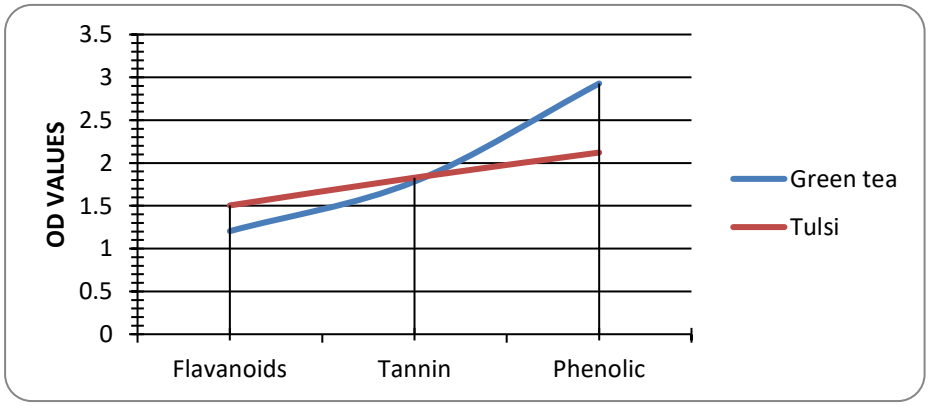
Flavonoids, Tannin and Phenolic content was measured by Aluminium chloride colorimetric method, Folic cioCalteau and Vannil in HCl method respective lt.

**TABLE: 3**

| Parameter | Green tea | Tulsi |
|---|---|---|
| Flavanoids | 1.204 | 1.504 |
| Tannin | 1.783 | 1.828 |
| Phenolic | 2.928 | 2.122 |

**Graph -2**

**Flavonoids, Tannin and Phenolic content**




**Antimicrobial Assay:**

Antimicrobial activity of Green tea curd, Tulsi curd and mixed curd by using antimicrobial assay technique the reading was taken by spectrophotometer at 540nm.

**TABLE:4**

| Organism | Control | Green tea curd | Tulsi curd | Mixed curd |
|---|---|---|---|---|
| *E.coli* | 1.481 | 1.413 | 1.402 | 1.398 |
| *Pseudomonas* sp | 0.816 | 0.701 | 0.754 | 0.684 |
| *Streptococcus mutans* | 0.990 | 0.898 | 0.876 | 0.856 |

**FT-IR Analysis**

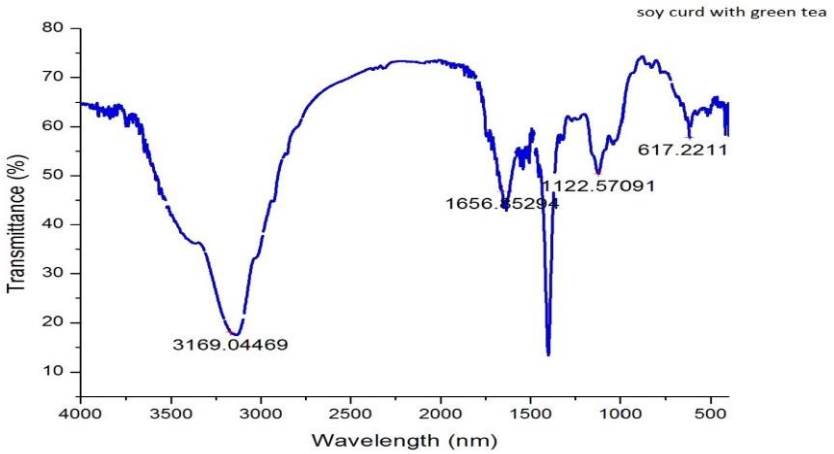



**Figure -10 FT-IR analysis for Green tea**

The peak at 617.2211 shows the presence of C-Br stretching and it indicates the presence of halo compound. The peak at 1122.57shows the presence of C-N stretching, and it indicates the presence of Aliphatic amines compound. The peak at 1656.85 shows the presence of –C=C- stretching and it indicates the presence of Alkenes. The peak at 3169.044 shows the presence of= C-H stretching, and it indicates the presence of Aromatics compound.

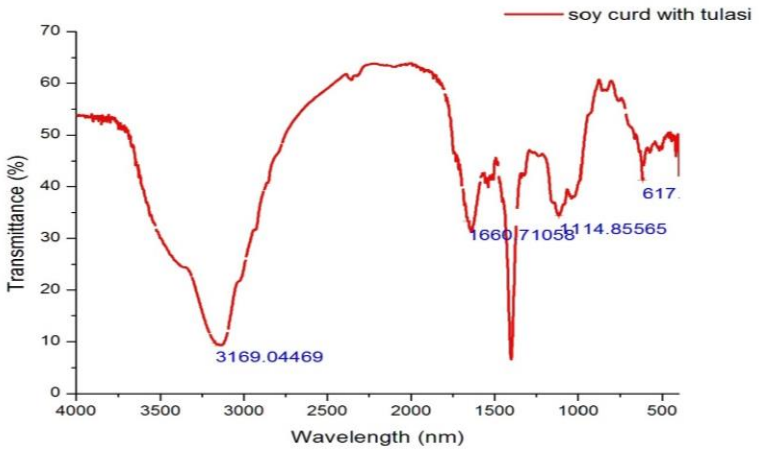



**Figure -13 FT-IR analysis for tulsi curd**

The peak at 617.2211 shows the presence of C-Br stretching and it indicates the presence of halo compound. The peak at 1114.85shows the presence of C-N stretching and it indicates the presence of Aliphatic amines compound. The peak at 1656.85 shows the presence of –C=C- stretching and it indicates the presence of Alkenes. The peak at 3169.044 shows the presence of= C-H stretching and it +indicates the presence of Aromatics compound.

**CONCLUSION**

Soymilk, a traditional oriental food beverage, is the water extract of soybean that provides a rich yet economical supply of protein and calories, contains no cholesterol or lactose, and only a small quantity of saturated fatty acid compared with cows' milk. Commercial Soy bean seeds are used to get milk by the process called socking, blanching and dehulling and as the result soymilk was prepared. To remove the beans like smell soymilk was boiled and this will also reduce

the microbial lode. The selected probiotic lactic acid producing bacteria was *Lactococcuslactis* was added to the soymilk to make fermentation.

Litmus test was done to identify the lactic acid production in the fermented milk product. After this phytochemical analysis was done. Estimation of Carbohydrate, protein, fat, ash content, solid content, amino acid was done by using standard procedure. To improve the taste and flavour green tea extract, thulasi extract were added to the fermented soy curd. After adding the extract phytochemical tests and flavanoid, anti-oxidant and tannin content was measured.

Soymilk is not so popular in India. Due to some health benefits of these products gradually awareness was created and medical fratemity is recommended to consume for Hyocholestremic patients. Flavoured Soymilk with sugar or vanillin flavour is marketed as beverage some parts of country. Soybean is very rich in protein and soluble fibre. Soymilk produced from beans is rich in protein more than or equal to cow milk. It has many health benefits. The is flavones in soymilk help fighting with heart disease, menopause, arthritis. It is a low fat high protein content and now recommended by Doctors. To increase the value of soymilk, the soymilk fermented by lactic acid producing probiotic organism Lactococcuslactis.

The soy curd was prepared and to increase the value and supress the soybean flavour green tea extract and tulsi was formulated. A cocktail of green tea, soy protein and probiotic organism from soyurd may help combat high cholesterol and blood pressure, laboratory studies by scientists at the National Institute of Technology (NIT), Rourkela, have suggested.

The work is mainly for create a single “nutrition–packed functional food” that would combine the benefits of probiotics from curd antioxidant from green tea and tulsi and protein subunits called peptides from soy. In this study the fat content of soy curd was estimated, but no validated through the experimental studies and animal studies. For the further or future study, the work has to be validated for the experimental analysis and animal studies for identify the cholesterol control ability of soy fortified curd.

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