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Optimization of Fermentation Conditions for the Development of Probiotic Soymilk Using Lactobacillus Paracasei Ssp. Paracasei 013 Strain

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Abstract

The present study was aimed at developing probiotic soymilk by fermenting soymilk with Lactobacillus paracasei ssp. paracasei 013 strains. The bacteria were grown on MRS media and Tofu whey (byproduct in tofu preparation) inoculated over soymilk with different inoculums volume (1 to 5%) and for different time period (1 to 5 h). Evaluation of microbiological, biochemical and different physico-chemical parameters of fermented soymilk revealed no significant changes in electrical conductivity, NaCl, and total solids, but pH was found to decrease and titrable acidity was found to increase with time period. The study indicated that the proper coagulation point could be achieved in 3 hours with 3% culture using MRS as growth culture media, whereas for tofu whey culture media the proper coagulation was reached in 3 hours with 4% at 37°C.

Keywords: *Coagulation, Microbiology, Probiotics, Soymilk, Tofu Whey*

1. Introduction

The soybean, *Glycine max* (L) Merrill, belongs to the family *Leguminosae* and subfamily *Papilionoidae* originated in Eastern Asia is composed of macronutrients such as lipids, carbohydrates and proteins. Carbohydrates make up about 30% of the seed, with 15% being soluble carbohydrates (sucrose, raffinose, stachyose) and 15% insoluble carbohydrates (dietary fiber). Protein content of soybean varies from 36 to 46% depending on the variety [8, 9]. Soybeans also contain micronutrients, which include isoflavones, phytate, saponins, phytosterol, vitamins and minerals. Soybeans are transformed into many different varieties of foods such as soymilk, tofu, soybean cheese and miso, which are traditional foods in Asia. Among these soy foods, soymilk has gained much popularity as a healthy food drink. Soymilk is a stable emulsion of oil, water, and protein, which is made from soaking, grinding and boiling soy beans with water.

Soymilk is high in protein, cholesterol-free, and low in calcium, fat and sodium. Soymilk looks like dairy milk except that it has its own peculiar odour and taste. Soymilk has been an important high quality protein source in the diet of Eastern people for a long time. In Western countries, soymilk has been used mainly as an important replacer of milk for lactose intolerant people, as well as a low cost source of good quality protein and energy, mainly in developing countries [11, 14]. However, the consumption of soymilk is limited because of the beany flavor and the presence of non-digestible oligosaccharides such as stachyose and raffinose. These non-digestible oligosaccharides are not digested by human beings and may cause flatulence. Further, soybeans are rich with phytic acid which is negatively charged, it complexes with positively charged ions and proteins and subsequently decreases the bioavailability of these compounds [15, 20].

Lactic acid fermentation is the way to overcome these limitations. Soymilk is also a suitable medium for growing lactic acid bacteria, thus improving the quality of fermented products. Lactic acid bacteria have attracted enormous attention for food manufacturer due to their potential associated with health-promoting effects. They are classified as probiotics which do not pose any health risk and some are designated as “GRAS” (Generally Recognised as Safe) organisms. Probiotic lactic acid bacteria provide substantial health benefits to human health by means of maintenance of normal intestinal flora. Some of probiotic strains have been shown to be effective in reducing the severity and duration of diarrhea, lowering cholesterol, increasing of turnover of enterocytes and neutralizing of dietary carcinogens [19]. The fermentation of soymilk with the probiotic cultures of lactic acid bacteria and bifidobacteria have been shown to exert beneficial effects on human hosts [21, 22]. Soy is considered as a good substrate for functional foods since

fermentation by probiotics has the potential to reduce beany flavor, reduction of oligosaccharides, release of free isoflavones, improves antioxidant activity and has the beneficial effect of using probiotics.

A foremost benefit of soy beverage fermentation is the reduction of ‘‘beany” flavor [4]. Fermentation of soymilk with various microorganisms, especially lactic acid bacteria, has been attempted to overcome the problem of beany flavour and increase acceptability [7]. Blagden and Gilliland [4] reported that methanol, acetaldehyde, ethanol and hexanal were the 4 major volatiles detected in unfermented soymilk. Soymilk contains oligosaccharides, principally sucrose, raffinose and stachyose, which are recognized as the flatulence factors. This can be reduced by lactic fermentation as shown by Wang et al. [22]. Study showed a reduction in stachyose and raffinose and increase in sucrose, fructose, galactose in fermented soymilk. In non-fermented soy food products, isoflavones predominantly exist as biologically inactive glycoside conjugates ranging from 83.90% to 98.37% [12]. In humans, the isoflavone aglycones are absorbed faster and in greater amounts than their glycosides counterpart. Aglycones are absorbed directly through the gut wall, while isoflavone glycosides are very poorly absorbed from the gut due to their higher hydrophilicity and larger molecular weight [10]. Microorganisms in soymilk may lead to a combination of benefits as probiotics as well as the transformation of isoflavone glycosides to bioactive isoflavone aglycones. In addition, aglycones have been reported to be more stable than isoflavone glycosides during the storage at different temperatures [18]. Antioxidant activity of fermented soy foods increases due to isoflavone bioconversion during fermentation. Wang et al. [23] reported that antioxidative activity increases as the fermentation period is extended. Considering all the advantages of soymilk fermentation, the study was undertaken to optimize fermentation conditions for the development of probiotic soymilk which can be further be made into powder form by drying.

2. Material and Methods

2.1 Raw materials

Soybeans variety particularly JS335 (JAWAHAR SOYBEAN 335) was used to prepare the aqueous extract with dehulled split soybeans. The probiotic bacteria specifically Lactobacillus paracasei ssp. paracasei 013 strains grown in MRS (de Man, Rogosa and Sharpe) broth and Tofu whey (byproduct in tofu preparation) was used as culture over the prepared soymilk.

2.2 Preparation of Soymilk

Soymilk was prepared by soaking split dehulled soybeans (JS335) in water in the ratio of 1:3 for 3-4 hours. Then the soaked soybeans were grinded with water in the ratio of 1:6 in a blender for 2 min. The blended mixture was cooked for 15 to 20 minutes with constant stirring which aims to reduce the beany flavor and destroys the anti-nutritional compounds in the soybeans. The mixture was strained through muslin cloth to collect the soymilk and the residue (okara) was retained in the muslin cloth.

2.3 Preparation of culture media with tofu whey for the preparation of fermented milk

Tofu whey, a by-product of tofu manufacturing, is discarded by most soybean processing industries which can be used as a good source of carbohydrates, some free amino acids, isoflavones and protein. Tofu whey contains valuable compounds like non-digestible oligosaccharides (NDO) and is recognized as prebiotics. For the preparation of tofu, soymilk was coagulated by heating, in combination with salts, acid or enzymes. This generates a liquid byproduct called tofu whey (TW). The tofu whey is highly perishable and needs effective utilization. The lactobacilli population obtained in MRS culture medium was found to be higher than that in TW alone, and supplementation of TW was thus examined. Addition of yeast extract (0.2%), glucose (1.5%) and buffer to TW was found to show higher populations up to 1.7×10^9 cfu/ml. Tofu whey was fermented for 24 hour which exhibited a potentially high antioxidant activity.

2.4 Preparation of Fermented Soymilk

For fermentation, 100 ml of soymilk in a 250 ml screw-cap Erlenmeyer flask was inoculated with 1 to 5 mL of the inoculum and incubated at 37 ^{0}C for a period of 1 to 5 hour. Fermentation study was carried using MRS (De Man, Rogosa and Sharpe) broth and tofu whey (byproduct from tofu preparation) as the culture growth media for growth of probiotic bacteria specifically Lactobacillus paracasei ssp. paracasei 013 strains.

2.5 Physico-chemical analysis of the fermented soymilk

2.5.1 Determination of pH

pH of the fermented soymilk was determined using a hand held pH meter (Model Ph 323,Ser.Nr.63260002, WTW 82362 Weilheim, and Germany) attached to a stainless

steel pH/temperature probe. pH meter was calibrated with at least two standard buffer solutions before each measurement. Then to measure the pH of samples, the probe was dipped into the sample, and reading was noted down.

2.5.2 Determination of Titratable acidity

It was determine by titrating a know volume of milk with standard alkali to the point of an indicator line phenolphthalein. Acidity is expressed as percent lactic acid. The burette was filled with 0.1N NaOH solution. Then the sample was mixed thoroughly by avoiding incorporation of air. 10 ml sample was then transfer with the pipette into the conical flask. Equal quantity of distilled water was added. 3-4 drops of phenolphthalein indicator was added and stirred with glass rod. Initially reading of the alkali in the burette at the lowest point of meniscus was taken. The content was rapidly titrate with 0.1N NaOH solution continuously by addition of alkali drop by the drop and stirring the content with glass rod till first definite change to pink colour was reached which remains constant for 10 to 15 seconds.

$$\% \text{ Titrable Acidity} = \frac{ml \times N \times 90 \times 100}{V \times 1000} \quad \cdots\cdots \quad (1)$$

Where,
ml= ml 0.1 NaOH used
N= Normality of 0.1 N NaOH
V= ml sample used

2.5.3 Determination of TDS & Conductivity

The total dissolved solid and conductivity of samples was determined by a microprocessor based conductivity meter (Model: H12300, Hanna Instrument Inc., Woonsocket, USA). A determination of a solution's conductivity provides a first indication of the concentration of electrolytes dissolved in the sample solution. A higher conductivity is related to a higher concentration of TDS in the solution. The conductometric probe was inserted in the sample solutions and necessary readings were noted down. The probe was rinsed and dried properly after every measurements.

2.5.4 Determination of Total Solid content

To measure total solids, clean petriplate was washed and dried in a hot air oven at 105°C for one hour. Then empty dish was weighed in analytical balance. 10mL of unfiltered sample was transfer into the petriplates using a pipette and its weight was noted down. . Petriplates were placed in the hot air oven at 105°C and dried for 5 hours to get constant mass. Then the container was cooled in a desiccator. The dish was weighed as soon as it was cooled to avoid absorption of moisture due to its hygroscopic nature. The total solid content was calculated by using the following formula

$$\%\text{Total solids} = \frac{[(\text{weight of dry sample} + \text{container}) - (\text{weight of empty container})]}{(\text{weight of wet sample} + \text{container}) - (\text{weight of empty container})} \times 100 \quad \cdots\cdots \quad (2)$$

2.6 Biochemical analysis of fermented soymilk

2.6.1 Proximate analysis

2.6.1.1 Determination of Crude Protein

Crude protein (N × 6.25) was determined by Kjeldahl method according to AOAC [2], AC-4-41. Crude protein is total nitrogen multiplied by protein factor. It is expressed in g per 100 g sample. Total nitrogen content includes nitrogen primarily from proteins and to a lesser extent from all organic nitrogen containing non-protein substances. For practical purposes, non-protein nitrogen is assumed to be of little significance. The method was based on the digestion of proteins and other organic food components in the sample (0.25g) with 15 ml sulfuric acid in the presence of 100g catalyst (sodium or potassium sulphate) to release nitrogen from protein. Then the Digestion tube was then place in a kelpus digestor (model: KES) and temperature finally increased to 420ºC for 3 hours or until the solution turned bluish green and retain it as ammonium salt. Then 50 ml of 4 % boric acid indicator solution was placed on the distillation unit (model: DISYL-EM). Ammonia gas is liberated upon addition of excess alkali (concentrated sodium hydroxide) and was distilled into a boric acid solution to form ammonium-borate complex. The ammonia liberated from the complex was titrated with 0.1 N sulphuric acid. The amount of nitrogen in the sample was determined from the milligram equivalent of the acid used. Crude protein was determined by multiplying the nitrogen content with a conversion factor specific to the food matrix. Formula used to calculate Crude Protein is as follows:

$$\% \text{ Nitrogen} = \frac{0.014 \times \text{burette reading} \times 100 \times \text{normality of sulphuric acid}}{\text{weight of sample}} \quad \cdots\cdots \quad (3)$$

$$\% \text{ Protein} = 6.25 \times \text{Nitrogen} \quad \text{.....} \quad (4)$$

2.6.1.2 Determination of Fat

Fat content in the products was determined using soxhlet apparatus according to AOAC standards using Iso-propanol as solvent. The boiling flask was rinsed with commercial grade acetone to remove any residual oil/fat and then was dried in hot air oven for an hour. The flask were weighed and labelled. 2 to 3g of samples were taken in the filter paper and folded firmly and kept in paper thimbles. The thimbles were placed in the soxhlet extraction tubes. The tubes were then filled with solvent, sufficient enough so that the siphon system start working. Finally the heaters were switched on and the system was allowed to run for 4 hours. The tubes were then removed from the assembly and the solvent was allowed to evaporate until the flask were left with the fat alone and then was weighed. Formula used for determination of fat content is as follows:

$$\text{Fat } \% = \frac{weight\ of\ Fat}{weight\ of\ sample} \times 100 \text{.....} \quad (5)$$

2.6.1.3 Determination of Moisture content

The moisture was determined by oven drying method as per AOAC. The empty dish was dried in oven and transferred to desiccators. Empty dish was then weighed with lid. Then 2g of sample was weighed in the dish and placed in the oven. Sample was dried for 3-5 hours at 105ºC for constant weight. After the sample was dried, it was transferred with the lid to the desiccator and reweighed with dried sample after cooling. Moisture content (% wet basis) was determination as follows

$$\text{Moisture } (\%) = \frac{W1 - W2}{W1} \times 100 \text{.....} \quad (6)$$

Where:
W1= Weight of sample before drying; W2= Weight of sample after drying

2.6.1.3 Determination of Ash

Ash content which is the measure of total amount of minerals present within a food, was determined using muffle furnace (make: SCIENTECH, Size-8×8×12). About 1-3 g sample was weighed in a crucible. Then the sample was heated over heater till no smoke is produced. The crucible was then transferred to the muffle furnace maintained at 650ºC for 2 hours. The muffle furnace was turned off and allowed to cool and was reweighed. Percentage ash content was determined as follows

$$\text{Ash } (\%) = \frac{Weight\ of\ ash}{Weight\ of\ sample} \times 100 \text{.....} \quad (7)$$

2.6.1.5 Determination of Carbohydrate

Carbohydrate content was determined by the formula

$$\text{Carbohydrate } (\%) = 100 - (\text{protein } \% + \text{fat } \% + \text{moisture content } \% + \text{ash } \%) \text{.....} \quad (8)$$

2.6.2 Determination of Anti-oxidant activity

2, 2- diphenyl-1-picrylhydrazyl (DPPH) assay method was used for the determination of antioxidant activity. This method was developed by Blois [5] with the viewpoint to determine the antioxidant activity in a like manner by using a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33). The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine [6]. DPPH is a stable free radical in a methanolic extract. The odd electron in the DPPH free radical gives a strong absorbion maximum at 517 nm and is purple in colour. In the oxidized form, the DPPH radical has an absorbance maximum centered at about 520 nm [16]. 0.1mM DPPH solution was prepared by addition of 0.0395 g of DPPH to 10 ml methanol (stock solution). Then 1 ml of stock solution was added to 99 ml methanol for the preparation of working solution. To determine the antioxidant activity 1 g of sample was taken in a test tube and mixed with 8 ml of methanol. The mixture was kept overnight. Then the extract was filtered using Whatman no.1filter paper and the volume of extract was maintained to 10 ml using methanol. 2 ml of the extracted sample was mixed with 2 ml DPPH reagent and kept in a dark container. Then the mixture was incubated for 30 minutes. Absorbance was measured at 520 nm by using UV-VIS Spectrophotometer

Model: SL 164 against blank and control (methanol and DPPH reagent).

Free radical scavenging activity was measured as the decrease in the absorbance of DPPH using following equation

$$\% \text{ Inhibition} = [(A0 - Ae)/ A0] \times 100 \text{.....} \quad (9)$$

2.7 Microbiological analysis of fermented soymilk

Enumeration and assessment of viability are crucial in this research and selection of fermentation starter bacteria and development of probiotics. MRS is the growth medium used which is so-named by its inventors: de Man, Rogosa and Sharpe, designed to favour the luxuriant growth of *lactobacilli* for lab study. It contains sodium acetate, which suppresses the growth of many competing bacteria. Thus to optimize the fermented soymilk Lactobacillus (MRS) agar media and Standard Plate Count Agar was used for cultivation of bacteria. For the preparation of media, weighed amount of media was dissolved in distilled water, was made up to required volume and then autoclaved at 121ºC, 15 lbs pressure for 15-20 minutes.. Then repeated dilution of the solution was performed to achieve a geometric dilution of the original solution that is required in experiments requiring highly dilute solutions with great accuracy. To obtain direct counts, 15 - 20 ml sterile, molten (45 - 50°C) sterile media was poured into sterile petridishes containing 1 ml volumes of diluted test sample. Inoculum was distributed throughout medium by rotating the plate in one direction, then in the reverse direction. The plate was allowed to cool and the lid was closed. This was carried out in a sterile condition in the laminar air flow. Then the agar plates were incubate at 37°C for 36 hour. The colonies were counted under the colony counter.

3. RESULTS AND DISCUSSION

Soymilk was prepared by wet grinding of soaked dehulled soybeans (JS335) for 1-2 minutes in blender, followed by cooking for 15-20 min and then by filtering to separate the soymilk and the byproduct okara. The prepared soymilk was then filled in 500 ml screw cap Erlenmeyer flask and autoclaved at 121°C for 15 min for effective sterilization by moist heat. Soymilk was then subjected to

physiochemical analysis before fermentation. The total solid content was experimentally found to be 7.4 %, conductivity (mS), total dissolved solid (ppm) and pH was found to be 1.54, 776 and 6.75 respectively. The antioxidant activity (%) was determined to be 35 % and the total plate count (cfu/ml) of raw and pasteurized soymilk were analyzed. There was no total or viable bacterial growth in pasteurized soymilk as there was in raw soymilk (4.5 x$10^{5)}$. This condition is required here as the study aimed at growing specific bacteria *Lactobacillus paracasei ssp. paracasei* 013 strains.

The tofu whey which is the byproduct of tofu was prepared by coagulating soymilk using 0.3 % of citric acid. The liquid separated from the coagulum is centrifuged to obtain clear liquid called tofu whey. Recently, tofu whey (TW) has been proposed as a potential alternative for the production of starters for the fermentation of soy products [17]. The pH of Tofu whey was found to be 5.83 which is not optimum for the growth of lactobacilli, thus the pH was maintained to desired level by using strong base sodium hydroxide. Conductivity (mS) and total dissolved solid (g/l)) was found to be 3.69 and 1.83 respectively.

3.1 Viability of bacteria culture in soymilk

Ben Ounis [3] reported that demineralized TW supplemented with yeast extract and sucrose was relatively good growth media for lactobacillus. Comparative study between MRS culture media and the prepared tofu whey incorporated with nutritional additives (glucose and yeast extract) was carried out in order to develop conditions for the utilization of tofu whey, which otherwise is discharged off as waste. The lactobacillus was inoculated in a sterile condition into the MRS media and fortified tofu whey. Then both the media were incubated at similar conditions and the concentration of their viable cells were studied as shown in the Table 1, which shows that tofu whey can be used as a good media for the growth of probiotic bacteria.

Table1. Concentration of viable cells

Time (hour)	*MRS Culture (%)		*TW Culture (%)	
	Viable cells concentration N (cfu/ml)	pH	Viable cells concentration N (cfu/ml)	pH
1	2 x10^9	6.4	2 x10^9	6.5
2	4 x10^9	6.2	2 x10^9	6.4
3	6x10^9	5.8	2 x10^9	6.3
4	8 x10^9	5.8	11 x10^9	6.2
5	10 x10^9	5.7	12 x10^9	5.9

*MRS (de Man, Rogosa and Sharpe) culture media,
*TW- tofu whey

Ben Ounis [3] reported in his study that lactic acid production during fermentation is proportional to sugar catabolism. Effectively, the mean total sugar consumed in these media during fermentation corresponds to the lactic acid produced. Thus the variation of pH as shown in Table1 may represent the sugar consumption during fermentation. Whereas, according to the study the positive acceleration phase, which is necessary for adaption of cells to new environment lasts longer up to 3rd hour in case of TW, may be due to different environmental conditions or stresses eg. fluctuation in the biomass distribution, oxygen availability, salt concentration, pH, glucose limitation, etc. During this phase the bacteria grow and the size increases;

but the population density is almost constant. This phase is then followed by exponential growth phase starting at the 4th hour.

3.2 Coagulation study with MRS and TW as culture media

Coagulation of the soymilk during fermentation represents the increase in concentration of bacteria. It was found during the study that the sample incubated for more time showed more coagulation as compared to sample incubated for lesser time. On the basis of the study several stages of coagulation based on the fermentation conditions were identified. Basically four stages were considered in this study – no coagulation, slight coagulation, moderate coagulation and proper coagulation stage as shown in the Figure1. The purpose of this categorization was to compare the MRS culture and TW as culture media on the coagulation rate with the variation in inoculum volume (1%, 2%, 3%, 4% and 5%) and incubation time (1h, 2h, 3h, 4h and 5h) as shown in Table 2. Optimum condition for proper coagulation with MRS culture media was achieved in 3 hours with 4% culture volume, whereas for tofu whey as culture media the optimum condition for proper coagulation was achieved in 4 hours with 4% culture volume at 37°C.

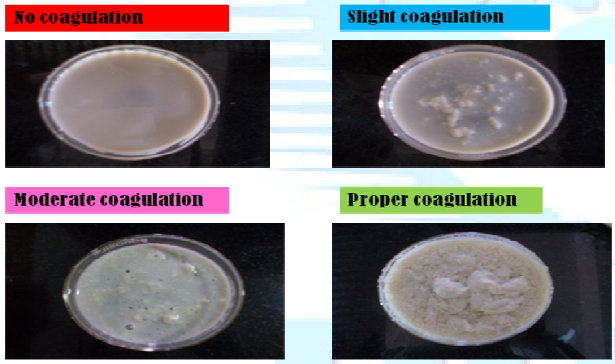

Figure 1 Different stages of coagulation

Table 2. Shows the different stages of coagulation with MRS and TW as culture media at different inoculation volume and at different time period of incubation

Time (hr)	culture type	Observation				
		1%	2%	3%	4%	5%
1	MRS	NC	NC	NC	SC	MC
	TW	NC	NC	NC	SC	MC
2	MRS	NC	NC	SC	MC	MC
	TW	NC	NC	SC	SC	PC
3	MRS	SC	SC	MC	PC	PC
	TW	NC	NC	SC	MC	PC
4	MRS	SC	SC	PC	PC	PC
	TW	NC	SC	MC	PC	PC
5	MRS	SC	MC	PC	PC	PC
	TW	NC	SC	PC	PC	PC

3.3 Physiochemical analysis of fermented soymilk

Physiochemical analysis of the non fermented soymilk and soymilk fermented for the period of 1 to 5 hour shown in Table 3. The study shows that incubation time affects the pH and titrable acidity of the sample as the organic acid in the sample increases due to the action of lactobacilli. The production of organic acid(mainly lactic acid) contributes to the flavor of the final product. It is clear that organic acids are one of the factors to prolong shelf life of the final product. The inhibition of pathogenic and spoilage flora is dependent on concentration of organic acids [1] .No significant changes was observed in the electrical conductivity, NaCl, TDS and total solids content in fermented soymilk during fermentation up to 5 hour. But with 1% culture of MRS, coagulation started at 3rd hour whereas with TW no coagulation was seen for the test period of 5 hour. Graphical representation of the Table 4.5 is given in Figure 2.

3.3 Biochemical analysis

The Figure 3 shows the antioxidant activity of the non fermented soymilk and fermented soymilk incubated for 1 to 4 hour. Antioxidant activity (DPPH inhibition %) increased as the incubation time increases. The antioxidant activity was found to increase with the increase in fermentation hour. However the probiotics incorporated in soymilk via MRS as culture media showed more antioxidant activity in comparison to probiotics incorporated via tofu whey.

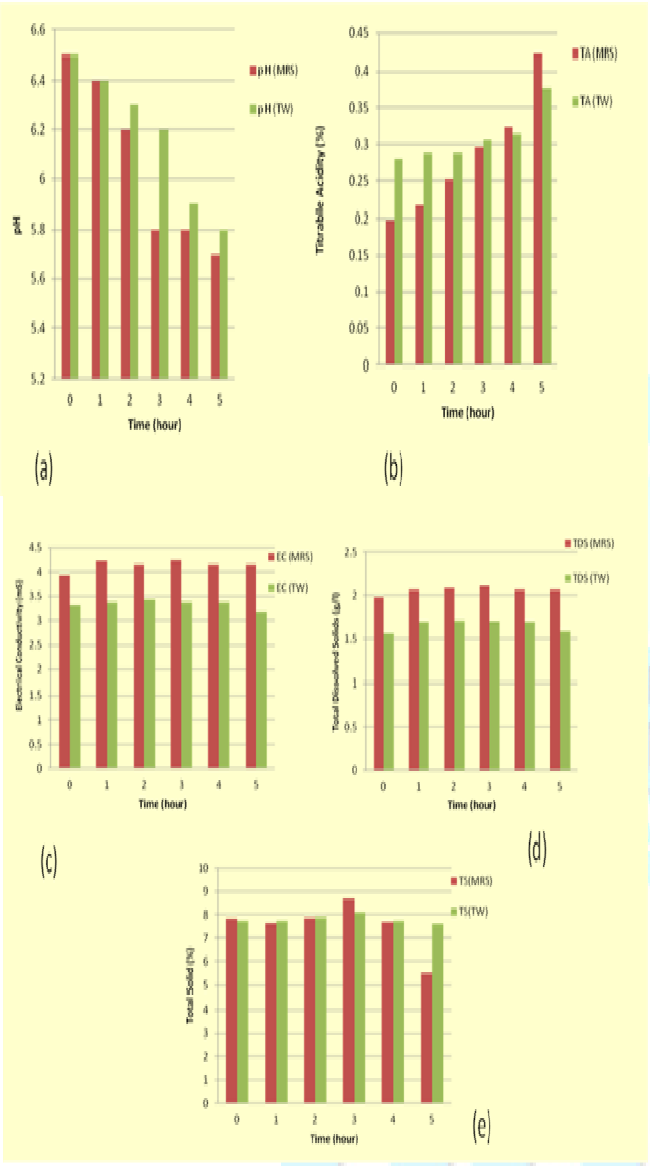

Figure 2. Physiochemical analysis-where (a), (b), (c), (d) and (e) represents time versus pH, Titrable acidity, electrical conductivity, total dissolved solids and total solids respectively

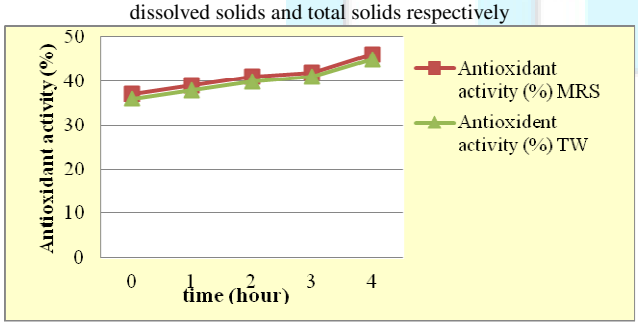

Figure 3. Antioxidant Activity (%) of fermented soymilk with MRS & TW as culture media

4. CONCLUSION

Based on the results obtained, several conclusions have been drawn in the present investigation stating that Tofu whey can also be used as a good media for the growth of probiotic bacteria. Optimum condition for proper coagulation with MRS culture media was achieved in 3 hours with 4% culture volume, whereas for tofu whey as culture media the optimum condition for proper coagulation was achieved in 4 hours with 4% culture volume at 37°C with acceptable viability. No significant changes was observed in the electrical conductivity, NaCl, TDS and total solids content in fermented soymilk during fermentation up to 5 hour. The antioxidant activity in fermented soymilk was found to increase with the increase in fermentation hour. The probiotics incorporated in soymilk via MRS as culture media showed more antioxidant activity in comparison to probiotics incorporated via tofu whey. The coagulation study can also be used to optimize coagulation state of the feed used for spray drying as it cannot accept highly coagulated slurry which may clog the nozzle.

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