



## **Production of Fermented Soymilk Drink Containing Probiotic *Bacillus coagulans***

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### **Authors' contributions**

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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### **ABSTRACT**

The aim of this study is to produce a fermented soymilk drink using *Bacillus coagulans*. This was performed in the Microbiology laboratory of Nnamdi Azikiwe, University. 20ml aliquots of soymilk containing *Bacillus coagulans* was fermented at 28°C, 37°C, 42°C and 50°C for period of 9 h. The pH of the soymilk and growth of *Bacillus coagulans* was checked during the fermentation period. The effect of sugar supplementation and adjustment of initial pH on soymilk fermentation was also checked. A 9-point hedonic scale was used by the sensory panelist for the sensory evaluation of the fermented soymilk. At 28°C, pH of soymilk did not decrease and cell count did not increase throughout the fermentation period. Fermentation at 37°C, 42°C and 50°C recorded decrease in pH and increase in cell count. Addition of 0.5% sucrose improved acid production and maintained a good cell count. Concentrations above 0.5% sucrose saw a slight decline in cell count. Glucose concentration of 0.5% to 2% improved acid production. Glucose concentration of 0.1% to 1% improved the growth of the probiotic cells. Concentration above 1% caused a drop in probiotic cell count. Adjustment of soymilks initial pH and addition of 0.5% glucose resulted in pH drop to 4.5 after 9h fermentation at 50°C. The fermented soymilk had moderate overall acceptability by the sensory panelist. *Bacillus coagulans* can be used as probiotic of choice to produce a fermented soymilk.

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## 1. INTRODUCTION

Soymilk is an aqueous, white, creamy extract produced from soybeans, which is similar to cow milk in appearance and consistency. It is a very rich source of highly valuable proteins, unsaturated fatty acids, soluble and insoluble dietary fibers (prebiotics) and isoflavones, whose presence in everyday diet is very important [1]. Soymilk's comparative low cost and high nutritive value as good source of protein, has placed it as a common food among people, especially children, in most developing countries [2].

Probiotics are defined as "live microorganisms that, when taken in adequate amounts, confer a health benefit on the host" [3]. In order to obtain the benefits of probiotic, the probiotic products should contain at least  $10^6$ – $10^9$  cfu/g probiotic microorganism and should survive until the end of shelf life. Probiotic microorganisms, some of which are naturally among the intestinal microbiota, could protect humans from diseases, modulate and strengthen the immune system, prevent tooth decay, have anti-carcinogenic properties, and be effective against coronary heart disease [4]. Probiotic microorganism can be classified into two groups, the spore forming probiotics and the non-spore forming probiotics. Though most probiotic microorganisms fall under the non-spore formers, the spore forming probiotic microorganisms have the advantage of being less susceptible to adverse environmental conditions such as high temperatures.

Dairy products are often used as vehicles to carry probiotics for the development of functional food products with *Lactobacillus* and *Bifidobacterium* being the most commonly used microorganism in probiotic food production [5,6,7]. Fermenting soy milk with lactic acid bacteria considerably increases its health value such as, low cholesterol and lactose, ability to reduce bone loss and menopausal symptoms, prevention and reduction of heart diseases and certain cancers as well as increased oxidative activity [8].

*B. coagulans* is a gram-positive, facultative anaerobic, nonpathogenic, spore-forming, lactic acid-producing bacterium. It is resistant to heat; the optimum growth temperature for *B. coagulans* is 35 to 50°C and the optimum growth pH is 5.5 to 6.5 [9]. It has the characteristics of microorganisms used as probiotics. Unlike other lactic acid bacteria added to food as probiotic, *B.*

*coagulans* can survive in food at room temperature for long period [10]. Several *Bacillus* sp. have received GRAS status and Grasp notification number (GRN) for use in food as probiotics from FDA. Examples include *B. coagulans* MTCC 5856 (GRN 000601), *B. coagulans* GBI-30, 6068 (GRN-000660), *B. coagulans* DSM 17654 (GRN000949), *B. coagulans* 70258 (GRN 000691) and *B. coagulans* SNZ 1969 (000864).

Milk and milk products have been used as the vehicle to deliver probiotics. Nevertheless, milk is quite an expensive commodity to the common person especially in developing countries. Soymilk which is an alternative to milk, is more affordable to the poor compared to milk but has hardly been used as a means of conveying probiotics. Therefore, producing a probiotic soymilk drink using *B. coagulans* will be of immense benefit.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Phosphate buffered saline, glucose yeast extract agar, DeMan Rogosa Sharpe agar, DeMan Rogosa Sharpe broth, distilled water, acetic acid, sodium hydroxide, Phenolphthalein, glucose, sucrose, lactose. All chemicals used were analytical grade.

### 2.2 Procurement of Probiotic *Bacillus coagulans* and Activation of Spores

Lactospore<sup>®</sup>, a commercial probiotic preparation containing L (+) lactic acid producing microbial preparation from *Bacillus coagulans* MTCC 5856 was acquired from Sabinsa Corporation, New Jersey, USA.

One gram of lactospore ( $6 \times 10^9$ /g) was suspended in 100 ml of sterile 0.2 M phosphate buffered saline (PBS, 7.0) and incubated in a water bath for 30min at 75°C, followed by immediate cooling to below 45°C. The suspension was then serially diluted in sterile 0.2 M PBS, pH 7.0 distilled water and plated out on GYE agar [10]. The activated spores were then stored in agar slant.

### 2.3 Preparation of Soymilk

The preparation of soymilk is shown in Fig. 1. Briefly, soybeans (*Glycine max* L.) was soaked in

water for 8 hours, it was drained and boiled in fresh water until a soft cotyledon is obtained, it was then washed to remove the hulls. The blanched soybean was ground using a clean blender (Binatone) with the addition of water to give a water-to-dry-soybeans ratio of 4:1 on a weight basis. The resultant slurry soybeans were filtered through cheesecloth. The resulting filtrate soymilk was boiled for 30 minutes at 80°C to obtain a pasteurized soymilk.

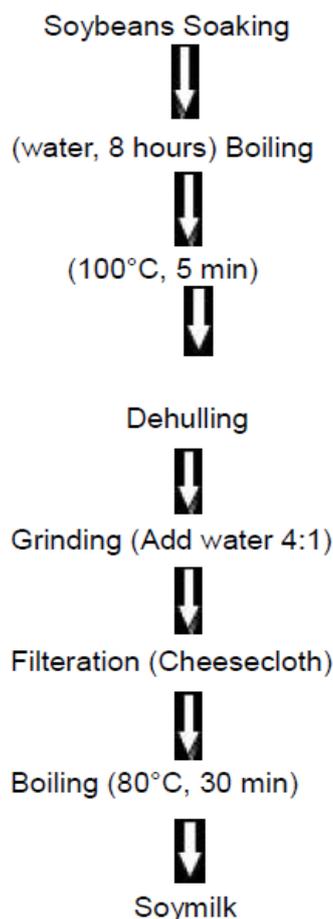


Fig. 1. Flow chart for the preparation of Soymilk

## 2.4 Preparation of Inoculum

Activated spores of *Bacillus coagulans* MTCC 5856 was inoculated into MRS broth using a sterile wire loop and incubated at 37°C for 48h.

25ml of MRS agar was inoculated aseptically with 1% (v/v) ml of broth culture of the probiotic strain and incubated at 37°C for 24 h. After the incubation period, 10-fold dilution was carried out to obtain a culture containing 10<sup>8</sup>CFU/ml of *B. coagulans*.

Optical density at 600nm (OD600) was carried out on the several dilutions and 1ml from the several dilutions was pour plated onto MRS agar and incubated at 37°C for 48 h. The plates containing 30-300 colonies were taken as ideal.

## 2.5 Fermentation of Soymilk

A measured volume, 150 ml of soymilk was dispensed into 250 ml conical flask and sterilized at 121°C for 15 mins. Soymilk was cooled and then inoculated with 1% v/v (1.5 ml) of active *B. coagulans* MTCC 5856 culture. The mixture was shaken properly and 20 ml aliquot dispensed into sterilized 100 ml conical flask aseptically. The inoculated soymilk was fermented for 9 h at room temperature (28°C).

To determine the optimum temperature for increase in acid production and cell count, fermentation of soymilk was done at 37°C, 42°C and 50°C. During the fermentation period, pH and viable cell counts were monitored at 0, 3, 6 and 9h. The CFU/ml was determined by plate count on MRS agar.

Fermentations were carried out in triplicate.

## 2.6 Fermentation of Soymilk Supplemented with Sugar

To determine the effect of supplementation with sugar on increased acid production, 150 ml of soymilk was dispensed into 250 ml conical flasks and 0.1%, 0.5%, 1%, 1.5%, 2% w/v of glucose, lactose and sucrose added to them respectively and heated for 121°C for 15 min. 20 ml aliquot of soymilk containing each sugar concentration was dispensed into 100 ml conical flask and fermented at 50°C for 9 hours. pH and cell count was monitored at 0, 3, 6 and 9h.

## 2.7 pH Adjustment and Supplementation with Sugar on Soymilk Fermentation

Taking further steps to improve acid production, pH of soymilk was first adjusted to 5.5 using acetic acid. A volume of 150 ml of the pH-adjusted soymilk was dispensed into 250 ml conical flasks and 0.5% glucose and 0.5% sucrose added to each flask separately. The soymilk was then corked tightly before heating at 100°C for 6 min. 20 ml of the soymilk was dispensed into 100 ml conical flasks and fermented at 50°C for 9 hours. pH and cell count was monitored at 0, 3, 6 and 9h.

## 2.8 Analysis of Fermented Soymilk

The pH of fermented and unfermented soymilk were determined using a single electrode pH meter (Hanna instruments) at 28°C ±2. The pH meter was calibrated using buffer 7.0, 4.0 and 10.0 according to the manufacturer's instructions. 20ml of the sample was placed in a beaker and the electrode of pH meter dipped into the sample. The pH meter was calibrated between sample testing to ensure accuracy. The reading displayed on the screen of the pH meter was taken and the process repeated two more times to ensure a consistent result.

Amount of acid formed during fermentation was determined by titrating the fermented soymilk sample against 0.1N NaOH using phenolphthalein as indicator and expressed as lactic acid g/100 ml.

## 2.9 Sensory Evaluation

Sensory quality of the fermented soymilk was determined using a 9-point hedonic scale.

A 10-member panel consisting of 20-assessors who are familiar with sensory description and attribute intensity [11] carried out sensory evaluation. The sample was presented at 20°C to the assessors. The assessors recorded their observation on sensory sheet using the scale

- 9 (Like Extremely)
- 8 (Like very much)
- 7 (Like moderately)
- 6 (Like slightly)
- 5 (neither like nor dislike)
- 4 (dislike slightly)
- 3 (dislike moderately)
- 2 (dislike very much)
- 1 (dislike extremely).

## 2.10 Statistical Analysis

Data obtained from the study was analyzed using SPSS (Statistical package for social sciences),

version 23.0 New York, U.S.A, 2016. Mean and standard deviation was used to represent different variables of sensory evaluation.

## 3. RESULTS AND DISCUSSION

### 3.1 Fermentation of Soymilk

The result as shown in Figs. 2 and 3 indicates that at 28°C there was neither a decrease in pH nor increase in cell count throughout the fermentation period. Fermentation at 37°C, 42°C and 50°C showed decrease in pH and increase in cell count. This complies with the study conducted by Bozanic et al. [12] wherein soymilk was fermented at two different temperatures (37°C and 42°C) using ABTS culture (*Lactobacillus acidophilus*, *Bifidobacterium* spp, *Streptococcus thermophilus*). Their result showed that *Bifidobacterium* grew better during the fermentation at 42°C than at 37°C. Nicoleta-Marcica et al. [11] showed *Lactobacillus casei*<sup>®</sup>431 grew significantly better at 37°C than at 30°C during a 12 h of fermentation.

*Bacillus coagulans* grows at relatively high temperatures. Fermentations carried out at high temperatures are less susceptible to microbial contamination.

### 3.2 Effects of Soymilk Supplementation with Sugar during Fermentation with *Bacillus coagulans*

The addition of sucrose at 0.1% did not increase acid production. Addition of 0.5% sucrose increased acidity. Higher concentration than 0.5% up to 2% tested did not significantly improve acidity over the rate at 0.5%. At 0.1% to 0.5% cell count increased. Concentration above 0.5% sucrose caused decrease in cell count.

Addition of lactose did not improve acid production. Also, increase in concentration led to decrease in cell count.

Glucose at 0.1% concentration did not improve acid production. Higher concentration at 0.5% up to 2% improved acid production significantly. 0.1% to 1% concentration increased the growth of the probiotic cells. Concentration above 1% caused a decrease in probiotic cell count.

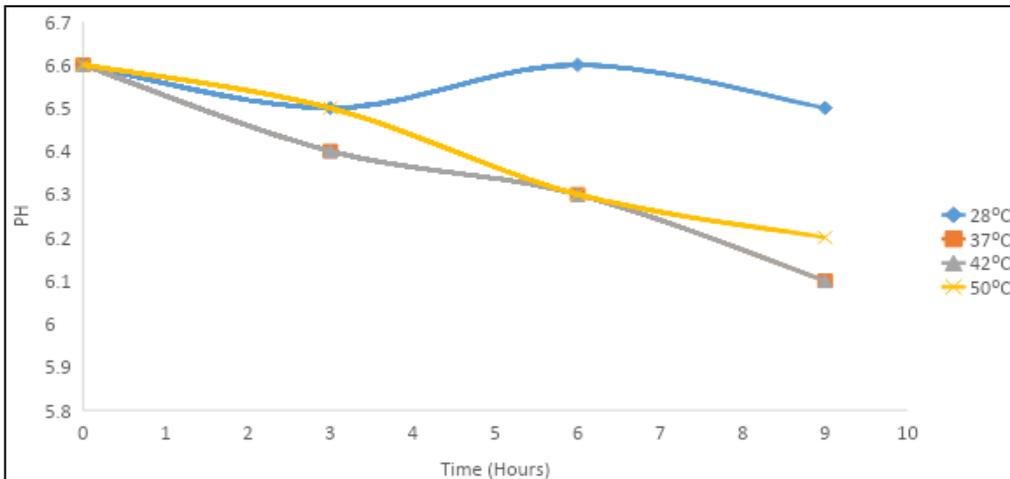


Fig. 2. Effects of fermentation temperature on pH change

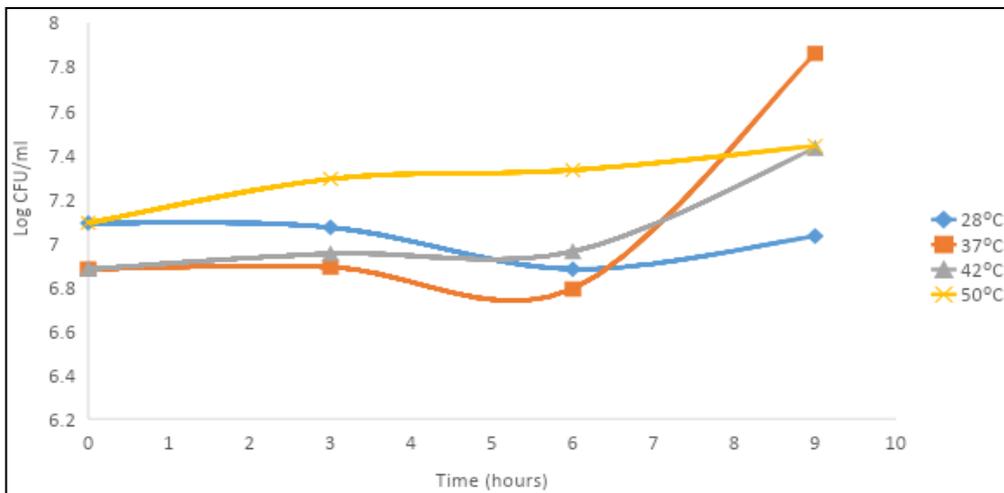


Fig. 3. Effect of fermentation temperature on cell count

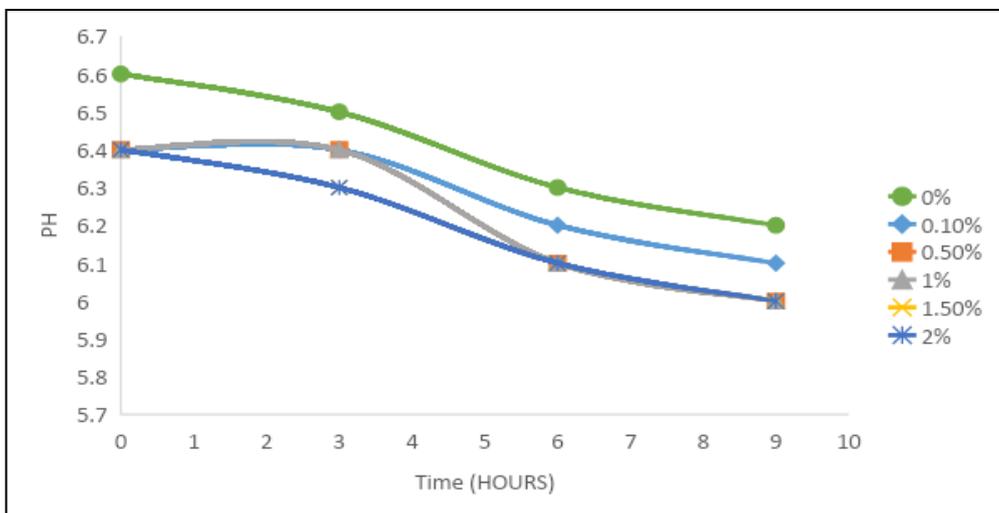


Fig. 4. pH change during fermentation with sucrose at 50°C

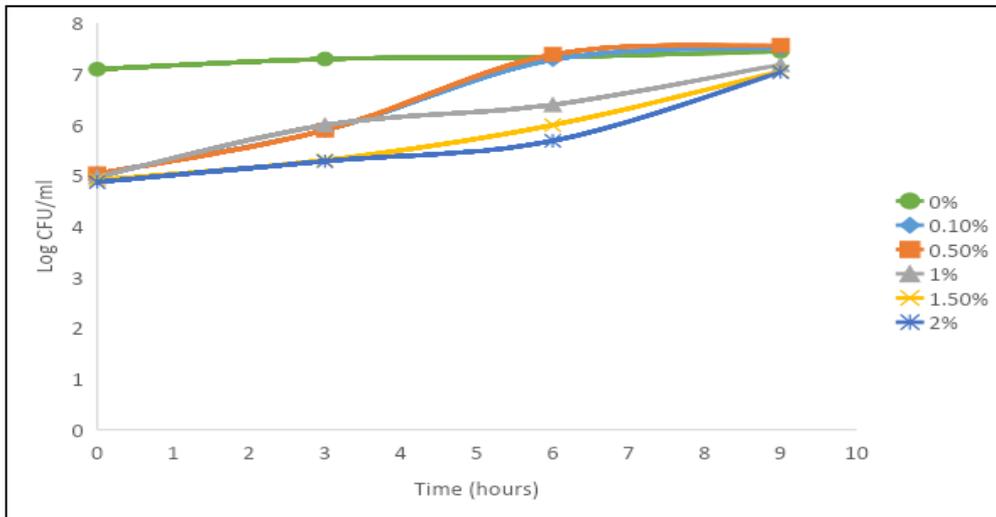


Fig. 5. Cell count during fermentation with sucrose at 50°C

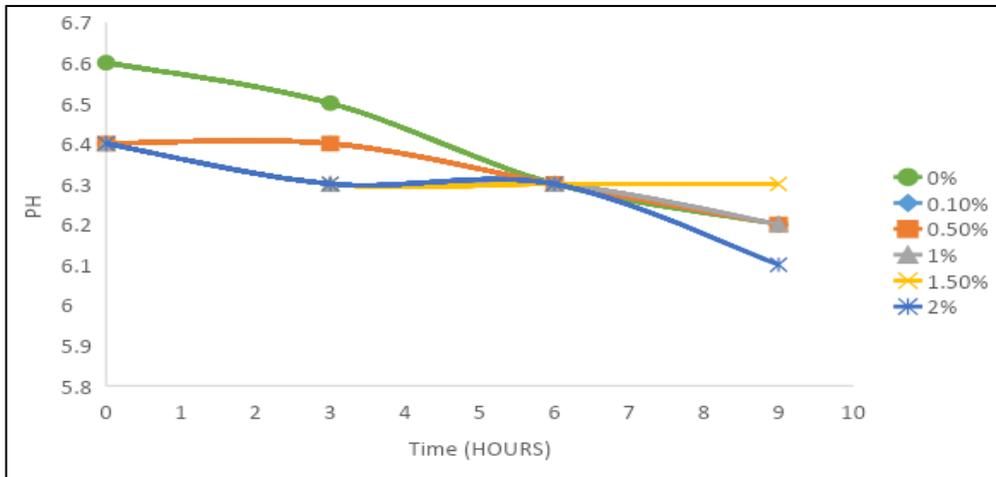


Fig. 6. pH change during fermentation with lactose at 50°C

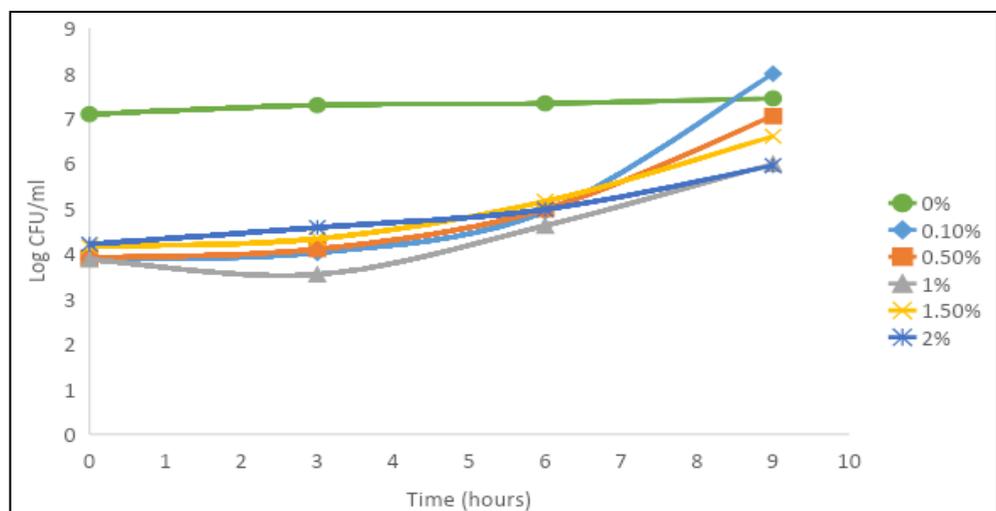


Fig. 7. Cell count during fermentation with lactose at 50°C

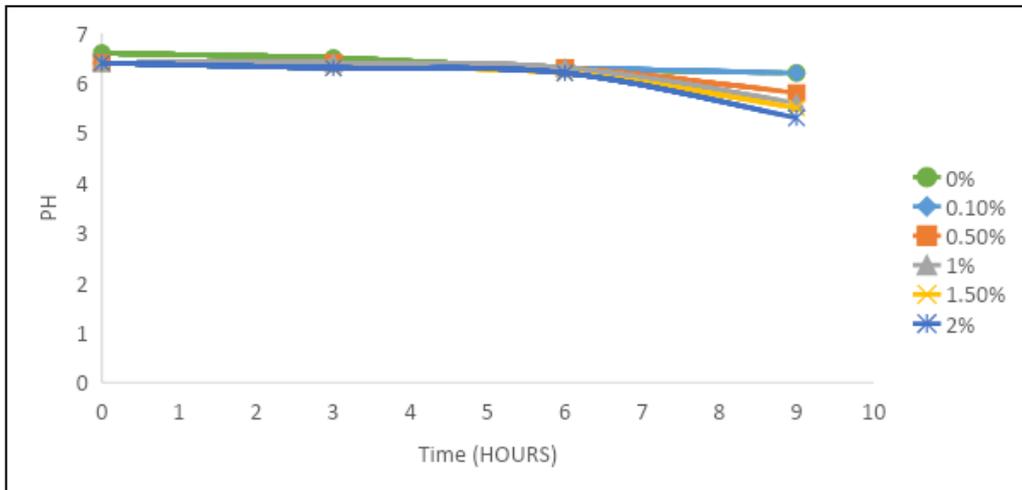


Fig. 8. pH change during fermentation with Glucose at 50°C

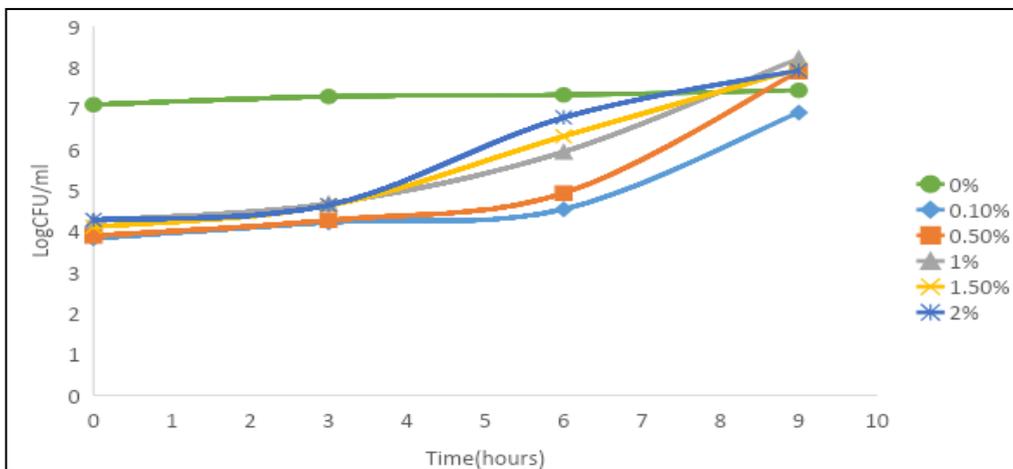


Fig. 9. Cell count during fermentation with Glucose at 50°C

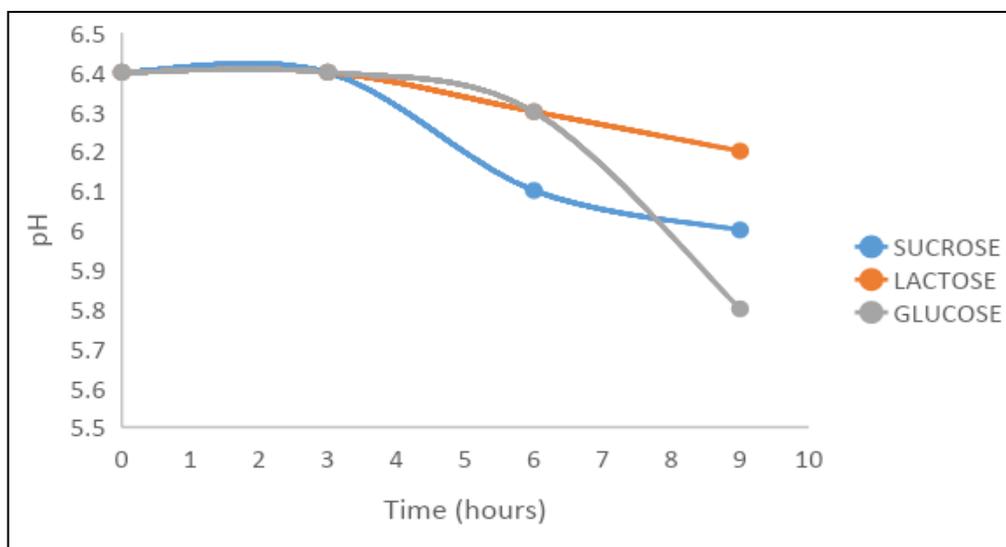
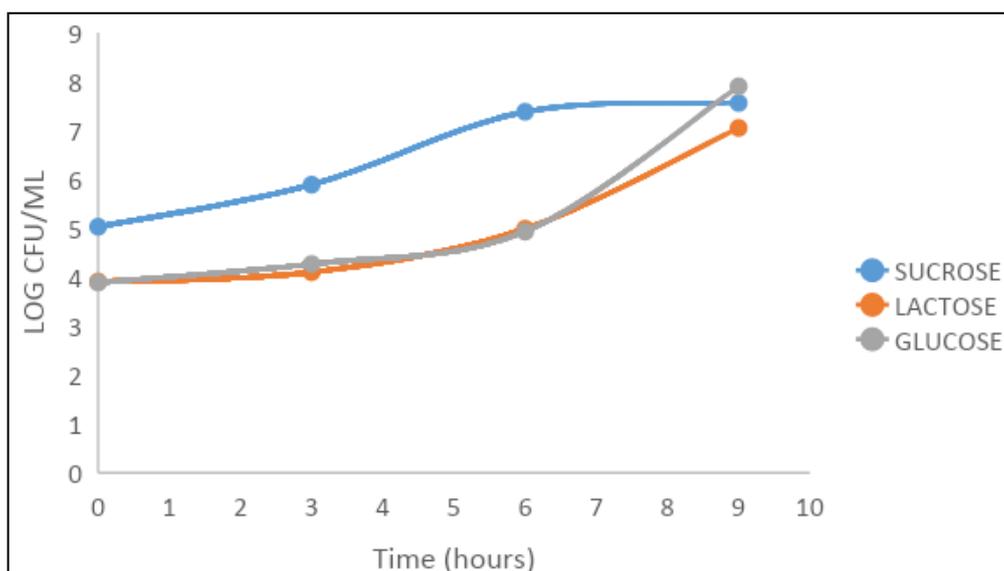


Fig. 10. Showing pH change during fermentation with 0.5% sugar



**Fig. 11. Showing cell growth during fermentation with 0.5% sugar**

In this study Figs. 4-11, fermentation process was optimized to maximize the amount lactic acid produced, which is equivalent to lower pH and also good probiotic spore count (>6 log CFU/ml) of the final fermentation product. In the presence of hexose sugars, (sucrose, lactose and glucose) soymilk pH decreased and had a high probiotic spore count with glucose showing the best response in terms of pH and probiotic count. The result showed that at sucrose and lactose concentration above 0.5%, pH decrease resulted in a simultaneous decrease in probiotic spore count. With glucose, increase in concentration resulted in decrease in pH and increase in probiotic spore count but above 1% concentration the spore count began the decrease. This observation showed that at concentration above 0.5% to 1% Sucrose, lactose and Glucose, growth-limiting effect is expressed. The result equally indicated that *Bacillus coagulans* preferred to utilize glucose than sucrose or lactose for lactic acid production. This finding is similar to the finding of Zhang et al. [13] who found that pH values of fermented turi-milk with added glucose were lower (3.64 – 3.67) than that with added sucrose (3.71 – 3.75). Sanadi et al. [14] grew *Bacillus coagulans* using molasses as nutrient source, the result of their finding showed that 1% (w/v) molasses served as viable concentration to grow *B. coagulans*. The addition of sugar (sucrose, lactose and glucose) to soymilk prior to fermentation had varying effect on acid production. Lactose had the least influence on acid production. There was no difference in pH between soymilk with and without lactose supplementation. After

fermentation of soymilk with 0.5% lactose for 9h at 50°C, pH value was 6.2. Sucrose supplementation decreased the pH of soymilk, though poorly. pH value of soymilk with 0.5% sucrose was 6.0 after 9 of fermentation. For soymilk with 0.5% glucose, pH value was 5.8 after 9 h of fermentation (Fig. 10). Probiotic cell count was high (7 log cfu/ml) after fermentation irrespective of the sugar added (Fig. 11). In the study by Bozanic et al. [15] soymilk with glucose had a lower pH than soymilk with sucrose. Soymilk with glucose and soymilk with sucrose were fermented with *Lactobacillus casei* Lc-01. pH value of soymilk with glucose after 14 hours of fermentation was 4.62 while that of soymilk with sucrose was 5.7 after 34 hours of fermentation but viable cell counts was high. in both samples.

### 3.3 Effects of Initial pH Adjustment and Supplementation with Sugar on Soymilk Fermentation

Soymilk with pH adjusted to 5.5 using Acetic acid (pH 3.2) fermented for 9 hours at 50°C did not improve acid production. The addition of 0.5% Sucrose did not improve acid production. With the addition of 0.5% glucose, acid production was significantly improved.

The combined effect of low pH (5.5) and 0.5% glucose on fermentation of soymilk as seen in Figs. 12 and 13 showed to be the most preferred condition for a probiotic soymilk drink. Comparable with our findings is the work of Park et al. [16] who found that strawberry soy-based

yogurt produced more lactic acid than plain soy-based yogurt. (Strawberry has a pH of 3.0 to 3.5 and its major sugars include Glucose, fructose and sucrose).

of a fermentation medium shows that there has been an accumulation of organic acid (lactic acid) because of utilization of fermentable sugars by the fermenting strain.

### 3.4 pH and Titratable Acidity

As presented in Fig. 12, *Bacillus coagulans* was able to bring down the pH soymilk from 5.5 to 4.5 after 9h of fermentation. The titratable acidity increased to 0.634 g lactic acid/100ml from 0.333 g lactic acid/100ml, showing that *Bacillus coagulans* is capable of utilizing fermentable sugars in soymilk to produce lactic acid. Similar to our result, Aishwarya et al. [17] showed that *Bacillus coagulans* reduced the pH of tender coconut water from 5.02 to 4.44. Decrease in pH

### 3.5 Sensory Evaluation

Sensory properties of a fermented food product are important for its acceptability by consumers. Based on the assessment of the panelist, the fermented soymilk is moderately acceptable on a 9-point Hedonic scale. The beany flavour associated with soymilk was absent in the fermented soymilk. The aroma/ smell of the fermented probiotic soymilk can be enhanced by the addition of flavours.

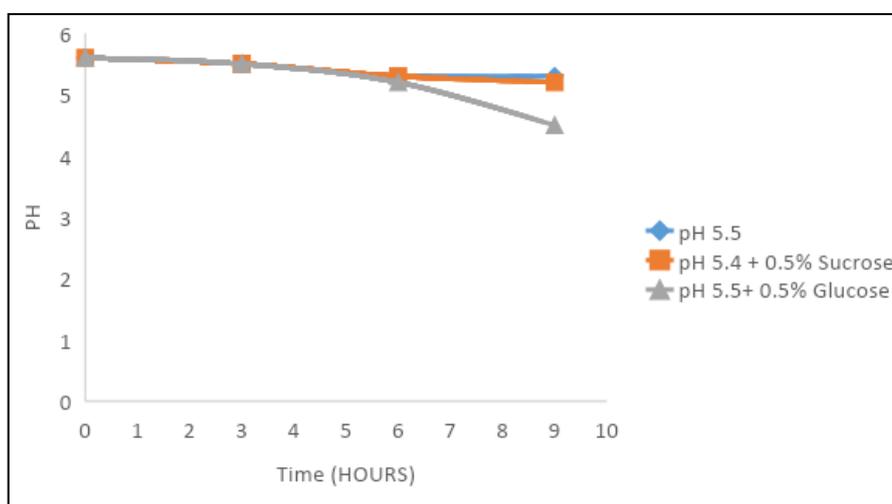


Fig. 12. pH change during Fermentation of Soymilk with pH 5.5, pH 5.5 + 0.5% Sucrose and pH 5.5+ 0.5% Glucose

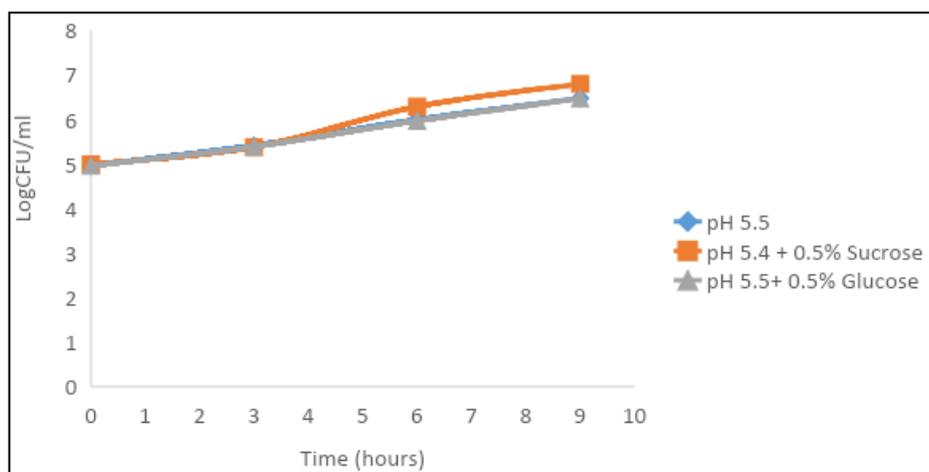


Fig. 13. Cell count during fermentation of soymilk at pH 5.5, pH 5.4 + 0.5% sucrose and pH 5.5 + 0.5% glucose

**Table 1. Evaluation of sensory properties of fermented probiotic soymilk drink**

Sensory variables	Measure (Mean ± STD)
Appearance	7.90±0.73
Taste/Flavour	7.0±0.67
Texture/Consistency	7.80±0.42
Aroma/Smell	6.7±0.94
Overall Acceptability	7.2±0.63

#### 4. CONCLUSION

In this study, *Bacillus coagulans* adapted to the soymilk environment, bringing down the pH of the soymilk to a level depicting fermented food and also increasing in cell count to a level that is acceptable for a probiotic food product. It will also be noted that addition of Glucose and adjustment of the soymilk pH led to an improved fermented soymilk. In conclusion, the results of the study shows the feasibility of producing a fermented probiotic soymilk using *Bacillus coagulans* as the choice probiotic.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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