EFFECT OF COMPOSITION OF SOYMILK ON ACID PRODUCTION BY *LACTOBACILLUS* FERMENTATION

by

CHUN-YEN CHANG

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Martha B. Stone

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INTRODUCTION

Soybeans are an excellent source of oil and protein. Soybeans supply about 80% of the edible vegetable oils used in the U. S., and the meal residue is utilized as feed for livestock and poultry (Wolf and Cowan, 1975). Because of the increasing gap between food supply and world-wide demand for high quality food products, shifts from animal to vegetable sources of protein have gained significant recognition. It is more efficient to utilize soybeans as an direct source of edible protein than as a feed.

However, direct consumption of soybeans has been limited by their flavor and flatus-inducing problems. The undesirable flavor of soybeans is described generally as beany, bitter, grassy, and astringent, and is attributed partially to the degradation of lipids. Lack of α-galactosidase for complete hydrolysis of carbohydrates in soybeans (low molecular weight oligosaccharides including sucrose, raffinose and stachyose) results in the production of flatus gases in the gastrointestinal tract of humans (Gitzelmann and Auricchio, 1965). Some lactic acid bacteria that possess the ability to utilize sucrose and galacto-oligosaccharides for growth and acid production can be used to manufacture a fermented product from soymilk, a water extract of whole soybeans. This helps remove flatus-inducing sugars and improve the flavor (Mital and Steinkraus, 1975). In order to make a desirable yogurt-like fermented soymilk, the level of total solids in the soymilk must be considered.

In this study, five different extraction ratios of soybeans to water were used to prepare soymilks with five different total solids levels. The contents of protein and carbohydrate, two major components of total solids in soymilk, were determined, and their effects on acid production by *Lactobacillus fermentum* NRRL B-585, *Lactobacillus acidophilus* NRRL B-1910 and B-2092 were investigated to provide a better understanding of the relationship between lactobacilli growth and soymilk composition.

LITERATURE REVIEW

Soybeans are the most important cash crop and the leading agricultural export in the United States. Soybeans used as food date back to ancient times in the Far East, however, their history in the United States is more recent. Before soybeans were well recognized for their exceptionally good nutritive values, they were used as hay and silage for animals. Now people have found an extremely wide area of utilization in human foods, animal feeds, and industrial applications (Smith and Circle, 1978a).

Considerable interest in protein from soybeans has developed for several reasons. Soybeans are abundant sources of protein of high nutritive quality, especially from the point of view of their low cost. The proximate composition of soybeans is shown in Table 1. Soybeans are the best plant source of protein, and their quality of protein is only inferior to animal protein because of their deficiency in sulfur-containing amino acids.

Soybeans can grow well in a variety of soils even if they are too depleted to support other crops. Soybean agriculture also is less labor-intensive than other crops, which means that more of the agricultural labor can be done by machine.

Production of protein from soybeans is more efficient than producing protein from animal sources. To produce

Table 1 - Average composition of soybean seed parts of six U.S. varieties[a]

	Whole Soybeans (%)	Full-fat Cotyledons (%)	Full-fat Hypocotyl (%)	Hull (%)
Protein	40.4	43.4	40.8	9.0
Fat	22.3	24.3	12.0	0.9
Carbo-hydrate[b]	31.9	27.4	42.7	86.2
Ash	4.9	5.0	4.5	4.0

[a]Moisture-free basis.
[b]Includes crude fiber.

Source: Kawamura, 1967.

enough chicken meat for the minimum daily protein requirement for one human, the chickens must first have been fed enough protein to satisfy the minimum protein requirement for six people. For beef production, a minimum protein requirement for 15 people must be met.

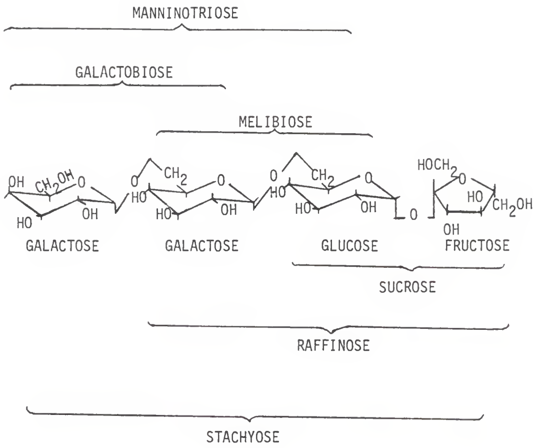
Other than the news stories of famines, there is a general deterioration in the diet of people in developing countries. Their diets are especially lacking in protein. The prodigious soybean is viewed as a potential solution to some of our food problems in the world (Hapgood, 1987).

Oriental people have consumed fermented and unfermented soy products such as soymilk, tofu, kinako, miso, natto, soy sauce, and tempeh for hundreds of years (Saint, 1970; Hesseltine and Wang, 1978; Liener, 1978). However, utilization of soybeans in Western food patterns has been limited because of problems with flatulence, biologically active components, and flavor.

Flatulence is often experienced by persons consuming soybean foods. Several researchers indicate that the interaction of carbohydrates in soybeans with intestinal microflora is the primary factor in gas production (Gitzelmann and Auricchio, 1965; Calloway et al., 1966; Hellendoorn, 1969). The fermentable carbohydrates in soybeans and soy products are low molecular weight oligosaccharides such as sucrose, raffinose and stachyose. Stachyose, a tetrasaccharide, is made up of three simple sugars fructose, glucose, galactose (Figure 1). The stachyose molecule can be considered to contain the nonreducing sugars sucrose and raffinose and reducing sugars galactobiose, melibiose and manninotriose (Mital and Steinkraus, 1975). When soybeans are ingested, an invertase and α-galactosidase are required to completely hydrolyze these oligosaccharides. Gitzelmann and Auricchio (1965) have shown that α-galactosidase activity was not present in human intestinal mucosa. Microflora in the ileum and colon hydrolyze raffinose and stachyose to simple sugars which can then be broken down to carbon dioxide, hydrogen, and methane (Cristofaro et al., 1974). Clostridium perfringens probably is the principal intestinal anaerobe responsible for the production of flatus gases in the gastrointestinal tract of man and animals (Bornside and Cohn, 1965).

Raw soybeans possess many biologically active components including enzymes, proteinase inhibitors, hemagglutinnins, allergenic factors, saponins, sterols and triterpene alcohols, goitrogens, growth-vitamin-mineral factors, and phenolic constituents (Smith and Circle, 1978b). Among these components, trypsin inhibitors are highly related to protein digestibility. Several reviews are available on the relationship between animal nutrition and soybean trypsin inhibitors (Rackis, 1965; Liener and Kakade, 1969).

Undesirable flavor is another factor to limit the greater use of soybean products. Flavor of soybeans has



Source: Mital and Steinkraus, 1975.

Fig. 1 - Chemical structure of stachyose

been described as beany, bitter, grassy, and astringent. Development of off-flavors in some foods, during storage or processing, has been attributed to the degradation of lipids. This reaction can be enzymatically catalyzed or non-enzymatically initiated by air oxidation. Researchers show that n-hexanal, acetaldehyde, and acetone representing the major volatile carbonyl compounds can contribute to the distinctive and undesirable flavors of soybeans (Teeter et al., 1955; Fujimaki et al., 1965; Honig et al., 1969; Sessa et al., 1969).

A number of biochemical changes are known to occur during fermentation of soybeans. These changes in fermented soymilk theoretically could lead to a solution of these problems. Soymilk, the simplest soy food to prepare, is a water extract of whole soybeans. Soymilk is used extensively as a substitute for bovine or human milk in areas where bovine milk is expensive or unavailable and also for infants who are allergic to animal milk (Johnson et al., 1981). A comparison of the essential amino acid composition of soymilk with cow and human milk is shown in Table 2. Lactic acid bacteria possessing the ability to utilize sucrose, the major sugar found in soybeans, can be successfully used to manufacture a fermented product from soymilk (Mital et al., 1974). Some lactic acid organisms also possess α-galactosidase and can thus utilize galacto-oligosaccharides for growth and acid production. This would help to remove

Table 2 - A comparison of the essential amino acid composition of soymilk with cow and human milk

Essential Amino Acid	Source of Milk Soybean[a]	Cow[b]	Human[c]
Isoleucine	5.1	7.5	5.5
Leucine	8.3	11.0	9.1
Lysine	6.2	8.7	6.6
Methionine	1.4	3.2	
Cystine	1.7	1.0	
Total Sulfur AA	3.1	4.2	4.0
Phenylalanine			
Tyrosine			
Total Aromatics	9.0	11.5	9.5
Threonine	3.8	4.7	4.5
Tryptophan	1.3	1.5	1.6
Valine	4.9	7.0	6.2

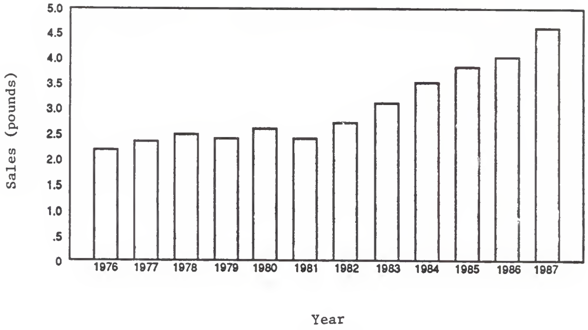
[a]Based on studies of Hackler and Stillings, 1967.
[b]Based on studies of Subrahmanyan, 1961.
[c]Based on studies of Rice, 1969.

flatus-inducing sugars. Production of volatile diacetyl and a reasonable level of acidity by lactic acid bacteria would also help to improve the flavor and keeping quality of soymilk (Pinthong et al., 1980; Daeschel, 1989). Also, the growth of yogurt consumption in the market has increased greatly in the recent years. Data from the Milk Industry Foundation (1987) indicate that per capita yogurt consumption in the U.S. has increased by 81% every 5 years since 1960. Information from U.S. Department of Commerce also shows increasing per capita yogurt sales since 1976 (Fig. 2). Use of an inexpensive milk analogue to make a yogurt-like product also has potential.

Soymilk Preparation Methods

Traditional Method. Traditional soymilk is made from good quality whole soybeans. Beans are washed thoroughly, soaked overnight, ground with enough water to give the desired solids content in the final product, and filtered to remove the insoluble residue. Usually, the ratio of water to beans is approximately 10:1 on the weight basis (Liener, 1978). The slurry is then boiled for 15-20 min. Since trypsin inhibitors are inactivated by heat, an effect of enhancement of nutritive value of the protein generally is accompanied (Liener, 1962). This method needs only simple equipment with little investment, but the product has a beany flavor.

Hot Water Grind Method. The hot water grind method



Source: Anon., 1988.

Fig. 2 -- Per capita yogurt sales in the United States

was developed by researchers at Cornell University (Bourne, 1970). Soybeans were soaked overnight in water at room temperature at a 1:3 soybeans to water ratio. Beans were then drained, rinsed, and ground with boiling water. Maintenance of the whole grinding process above 80^{o}C was critical, so the grinder had to be preheated before grinding. After grinding, the slurry was boiled in a steam-jacketed kettle for 10 min, and then filtered to remove the residue. It is advantageous to use this method to prepare soymilk having a greatly improved, bland flavor by inactivating the soybean enzyme lipoxygenase with moist heat during grinding. Otherwise, either heating the beans before grinding or after grinding will decrease the protein extractability or induce an off-flavor.

Whole Soybean Process. This Illinois process (Nelson et al.,1976) produced soymilk from whole soybeans. Soybeans were soaked overnight in tap water containing 0.1% $NaHCO_3$ (1:3 beans to water), drained, and blanched in a fresh boiling tap water solution of 0.5% $NaHCO_3$ for 30 min (1:3 original dry beans to water). Blanched beans were drained, rinsed, and wet-ground through Fitzpatric mill. The slurry was heated to 200^{o}F in a steam jacketed kettle and homogenized at 3500 psi (first stage) and 500 psi (second stage). By this method, about 89% of the total solids and 95% of the protein in raw soybeans were recovered. The disadvantage of this process was the high viscosity for a beverage, and high cost of a powerful homogenizer to produce a smooth, stable suspension.

Direct Steam-Infusion Cooking Method. Johnson et al. (1981) prepared soymilk from whole soybean flour by a continuous steam-infusion cooking process (Rapid hydration hydrothermal method) at 154^{o}C, neutral pH and a holding time of 34 sec. Solids yield was 86% and protein yield was 90% with this method. They explained their high yields as a result of optimum heat treatment and extreme shear encountered in steam-infusion and flashing since a certain amount of heat is required to dissociate the protein bodies leading to increased solubility and emulsification.

Soymilk prepared by different methods yield different amounts of total solids. For the same preparation method, a change in extraction ratio of beans to water can give a different total solids content. Various extraction ratios of beans to water ranging from 1:5 to 1:10 have been used to prepare soymilk in research studies. In general, total solids yield increases when the extraction ratio is increased, although extractability of proteins decreases. A ratio of 1:10 (dry soybeans:water) is most commonly used for many laboratory studies. A 1:20 or 1:40 ratio can yield a greater amount of the total protein but also results in a more dilute solution (Wolf, 1978).

Yield of soymilk solids is affected by water temperature during soaking and grinding (Wilkens et al., 1967). Soaking soybeans softens the beans for easy grinding and extraction. However, when a high temperature, rapid hydration grinding process and dehulled beans were used, soaking before grinding offered no advantages for the overall solids yield in soymilk.

Preheating soymilk can prevent coagulation of soy proteins or gel-formation for autoclaving later (Fukushima and Buren, 1970). When the solids content of soymilk is high, the effect is especially significant. Since gel-formation or increase of viscosity in soymilk is desirable in yogurt making, preheating soymilk before autoclaving was not considered in this study.

Composition of soymilk is changed when the preparation method is varied. A comparison of composition of soymilk made from different methods is shown in Table 3.

Lactic Acid Bacteria

Some of the most frequently used lactic starter cultures for the dairy industry are listed in Table 4. They are nonpathogenic bacteria, and their primary function is to convert lactose to lactic acid. Additionally, some cultures produce subtle flavor and aroma compounds that are unique and characteristic to that product. Some starter cultures used are single strain (one strain of a particular bacterium) or as multiple or mixed strains. A "mixed culture" of two bacteria, _Lactobacillus bulgaricus_ and

Table 3 - Comparison of soymilks prepared by five different methods

Method[a]	Extraction ratio	Solids (%)	Protein[b] (%)	Carbo-hydrate[b] (%)	Lipid[b] (%)
A	1:6.8	8.0	30.4	45.5	18.1
B	---	10.7	56.1	36.6	2.1
C	1:6	4.6	31.6	29.1	33.8
D	1:6	11.5	43.4	27.7	23.1
E	1:6	10.2	45.4	25.7	23.5

[a]A: Traditional method (Miskovsky and Stone, 1987).
B: Rapid hydration hydrothermal cooking method (Miskovsky and Stone, 1987).
C: Heating soybeans at 100°C for 30 min before grinding (Johnson, 1978).
D: Heating soybeans to 80°C or above during grinding (Johnson, 1978).
E: No heat applied to soybeans before or during grinding (Johnson, 1978).

[b]Based on percentage of total solids.

Table 4 - Examples of bacterial starter cultures used in fermented milk products

PRODUCT	CULTURE
Yogurt	Lactobacillus bulgaricus and Streptococcus thermophilus
Acidophilus milk	Lactobacillus acidophilus
Cottage cheese	Streptococcus lactis and/or Streptococcus cremoris
Cultured buttermilk	Leuconostoc citrovorum and Streptococcus lactis
Swiss cheese	Propionibacterium freudenreichii and Streptococcus lactis
Cultured sour cream	Streptococcus lactis and Leuconostoc citrovorum
Cheddar cheese	Streptococcus lactis and /or Streptococcus cremori

Source: Helfetich and Westhoff, 1980.

Streptococcus thermophilus, is suggested currently to make good yogurt (Helferich and Westhoff, 1980). There have been many lactic acid bacteria used to ferment soymilk including S. citrovorus, S. paracitrovorus, S. lactis, L. bulgaricus, S. thermophilus, S. faecalis, S. cremoris, L. acidophilus, L. delbrueckii, L. plantarum (Kellogg, 1934; Gehrke and Weiser, 1948; Ariyama, 1963; Hang and Jackson, 1967; Matsuoka et al., 1967; Obara, 1968; Yamanaka et al., 1970; Kim and Shin, 1971; Angeles and Marth, 1971; Mital et al., 1974). Some results were satisfactory, but others were not.

U.S. whole soybeans contain 4.5% sucrose, 1.1% raffinose, 3.7% stachyose, and traces of arabinose and glucose for a total of 9.3% sugars on a dry basis (Kawamura, 1967). There are about 1% fermentable sugars in soymilk with 1:10 beans to water ratio. If any lactobacillus cannot utilize raffinose and stachyose, then its growth in soymilk in terms of acid production will not be good enough to make a successful fermented product, unless it is fortified with additional sugar. Hull and Roberts (1984) studied 59 strains of L. acidophilus and yogurt starter bacteria and found that L. acidophilus was able to ferment and grow on a large number of sugars including raffinose and melibiose (intermediate hydrolysis product of raffinose and stachyose) in contrast to the yogurt starter strains. One study showed that L. fermentum could utilize raffinose and stachyose pretty well, but its utilization of sucrose was poor (Mital and Steinkraus, 1975)

Culturing conditions. Research studies have established culturing conditions such as the inoculum rate, incubation temperature and incubation time for the growth of lactic acid organisms in cow's milk. Since soymilk represents a different medium, these conditions are expected to change. Few efforts, however, have been made to investigate these aspects. Usually the conditions employed for cow's milk have been adopted for soymilk per se.

In general, the rate of inoculum has been 1% for most of the culture organisms, though at times higher rates, 2.5%, 3% and even 5% have been reported for certain cultures. The incubation period has been varied from a few hours to 48 hr with the most frequently accepted ones 16-18 or 24 hr. With respect to the incubation temperature, soymilk has been cultured more or less under the same conditions as used for cow's milk, i.e. 30^{o}C for mesophilic streptococci and 37 or 40^{o}C for Streptococcus thermophilus as well as most lactobacilli (Petal et al., 1980).

α-Galactosidase activity of lactobacilli. The lactobacilli enzymes are active within a range of pH 4.5 to 8.0. The optimal pH for enzyme activity is in a rather narrow range (5.2 to 5.9), although a broad range between pH 3 and 6 has been reported for α-galactosidases from yeasts, molds, and plants. The α-galactosidases in most organisms exhibit optimum activity between 38 to 42^{o}C. In order to allow lactic bacteria grow well and produce a desirable amount of acid, the optimum condition for α-galactosidase to utilize oligosaccharides of soymilk is important (Mital et al.,1973). The activity of this enzyme is strongly inhibited by Ag^{+}, Hg^{++}, chloromercuribenzoate, galactose, and melibiose. This enzyme also seems to catalyze a glycosyl-transfer reaction (Ohtakara et al., 1984).

Soymilk as Substrate for the Growth of Lactic Cultures

Total solids. In order to produce a desirable yogurt-like soy product, the solids contents of soymilk must be considered. The level of total solids in cow's milk is significant for both the consistency and aroma of the manufactured yogurt. In general, an increase in total solids will enhance these properties. Level of total solids also affects the titratable acidity of the milk because of the buffering action of the proteins, phosphates, citrates, lactates and other miscellaneous milk constituents. An increase in total solids results in an increase in the titratable acidity and a reduction in coagulation time (Tamime and Deeth, 1980). This also can occur in fermented soymilk.

One easy and inexpensive way to increase the level of total solids in soymilk is to increase the extraction ratio of soybeans to water. However, Tamine and Deeth (1980) cited studies on the effect of total solids on starter activity in yogurt manufacture which concluded that levels in excess of 25% total solids adversely affected the availability of moisture and hindered starter activity. The proteins and carbohydrates in the total solids of soymilk are different from those in cow's milk. Therefore, different effects of total solids on starter activity was expected in soymilk.

Two studies were done on yield of extracted solids in soymilk as affected by temperature of water and various pre-treatments of beans (Wilkens et al., 1967; Lo et al., 1968). Soybeans soaked 8 hr and extracted at temperatures between 45 to 80°C yielded higher milk solids than unsoaked beans with other extraction temperatures. Temperatures of extraction above 85 °C for soymilk resulted in substantial decreases in the solids extracted. A gradual rise in pH of soymilk was observed as the temperature of extraction was increased from 30 to 95°C. Soymilk prepared from pre-soaked beans had higher average pH values than milk prepared from bean flour or non-soaked beans.

Pre-soaked beans yielded a higher average volume of soymilk than either non-soaked beans or bean flour, although additional water was added to the non-soaked beans to compensate for the water taken up during pre-soaking. When beans were ground at temperatures above 85°C, additional difficulties were encountered in filtering the soymilks, which resulted in decreased volumes in the soymilks produced.

The feasibility of soymilk as a growth medium for lactic organisms is governed by two other factors, the heat treatment of soymilk during production and processing, and the presence of carbohydrates that the culture bacteria can utilize. In order to provide a suitable environment for lactic acid bacteria growing in soymilk and producing enough acids to coagulate soy protein, it is important to consider total solids. Additional factors, such as effect of heat treatment and presence of fermentable carbohydrates also should be considered.

Effect of heat treatment. The heat treatment given to soymilk at any stage during its preparation before inoculation with the organisms is the most important processing factor affecting the ability of soymilk to support the growth of the lactic-acid bacteria. Angeles and Marth (1971) studied heat treatment of soymilk and its influence on culture growth, and observed that unheated soymilk elicited optimal or nearly optimal activity from most test cultures. Heating the medium to 60°C resulted in increased acid formation by Streptococcus and Leuconostoc species and in a reduction of acid production by Pediococcus cerevisiae and Lactobacillus species. Extended heating of soymilk at 60°C reduced its suitability as a substrate for acid development by lactic acid bacteria. Acid formation by all cultures was minimal in soymilk heated at 80°C from <1 to 60 min. Responses in soymilk heated at 100°C for short durations were similar to those obtained when soymilk was heated at 80°C. More severe heating at 100 or 120°C progressively improved the quality of soymilk as a substrate. Inhibitory effects noted when soymilk was heated at 80°C coincided with development of a markedly higher concentration of sulfhydryls and /or toxic volatile sulfides in the medium during heating. Beneficial effects of more severe heating were attributed to expulsion of sulfides, a concurrent decrease in concentration of sulfhydryls, and a decrease in the oxidation-reduction potential of the medium (Angeles and Marth, 1971).

Fermentable carbohydrates. Lactic acid bacteria are known to utilize sugars such as lactose, glucose, fructose, galactose and maltose. Acid production in the medium depends upon growth of the organisms and their ability to ferment the available carbohydrates (Mital et al., 1974). Soymilk contains approximately 1% fermentable carbohydrates (Mital and Steinkraus, 1975). In addition, all carbohydrates in soymilk are not fermented by the lactic acid bacteria (Mital et al., 1974).

MATERIALS AND METHODS

Data from this experiment were analyzed with a strip-strip plot design. The principal factors investigated in the experimental design were: extraction ratio of soybeans to water (1:6, 1:7, 1:8, 1:9, and 1:10) and strain of lactobacilli (*Lactobacillus acidophilus* NRRL B-1910, B-2092, and *Lactobacillus fermentum* NRRL B-585). Fifteen treatment combinations were assigned randomly to the experimental units. The entire experiment was replicated four times.

Preparation of Soymilk

Dry, mature, whole Williams 82 soybeans obtained locally were used. Fifty grams of beans were soaked in tap water overnight at room temperature. The beans were washed, drained, and hot-ground with tap water (80-90^{o}C) for 3 min in an Osterizer blender (Model Imperial VIII, Milwaukee, WS). Water (300, 350, 400, 450, or 500 ml) associated with extraction ratios of 1:6, 1:7, 1:8, 1:9, and 1:10 (dry beans:water w/v) was poured over beans in the blender. The resulting suspension was filtered through 3 layers of cheesecloth and the residue was discarded. The resultant soymilk was dispensed in Pyrex culture tubes, autoclaved for 20 min at 121^{o}C, and held overnight at 5^{o}C.

Composition of Soymilk

Total solids. Soymilk (2.5-3 grams) was weighed into 5-cm diameter aluminum pans (Fisher Scientific Co., Pittsburgh, PA), and heated at 88-90^{o}C in an air oven until a constant weight was obtained. Usually a period of 16 hr was required. Samples then were cooled to room temperature (22-25^{o}C) in a desiccator, and weighed quickly. Total solids were reported as the percentage of residue remaining (AOAC, 1984).

Protein content. Protein content was determined by the AOAC (1984) micro-Kjeldahl procedure. A 100 to 120 mg sample was digested in the presence of 1.9 g of a mixture of potassium sulfate and cupric sulfate with a ratio of 9:1 by weight, and 3 ml concentrated sulfuric acid. Digestion was completed in approximately 2 hr. Ammonia from the distillation was collected in Erlenmeyer flasks containing 20 ml of 2% boric acid. The quantity of nitrogen was determined by titration with 0.02 N hydrochloric acid. Methylene blue-methyl red (2:3) was used as the indicator to produce a violet end point. A nitrogen to protein conversion factor of 6.25 was used for the estimation of protein.

Total carbohydrate. One ml of soymilk was added slowly with agitation to 2.0 ml of 1.8% barium hydroxide solution followed by 2.0% zinc sulfate solution. After standing for 10 min, the mixture was transferred to a 15-ml graduated, clean, dry centrifuge tube, and centrifuged until a clear supernatant was obtained (Mital and Steinkraus, 1975). Then the colorimetric method used by Dubois et al.

(1956) was applied with modification of the supernatant to determine total carbohydrate. Modifications were that one ml of 5% phenol was pipetted to 1 ml of the supernatant, mixed in the Vortex test tube shaker, and then followed by 5.0 ml of ACG grade concentrated sulfuric acid. A stream of sulfuric acid was directed against the liquid surface rather than against the side of the test tubes in order to obtain better dispersion. Samples were held at room temperature (22-25 ^{o}C) for 30 min. Absorbance of the characteristic yellow-orange color was measured at 480 nm with Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, NY). Total carbohydrates were determined by reference to a standard curve previously constructed for glucose.

Cultures

Lactobacillus acidophilus NRRL-2092, NRRL-1910, and Lactobacillus fermentum NRRL B-585 were obtained from the USDA Northern Regional Research Laboratory in Peoria, IL. Cultures were maintained by weekly transfer in Lactobacillus MRS broth and held at 5 ^{o}C between transfers. Purity of cultures was checked by Gram's staining procedure periodically. All cultures were transferred in soymilk daily for 2 days before they were used to prepare 16-18 hr old inocula for soymilk fermentation. The incubation temperature was 37^{o}C.

Acid Production and Changes in pH

Soymilk (25 ml) was inoculated aseptically with 0.5 ml of 16-18 hr old inoculum at a rate of 2%. Three different strains of lactobacilli were used with soymilk with the five different extraction ratios, respectively, for 15 treatment combinations. The inoculated media were incubated at a temperature of 40°C for 16 hr for all treatment combinations. Acid development was measured by titration of 1 g fermented soymilk with 0.02N NaOH using phenolphthalein as the indicator. Changes in pH also were monitored with a pH meter (Corning Scientific Co., Medfield, MA).

Statistical analysis

Analysis of variance was conducted on the observations measured according to the following design.

Source of variation	Degrees of freedom
Replication (Rep)	3
Extraction ratios (Ext)	4
Rep * Ext	12
Strains of lactobacilli (Str)	2
Rep * Str	6
Ext * Str	8
Error	24
Total	59

To test differences in the treatments, Least Significant Difference (LSD) method was performed on the response variables. Differences in results significant at the five percent significance level are reported. Multiple regression analysis of each factor studied was done to prepare a predictive model for each strain of lactobacilli. These fitted models were used to illustrate three-dimensional response surfaces. All statistical analyses were made with the Statistical Analysis System software package (SAS Inc., 1985).

RESULTS AND DISCUSSION

This study was conducted to determine the composition of soymilk when extraction ratios were varied, and to study the effect of composition of soymilk on growth of lactic acid bacteria in terms of acid production.

Composition of Soymilk

Total solids in soymilk includes protein, carbohydrate, lipid and ash. Since lipid and ash have little effect on acid development during soymilk fermentation, soymilk prepared with extraction ratios of 1:6, 1:7, 1:8, 1:9, and 1:10 (beans:water) was analyzed for total solids, protein and carbohydrate contents, and data are presented in Table 5. Johnson (1978) reported that the solids, protein, carbohydrate, lipid and ash varied as the bean-to-water ratio changed when three different extraction methods and homogenization were used with extraction ratios of 1:5, 1:6, 1:8, and 1:10. Total solids of soymilk in the current study increased significantly when extraction ratios of soybeans to water were increased ($P<0.05$). Protein and carbohydrate contents also increased when soybeans to water ratios were increased, though no significant differences existed between 1:8 and 1:9 ratios.

Correlation coefficients were calculated for total solids and selected composition factors of soymilk and are shown in Table 6. Correlation analysis revealed that

Table 5 - Composition of soymilk from varying beans:water ratios[a]

Extraction Ratio Beans:Water	Solids %	Protein %	Carbo-hydrate %
1:6	9.73^{A}	4.85^{A}	3.79^{A}
1:7	8.66^{B}	4.41^{B}	3.38^{B}
1:8	7.77^{C}	3.90^{C}	3.09^{C}
1:9	6.98^{D}	3.61^{C}	2.88^{C}
1:10	6.40^{E}	3.21^{D}	2.61^{D}

[a]Mean values in the same column with different superscripts (A, B, C, D, E) are significantly different ($p<0.05$, $r=4$).

Table 6 - Correlation coefficients for selected components of soymilk

	Solids	Protein	Carbohydrate
Solids	---	0.94**	0.72**
Protein		---	0.51*
Carbohydrate			---

* Significant at the 5% level.
**Significant at the 1% level.

protein and carbohydrate contents were associated positively with total solids content ($p<0.01$). However, the correlation between protein and total solids contents was higher than the correlation between carbohydrate and total solids contents. Table 7 shows the correlation coefficients for composition of soymilk and acid production in fermented soymilk. Acid production by L. fermentum NRRL B-585 was correlated significantly with total solids, protein, and carbohydrate contents ($p<0.01$), while acid production by L. acidophilus NRRL B-2092 was correlated significantly only with carbohydrate content in soymilk ($p<0.01$). There was no significant association between acid production by L. acidophilus NRRL B-1910 and composition of soymilk.

pH Values

pH values of autoclaved soymilk were approximately 6.5. After 16 hours of incubation, acids developed in inoculated soymilk, and pH values of soymilk decreased as shown in Table 8. pH values of soymilk fermented by L. fermentum NRRL B-585, L. acidophilus NRRL B-1910 and NRRL B-2092 ranged from 5.70 to 5.89, 4.64 to 5.00, and 4.60 to 4.94. Because of the capability of utilizing oligosaccharides in soymilk, the pH decreases were different in soymilk fermented by different lactic acid bacteria.

Acid Production

Lactic acid bacteria are Gram-positive, non-spore-

Table 7 - Correlation coefficients for acid production by three strains of lactobacilli and selected components of soymilk

	Acid Production		
	NRRL B-585	NRRL B-1910	NRRL B-2092
Solids	0.73**	-0.19	0.35
Protein	0.74**	-0.30	0.13
Carbohydrate	0.54**	0.15	0.77**

** Significant at 1% level.

Table 8 - Final pH values[a] for fermented soymilk

Extraction Ratio Beans:Water	Starter Culture NRRL B-585	NRRL B-1910	NRRL B-2092
1:6	5.70	5.00	4.94
1:7	5.79	4.81	4.73
1:8	5.79	4.69	4.68
1:9	5.81	4.64	4.60
1:10	5.88	4.74	4.71

[a]Values are means of four replicates.

forming bacteria producing lactic acid as the major or sole product of fermentation. The homofermentative lactic acid bacteria produce two molecules of lactic acid from one molecule of glucose. The heterofermentative lactic acid bacteria produce one lactic acid, and ethanol, and one carbon dioxide molecule from one molecule of glucose (Fung, 1986). The carbon mass balance during fermentation may be summarized as the following mathematic equation (Buono, 1988):

$$\text{CONSUMED SUBSTRATE} = \text{GROWTH} + \text{LACTIC ACID FORMATION} + CO_2$$

When substrate is available, more lactic acid bacteria can grow, and, therefore, more acid is produced in the environment. L. acidophilus NRRL B-1910, and B-2092 are homofermentative organisms and L. fermentum NRRL B-585 is a heterofermentative organism.

Table 9 displays the amount of acid produced in soymilk fermented by each strain of lactobacilli. Mean values of acid production from L. fermentum NRRL B-585, L. acidophilus NRRL B-1910 and NRRL B-2092 ranged from 7.00 to 16.15, 32.30 to 39.10, and 27.20 to 34.85 (μ mole lactic acid/gram fermented soymilk), respectively. Apparently, NRRL B-1910 and B-2092 grew better than B-585, when soymilk without fortification was used as a substrate.

Data in Table 9 also show that the acid production by lactobacilli varied when the extraction ratio of beans to

Table 9 - Mean values[a] of acid production for fermented soymilk

Extraction Ratio	µ mole lactic acid/g soymilk		
Beans:Water	NRRL B-585	NRRL B-1910	NRRL B-2092
1:6	16.15^{A}	32.30^{A}	32.35^{AB}
1:7	10.85^{AB}	33.80^{A}	34.85^{A}
1:8	10.25^{B}	36.40^{A}	32.35^{AB}
1:9	9.35^{B}	39.10^{B}	31.25^{AB}
1:10	7.00^{B}	35.65^{A}	27.20^{B}

[a]Values are means of four replicates. Mean values in the same column with different superscripts (A,B) are signifcantly different ($p < 0.05$).

water or solids content was increased. *L. fermentum* NRRL B-585 had maximum acid production in soymilk with a 1:6 beans to water extraction ratio. *L. acidophilus* NRRL B-2092 produced more acid when the extraction ratio of soymilk was increased from 1:10 to 1:7, and had maximum acid production at 1:7. Acid development decreased when the soymilk with 1:6 extraction ratio was used as a substrate for B-2092. *L. acidophilus* NRRL B-1910 produced the maximum amount of acid in the soymilk with a 1:9 extraction ratio. Acid production decreased when either a higher or lower extraction ratio than 1:9 was used for the NRRL B-1910 organism.

It seemed that a higher total solids content than the optimal level inhibited the growth of lactic acid bacteria in terms of acid production in soymilk. Since soymilk has a different amino acid pattern in protein structure and different carbohydrate composition from those present in bovine milk, a different amount of acid production in fermented soymilk was expected. However, it was interesting that the activity of lactic acid bacteria was inhibited at such a low total solids level when soymilk was the only substrate, while 25% total solids content was the level reported to inhibit the activity of lactic acid bacteria in bovine milk (Pulay and Krasz, 1974).

In order to obtain additional information, the relationships between acid production and composition of soymilk from raw data were plotted (Fig. 3-8). Fig.3

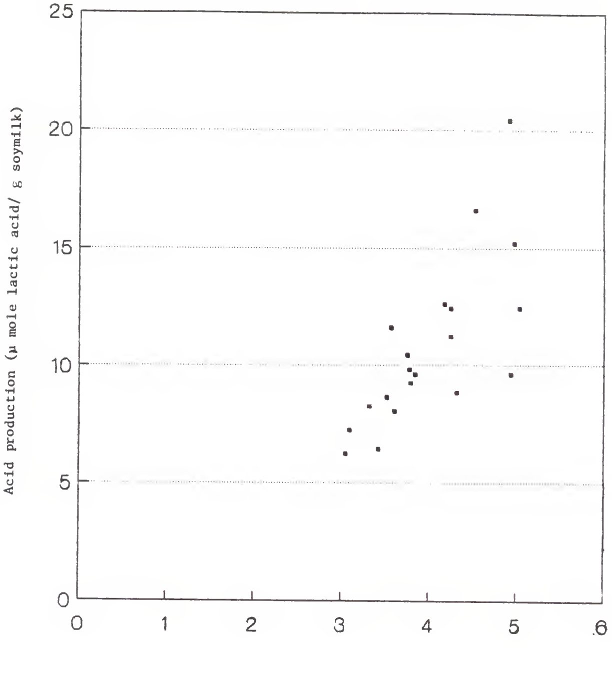


Fig. 3 -- Relationship between acid production by L. fermentum NRRL B-585 and protein content in soymilk

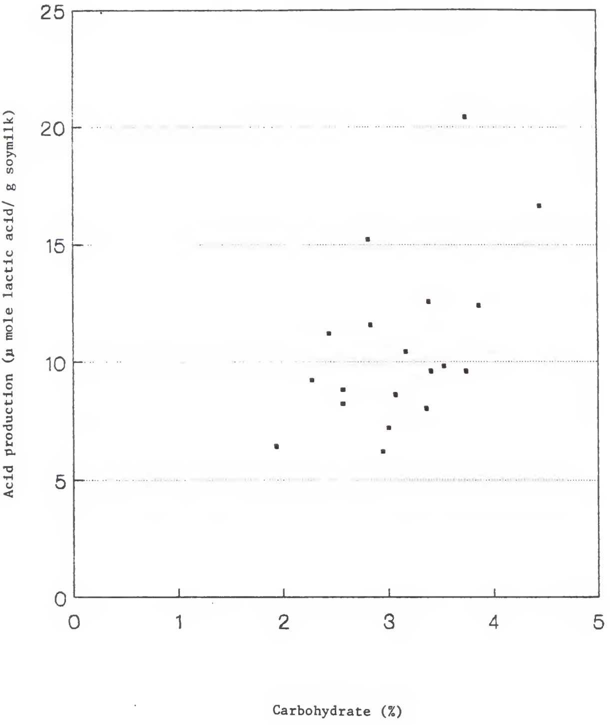


Fig. 4 -- Relationship between acid production by L. fermentum NRRL B-585 and carbohydrate content in soymilk

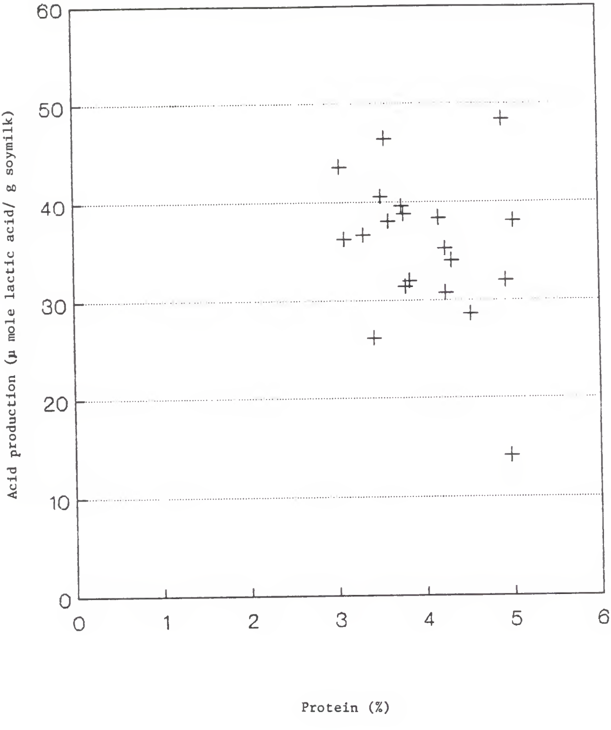


Fig. 5 -- Relationship between acid production by L. acidophilus NRRL B-1910 and protein content in soymilk

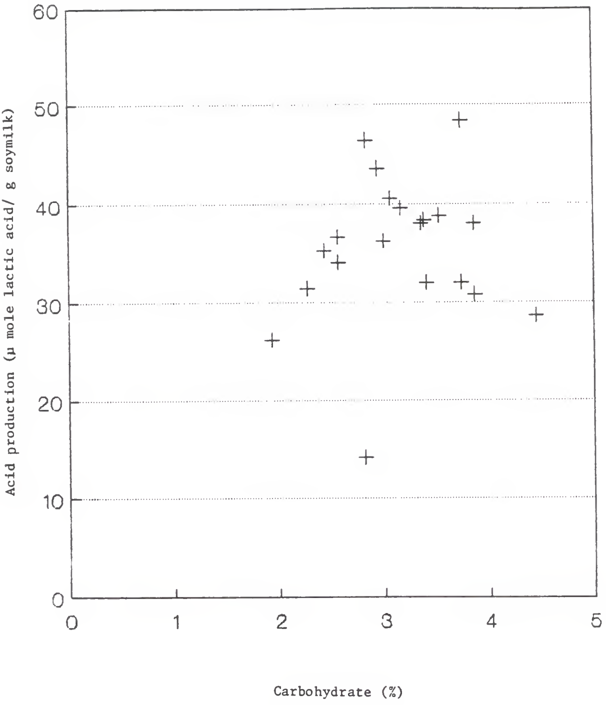


Fig. 6 -- Relationship between acid production by L. acidophilus NRRL B-1910 and carbohydrate content in soymilk

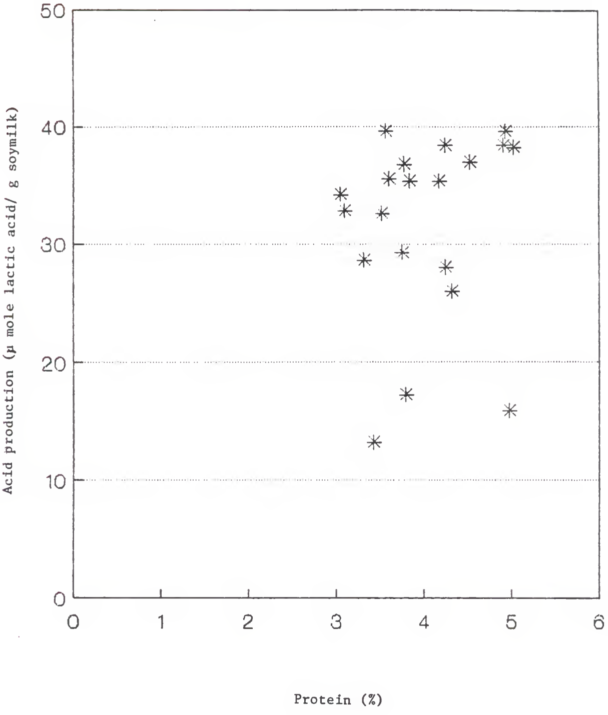


Fig. 7 -- Relationship between acid production by L. acidophilus NRRL B-2092 and protein content in soymilk

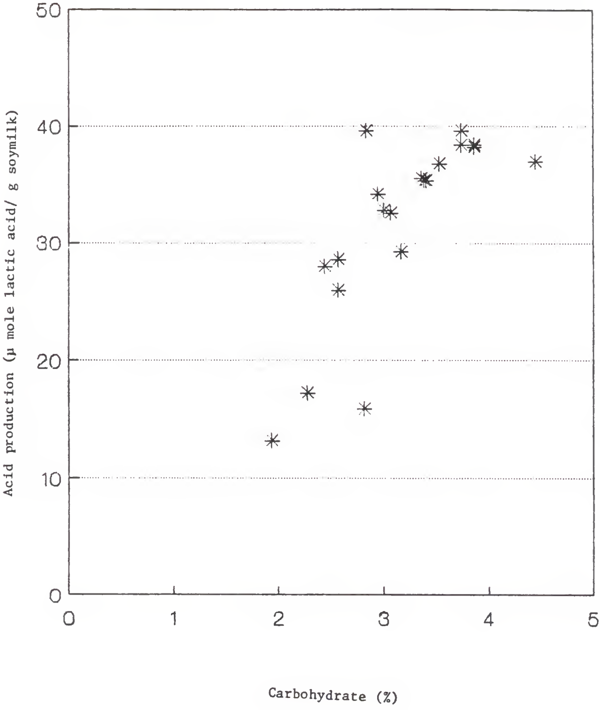


Fig. 8 -- Relationship between acid production by L. acidophilus NRRL B-2092 and carbohydrate content in soymilk

contains a plot of acid production by L. fermentum NRRL B-585 versus protein content in soymilk. It showed that acid production by B-585 increased when protein content in soymilk was increased. A similar effect was noted for carbohydrate content on B-585 acid production that is shown in Fig. 4. A level of total solids in soymilk as high as the 1:6 extraction ratio did not inhibit the growth of B-585. This indicated that B-585 could grow better and developed more acid in soymilk with a higher extraction ratio than 1:6 based on extrapolation of measured observations. However, the total amount of acid developed from B-585 was a lot less than from the two other strains of lactobacilli. Previous investigation (Mital and Steinkraus, 1975) showed that L. fermentum B-585 was not able to ferment sucrose, although it can completely utilize raffinose and stachyose by 12 and 25 hr, respectively. Since sucrose is the major sugar in soybeans, it was not surprising that B-585 could not grow well in soymilk.

L. fermentum NRRL B-585, L. acidophilus NRRL B-1910 and B-2092 all produced acid in their respective soymilks, but the effects of protein and carbohydrate contents on their acid production were different (Fig. 3-8). Fig. 5 showed that changes of protein content in soymilk had little effect on acid production by B-1910. Fig. 6 revealed that carbohydrate content in soymilk had a quadratic effect on acid production by B-1910. Acid production by B-1910 increased when the carbohydrate content in soymilk was increased until an optimal carbohydrate level was reached. Above this optimal level, acid production decreased when carbohydrate in soymilk was increased, as though excessive carbohydrate content inhibited the growth of B-1910.

L. acidophilus B-1910 grew relatively well in soymilk based on the amount of acid production (Fig. 5 and 6). Good utilization of oligosaccharides and subsequent accumulation of degraded products from oligosacchrides progressed during fermentation with this organism. The inhibition of lactobacilli activity might be attributed to pH decrement and excessive amounts of galactose and melibiose. Ohtakara et al. (1984) showed that the activity of α-galactosidase, which is present in lactic acid bacteria and is known to be able to hydrolyze α-galactosyl linkages in oligosaccharides, was inhibited greatly by galactose and melibiose. This inhibition was of a noncompetitive type.

Little effects were noted for protein content on acid production as shown in Fig. 8. Fig. 7 displays that acid production by *L. acidophilus* NRRL B-2092 increased when carbohydrate content in soymilk was increased.

Regression Analysis

Multiple regression analysis of acid production on solids content in soymilk was performed. The quadratic models for these three strains of lactobacilli were as follows:

NRRL B-585:

$$ACID = -29.9428 + 8.1969\ SOL - 0.3770\ SOL^2$$

$$R^2 = 0.5599$$

NRRL B-1910:

$$ACID = 65.5123 - 6.4139\ SOL + 0.3223\ SOL^2$$

$$R^2 = 0.0419$$

NRRL B-2092:

$$ACID = -27.3489 + 12.6089\ SOL - 0.6360\ SOL^2$$

$$R^2 = 0.1361$$

Quadratic models for B-1910 and B-2092 based on solids content were inadequate predictors for acid production. Using protein and carbohydrate contents as regressors improved the estimation of acid production by B-1910 and B-2092. Least squares equations containing quadratic and cross-product terms were:

NRRL B-585:

$$ACID = -20.0233 + 9.1474\ PRT + 2.1065\ CAR - 0.2840\ PRT^2 + 0.5773\ CAR^2 - 1.0767\ PRT*CAR$$

$$R^2 = 0.5952$$

NRRL B-1910:

$$ACID = 25.1734 + 0.8861\ PRT + 10.5233\ CAR - 9.2706\ PRT^2 - 15.9689\ CAR^2 + 22.6781\ PRT*CAR$$

$$R^2 = 0.5738$$

NRRL B-2092:

$$ACID = -36.0846 + 6.8468\ PRT + 29.1433\ CAR - 5.5145\ PRT^2 - 10.2592\ CAR^2 + 11.2994\ PRT*CAR$$

$$R^2 = 0.8202$$

Backward elimination procedure was employed in an attempt to remove all unnecessary terms from the full models without substantially increasing the size of the estimate of model variance. This procedure was used to select best fitted models (Draper and Smith, 1981).

NRRL B-585:

$$ACID = - 5.9514 + 4.1690\ PRT$$

$$R^2 = 0.5499$$

NRRL B-1910:

All variables were retained in the model.

NRRL B-2092:

$$ACID = -34.6686 - 3.4052\ PRT + 40.1261\ CAR - 4.5171\ CAR^2$$

$$R^2 = 0.7543$$

Three-dimensional distributions of predictive acid production by three strains of lactobacilli are shown in Fig. 9-11, when carbohydrate (2.00 to 4.50%) and protein (3.00 to 5.00%) contents of soymilk were varied. For easier observation, three-dimensional surface graphs were reduced to two-dimensional contour plots (Figures 12-14). To check fitness of these models protein and carbohydrate data from

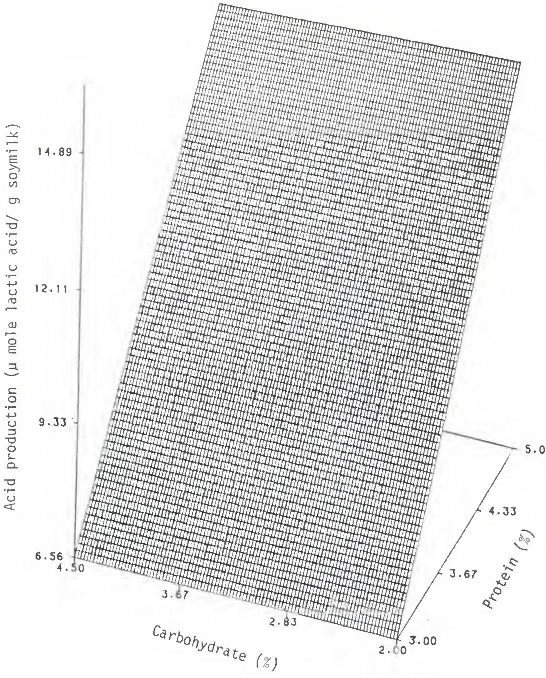


Fig. 9 -- Predictive acid production by L. fermentum NRRL B-585 as a function of protein and carbohydrate contents in soymilk

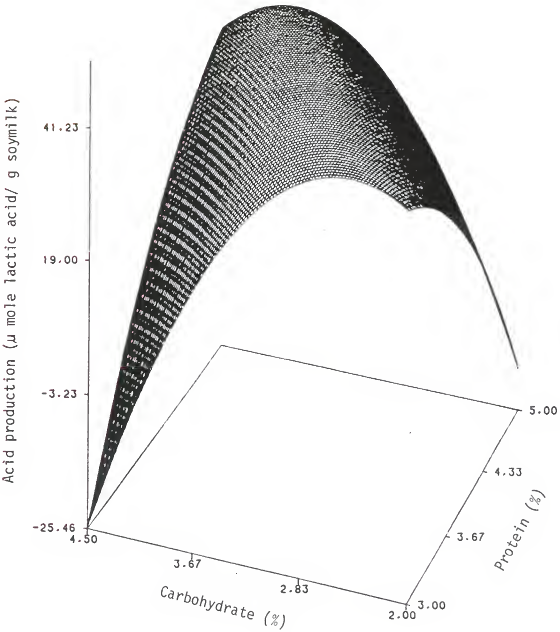


Fig. 10 -- Predictive acid production by L. acidophilus NRRL B-1910 as a function of protein and carbohydrate contents in soymilk

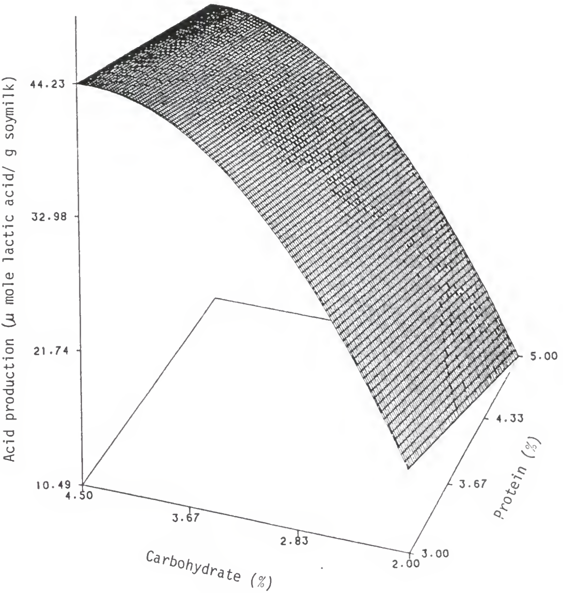


Fig. 11 -- Predictive acid production by L. acidophilus NRRL B-2092 as a function of protein and carbohydrate contents in soymilk

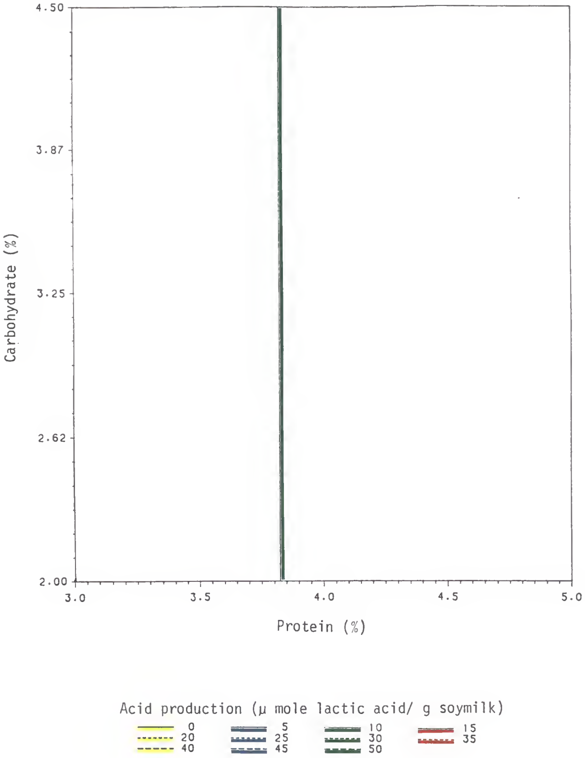


Fig. 12 -- Contour plot of acid production by L. fermentum NRRL B-585 in soymilk

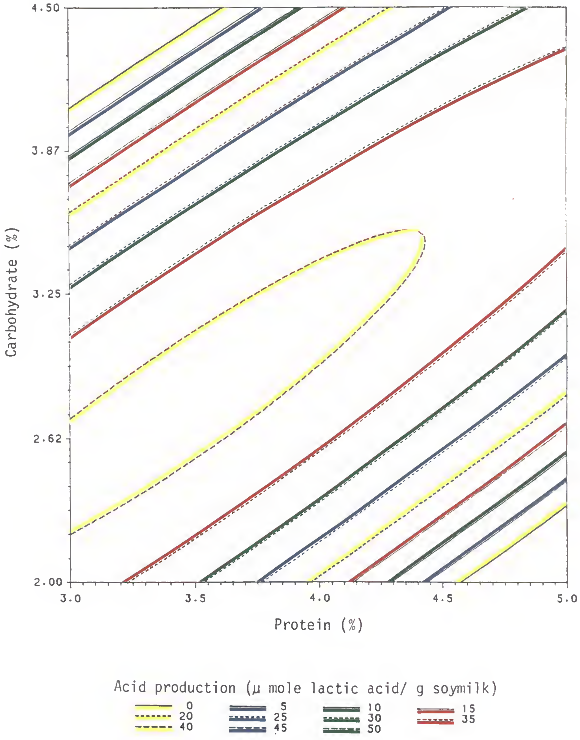


Fig. 13 -- Contour plot of acid production by L. acidophilus NRRL B-1910 in soymilk

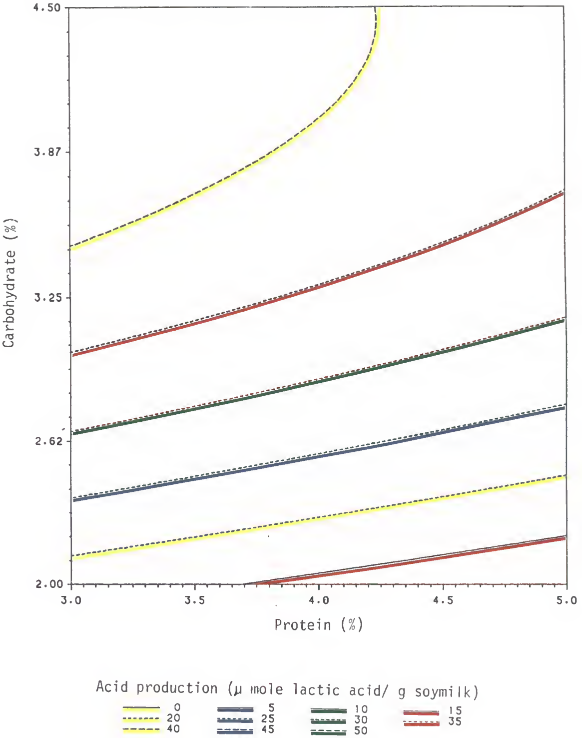


Fig. 14 -- Contour plot of acid production by L. acidophilus NRRL B-2092 in soymilk

Table 5 were plugged into the equations to calculate predictive acid production (Table 10). Some results in Table 10 were very close to experimental observations in Table 9, but some were not. This indicated that factors other than protein and carbohydrate contents in soymilk should be included in the model for better estimation, especially when NRRL B-585 and B-1910 were the organisms for fermentation.

Table 10 - Predictive values of acid production from regression models

Extraction Ratio Beans:Water	μ mole lactic acid/g soymilk NRRL B-585	NRRL B-1910	NRRL B-2092
1:6	14.27	38.77	36.01
1:7	12.43	39.95	34.33
1:8	10.31	40.96	32.91
1:9	9.09	41.19	31.13
1:10	7.43	41.18	28.36

CONCLUSIONS

Use of soymilk to make a yogurt-like product was investigated. In order to make an acceptable fermented soymilk, the consistency and acid development of the final product were critical. It was necessary to use soymilk containing resonable amounts of fermentable carbohydrate and protein, so lactobacilli could develop enough acid to produce a soft curd from soy protein. L. acidophilus NRRL B-1910 and B-2092 grew well in soymilk and produced sufficient acid to form a yogurt-like product from soymilk at five different bean to water extraction ratios. L. fermentum NRRL B-585 was not a good starter culture for fermented soymilk because of its poor utilization of sucrose.

In yogurt making, total solids of cow's milk is usually the only factor considered. However, when soymilk was used as a substrate for the growth of lactic cultures, factors other than total solids content, such as protein and carbohydrate, should be considered. Because each strain of lactobacilli showed a different pattern of growth in soymilk, adjustment of protein and carbohydrate contents in soymilk to optimize growth of a specific strain of lactobacilli was necessary.

Regression models including protein and carbohydrate content as variables were not adequate to predict acid production by lactobacilli. Since lactic acid fermention is a complicated biochemical process, further study of some other possible factors that influence fermentation such as incubation temperature, incubation time, and formation of degraded products that could be included in the model should be done.

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EFFECT OF COMPOSITION OF SOYMILK ON ACID PRODUCTION BY *LACTOBACILLUS* FERMENTATION

by

CHUN-YEN CHANG

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ABSTRACT

Soymilk was prepared from Williams 82 soybeans using five different extraction ratios of 1:6, 1:7, 1:8, 1:9, and 1:10 (dry beans:water). Total solids, protein and carbohydrate contents were determined from each soymilk extraction. Total solids, protein, and carbohydrate contents increased when the extraction ratios of soybeans to water were increased. Soymilk was fermented by Lactobacillus fermentum NRRL B-585, or Lactobacillus acidophilus NRRL B-1910 or B-2092, and evaluated as a substrate for acid production. Mean values of acid production from NRRL B-585, B-1910, and B-2092 ranged from 7.00 to 16.15, 32.30 to 39.10 and 27.20 to 34.85 (μ mole lactic acid/gram soymilk), respectively. Apparently B-1910 and B-2092 grew better in soymilk than B-585, when soymilk without fortification was used as a substrate. Effects of protein and carbohydrate contents of soymilk on acid production were different among strains of lactobacilli. Acid production by B-585 increased when protein and carhohydrate contents in soymilk were increased, but little acid developed in comparison to other samples. Carbohydrate content in soymilk had a quadratic effect on acid production by B-1910; changes in protein content had only little effect. Acid production by B-2092 increased when carbohydrate content in soymilk was increased. Little

effect was noted for protein content on acid production by B-2092. In order to make a yogurt-like product from soymilk, adjustment of protein and carbohydrate contents in soymilk to optimize growth of a specific strain of lactobacilli should be considered.

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