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PREBIOTICS SELECTION AND OPTIMIZATION OF CULTURAL CONDITION FOR PROBIOTIC *BIFIDOBACTERIUM LACTIS*

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Abstract: *Bifidobacterium lactis*, a well-known probiotic, was cultured in laboratory to observe the effect of substrate and mixing on cell growth. The cells were grown in MRS-IM media defined by Christian Hansen, Denmark, where carbohydrate sources were varied. Glucose, fructooligosaccharides (FOS), soy broth and commercial soymilk were used as carbohydrate sources. A one-liter bioreactor was used to grow the cells, where anaerobic condition was maintained by supplying nitrogen gas throughout the experiment. Fructooligosaccharides, and commercial soymilk were found effective on this cell growth. The overall cell growth was found to be almost indifferent, but growth rate was considerably higher due to mixing.

Keywords: *Bifidobacterium lactis*, probiotic, fructooligosaccharides

Introduction

The human intestine is one of the most complex habitats for microorganisms. Different kinds of microorganisms inhabit it. Some of them are beneficial and some are harmful. For maintaining sound health it is required to have the presence of friendly and helpful organisms in the colon (Tannock, 1995a). Scientists have been trying to find out the colonic organisms and differentiate those according to their merits. The helpful bacteria that inhabit the intestine are known as probiotics (Fuller and Gibson, 1997). These include lactic acid excretors as lactobacilli and bifidobacteria (Ziemer and Gibson, 1998). The probiotic *Bifidobacterium* is one of the most important organisms among them. The species *Bifidobacterium lactis* has been recently developed and is now used in some yoghurt production besides other commercial uses.

For their growth these probiotics use the food eaten by us. The nutritional foods, which help enhanced growth and development of the probiotics, are known as prebiotics. These are normally different types of oligosaccharides (Hidaka et al., 1986).

With increase in age of a person these bacteria are often reduced in human beings (Roberfroid, 1996). Consumption of probiotics is thus sometimes recommended for maintaining good health. There are various ways by which supplements of probiotics are made (Gibson and Roberfroid, 1995). These include yoghurt, produced or mixed with these organisms, and direct administration as bacterial capsules. An alternative way of increasing these organisms is the consumption of prebiotics. Prebiotics is a functional food that can reach the colon without much change of its original structure. These then used to increase these organisms. At present fructooligosaccharides (FOS) are known to be one of the best prebiotics and these are produced commercially (Hidaka et al, 1986; Mitsuoka et al, 1987).

The pathogenic microorganisms that cause disease like diarrhea and are found in the colon include *Salmonella, Shigella, Campylobacter, Yerisina, Aeromonas, Clostridium,* and *Escherichia* (Ziemer and Gibson, 1998). Several bacteria cause more chronic diseases in the colon. Carcinogenic products produced by colonic bacteria can be listed as, fecapentaenes, nitrosamines, heterocyclic amines, phenolic compounds, azo dyes, indolic compounds, secondary bile acids etc (Rowland, 1995).

Bifidobacteria inhibit functioning of many pathogenic organisms (Tannock, 1995b). In Japan dairy products containing bifidobacteria were fed to children to treat successfully some enteric infections (Tojo et al., 1987). It has an anticarciogenic effect, which is caused by direct and indirect removal of procarcinogens and activation of the immune system of the body.

Because of the low pH of the upper gastrointestinal tract (GIT), when direct administration or feeding yoghurt containing Bifidobacteria is made, the chance of survival of the bacteria is reduced. Due to the low efficiency of direct administration, feeding of prebiotics is recommended (Sultana, 2000). The probiotics can enhance the growth of existing bacteria present in the colon. But the available commercial FOS are costly

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and not well known to the people, though these selectively help to grow probiotics (Mitsuoka et al., 1987). Soya milk is thought to be one of the good prebiotics, which is cheap and familiar to the people because of its oligosaccharides content.

Bifidobacterium lactis is strictly anaerobic bacteria and according to theory they like to grow at the bottom of the test tube (Willis, 1991), where no oxygen can reach by diffusion.

The aim of this research was to grow probiotic *Bifidobacterium lactis* in the laboratory in order to check its various properties. As this organism was recently isolated (Miele et al., 1997), its biochemical properties are needed to be known. This work was done to see the effect of prebiotics and mixing in anaerobic condition on the growth of *Bifidobacterium lactis*.

Materials and Methods

Freeze-dried *Bifidobacterium lactis* (Bb-12) was cultured in the Bioprocess Technology laboratory of Asian Institute of Technology (AIT), Thailand. This strain was used throughout this work and this was obtained from Christian Hansen of Denmark through East Asiatic Company, Thailand. From the freeze-dried form colony was isolated on MRS-IM agar, then stored in the same agar stab in test tubes. The stock culture was then grown in MRS-IM media in 1-L bioreactor for several hours through stepwise fermentation. In every step of growth temperature of the media was kept 37^0C and anaerobic condition was maintained.

Carbohydrate source: To check the effect of different prebiotics the control was made by using 2% (w/v) glucose in MRS-IM media. For other prebiotics same carbohydrate concentration was used throughout the experiment. For liquid carbohydrate sources like soymilk and soy broth, 2% (v/v) was used in the media.

Fermentation Scheme: Two sets of fermentation were done throughout the experiment. In set-1, the pre-culture and subculture media were kept same as of the final bioreactor. To make an identical inoculation another fermentation scheme (set-2) was done, In set-2, all the pre and subcultures were prepared by using 2% (w/v) glucose, but in the final bioreactor different carbohydrate sources were used.

Mixing and Anaerobic Conditions: Sterile nitrogen was supplied to provide anaerobic condition and mixing was done by magnetic stirring. In every step of the work proper aseptic condition were maintained. Cell density was measured by measuring optical density (OD_{600}) by spectrophotometer. The OD values were then converted to cfu/ml with the help of standard curve ($R^2 = 0.9982$).

Results and Discussion

Growth of organisms with pre-culture seed grown on same substrate (set-1): Bifidobacterium lactis (Bb-12) was cultured in laboratory using different media and different fermentation conditions. It has been observed that with changing growth conditions, growth has also changed. This experiment was done to determine the best conditions for enhanced growth of *Bifidobacterium lactis*. Glucose was the main carbohydrate source, and in the various experiments carried out, instead of glucose different substrates have been used to observe the effect of various substrate on cell growth.

Initially growths of bacteria on different carbohydrate sources were studied. These included 2% (w/v) glucose, 2% (w/v) FOS, 1% (w/v) glucose + 1% (w/v) FOS, and 2 % (v/v) soy broth as carbohydrate sources (set1). For set2 2% (w/v) glucose, 2% (w/v) FOS, 2% (v/v) soymilk and 2% (w/v) glucose + 2% (v/v) soymilk were used as carbohydrate source. The growth results are shown in the following tables and figures.

From the table 1 it is seen that specific growth rate constant (μ) is highest in 2% FOS, but the overall growth is highest in media containing 1% glucose (w/v) + 1% FOS (w/v), though its specific growth rate constant is closer to that of control. Growth was poor in the medium containing only 2% soy broth. In soy broth the concentration of net glucose was very less, hence the growth was also less. As the bacteria were grown in 2% glucose medium, it needed minimum glucose of about 2% for its growth. In 1% glucose + 1% FOS the growth was more than the control, and it can be inferred that for its growth presence of glucose is essential, and FOS increased the growth.

The results of these initial experiments indicated that glucose seemed to be better substrate for growth of bifidobacteria. However, the higher specific growth with FOS indicates that when intestinal flora have to be increased quickly, consumption of FOS will be recommended. Also, one should remember that the glucose consumed would not reach the colon in the same concentration and form, while FOS, which is not affected by low pH, will.

The precipitation of soymilk was another problem when it was used in higher concentrations. The non-availability of the substances present in this substrate, include valuable FOS has to be overcome by using

lower dilutions when they may not denature and precipitate so easily. The soy broth experiment indicated that it was not a good source of sugar to enhance growth.

Table 1. Summary results of cell growth in bioreactor containing MRS-IM media.

Substrate	μ (hr^{-1})	*Initial* OD_{600}	*Highest* OD_{600}	*Initial conc.* (*cfu/ml x* 10^{-7})	*Final conc.* (*cfu/ml x* 10^{-7})
Control (2% glucose)	0.1142	0.825	4.9125	10.2118	60.3409
2% FOS	0.1822	0.321	3.3675	4.03074	41.3930
1% glucose + 1% FOS	0.1174	0.043	4.9500	0.62135	60.8008
2% soy broth	0.0625	0.030	0.2270	0.46192	2.87793

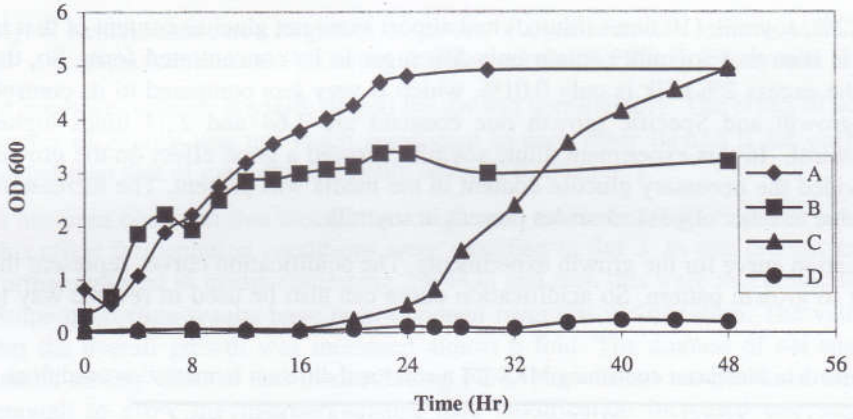

Fig. 1. Growth of *Bifidobacterium lactis* in MRS-IM media with different types of carbohydrate sources. A: Control (2% glucose); B: 2% FOS; C: 1% glucose + 1% FOS; D: 2% soy broth (set1).

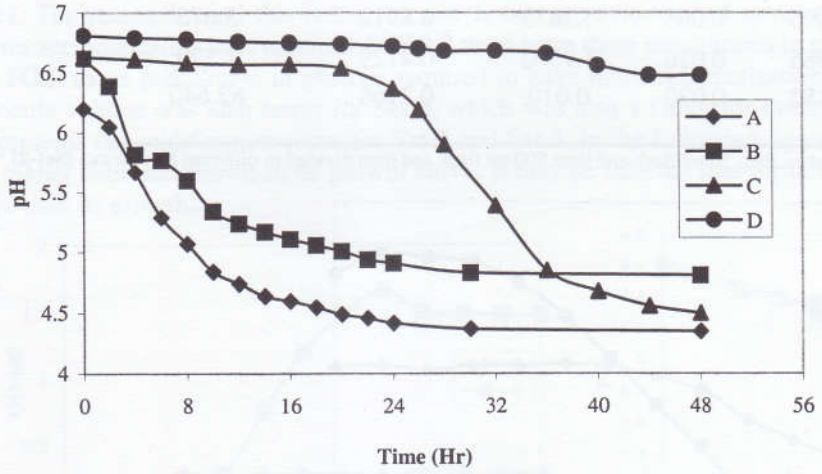

Fig. 2. Acidification curve of growth of growth of *Bifidobacterium lactis* in MRS-IM media with different types of carbohydrate sources. A: Control (2% glucose); B: 2% FOS; C: 1% glucose + 1% FOS; D: 2% soy broth (set2).

Effect of inoculum on growth of bifidobacteria: Figure 1 is the graphical representation of growth of in *Bifidobacterium lactis* in MRS-IM medium, where carbon source had been varied. Two growth curves have long lag phase, and for these two, their concentrations were lower than other two. So inoculum volume has a considerable effect on the lag phase of cell growth. This was observed from the initial absorbance (OD_{600}) values of these experiments, which were0.043 and 0.03 for 1% glucose + 1% FOS as compared to 0.825 and 0.321 with 2% glucose and only 2% FOS respectively. Growth could have gone high after the lag phase. However, as the overall productivity would have been reduced the experiment was not continued.

Figure 2 represents the pH values of the corresponding growth in different media. In these cases the curves have resemblance with its growth pattern. Higher the cell concentration higher the acid production hence, lower the final pH.

Effect of pre-culture on the growth of Bifidobacterium lactis: In the previous sections all pre-cultures and seed cultures were grown on the media containing same carbon source as that of the final media (**Set 1**). In the subsequent experiments all pre-cultures were grown in 2% glucose while the final cultures involved 2% glucose, 2% FOS, 2% soymilk and a combination of 2% glucose and 2% soymilk. Figure 4.5 represents the growth patterns in different media.

From figure 3 it is clear that both the values of μ and growth are highest for Glucose + 2% soymilk. The soymilk is 10 times diluted from its concentrated one. Media containing only 2% FOS or only 2% soymilk had very less growth. For these results it is clear that acclimatization is one important factor for good growth. This is clear from a comparison of these results that with the **Set 1** experiments. For good comparison, in this experiment (**Set 2**) the glucose experiment was repeated and found to be lower than **Set 1**. This was because of the lower level of inoculum used in **Set 2**.

While there was little growth with 2% soymilk alone, when this was added to glucose the increase in growth was much higher than the expected cumulative value. This showed again the need for acclimatization.

In the media with glucose and 2%, soymilk (10 times diluted) had almost same net glucose content of that in control. From the literature it is seen that soymilk contain only 5% sugar in its concentrated form. So, the increase of sugar content for the excess 2% milk is only 0.01%, which is very less compared to its control, but the net increase in cell growth and Specific growth rate constant are 1.64 and 2.27 times higher respectively than that of the control. In this experiment dilute soymilk showed a good effect on the growth of *Bifidobacterium lactis*, provided the necessary glucose content in the media was present. The increase to such high value was probably due to other oligosaccharides present in soymilk.

Figure 4 represents the acidification curve for the growth experiments. The acidification curves represent the lowering of pH corresponding to growth pattern. So acidification curve can also be used in reverse way to express the cell growth.

Tab. 2. Summary results of cell growth in bioreactor containing MRS-IM media* and different fermentation conditions.

Substrate	*μ (hr-1)*	*Initial OD_{600}*	*Highest OD_{600}*	*Initial conc. (cfu/ml x 10^{-7})*	*Final conc. (cfu/ml x 10^{-7})*
Control (2% glucose)	0.080	0.017	4.9125	0.3025	38.266
2% FOS	0.095	0.065	3.3675	0.8912	3.675
2% soymilk	0.085	0.026	4.9500	0.4129	1.529
2% glucose + 2% soymilk	0.182	0.030	0.010	0.2166	62.640

* All inocula were precultured in test tubes, then 50 ml flask and then 500 ml flask and then divided in different bioreactors (**Set-2**)

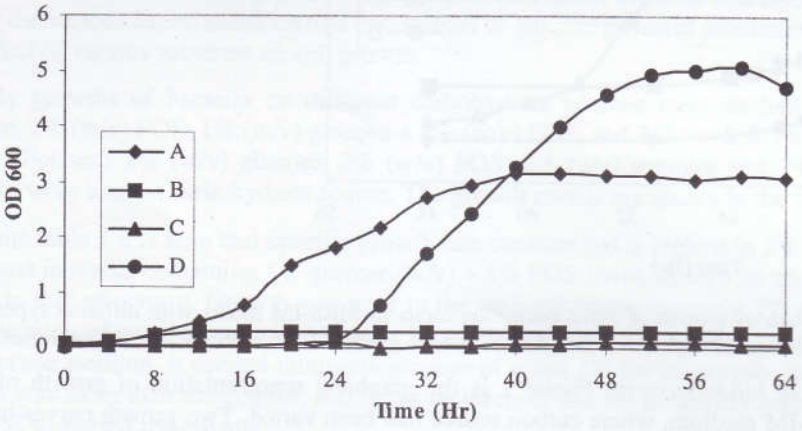

Fig. 3. Growth of *Bifidobacterium lactis* in MRS-IM media with different types of carbohydrate sources. A: Control (2% glucose); B: 2% FOS; C: 2% soymilk; D: 2% glucose + 2% soymilk (set2).

From the above tables and figures it is clear that only FOS or only soymilk is not effective for better cell growth, but with FOS or dilute milk addition increase the cell growth, what ever may be the culture conditions.

Essential sugar content is very much important for the growth. Fermentation conditions have also effect on cell growth. Growth in 2% FOS media for both the cases is its good example. Lower amount of milk sugar

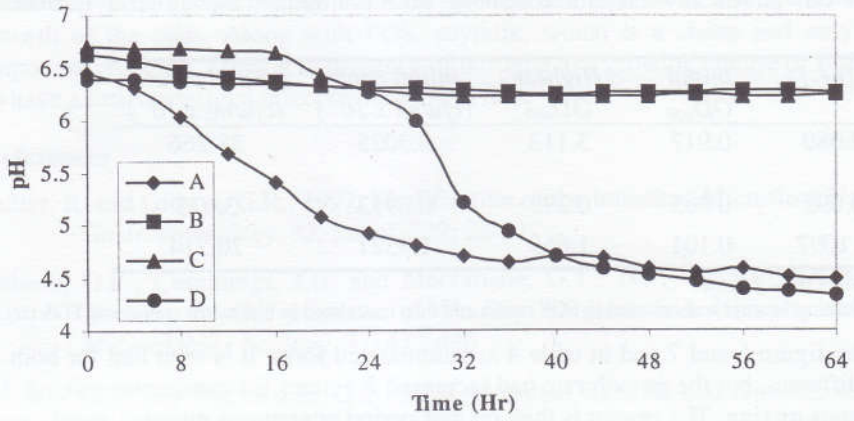

Fig. 4. Acidification curve of growth of growth of *Bifidobacterium lactis* in MRS-IM media with different types of carbohydrate sources. A: Control (2% glucose); B: 2% FOS; C: 2% soymilk; D: 2% glucose + 2% soymilk (set2).

or presence of dilute soymilk helped the growth considerably. The effect of soy broth or soymilk as the only source of energy was not much significant to each other though μ for only FOS (0.095 h^{-1}) is 1.5 times higher than that of media contain only soymilk (0.0625 h^{-1}).

It has been observed that inoculation of glucose media in 2% FOS media produced lower growth. To check this effect fermentation conditions were modified to **Set 3**. In this process the inocula were one more step further cultured in media containing 2% FOS and then this were inoculated in bioreactor containin2% FOS. Some interesting results have been obtained from this modification. The value of μ was increase not much but the overall growth was increased almost 6 fold. The amount of net sugar content were same both in media having glucose and media having FOS, but without modification the accessibility of FOS was not enough to grow the microorganisms. The modification increased one step growth, which helped it to acclimatize well in the final media, hence, the resultant increase of growth. The resultant data are presented in table 3, and figure 5(a) represents the growth curve for the different two sets of fermentation system.

Again, if the growth in 2% FOS for **Set 1** and **Set 3** are compared, the value of μ and growth were higher in set1. The reason behind this is that for **Set 1**, two pre-cultures had same media as in fermenter, where no extra acclimatization was required. In **Set 3** there were three pre-cultures in glucose and only one culture was in FOS, these precultures in glucose required to have more acclimatization, so the resultant decrease. The inocula volume was also lower for **Set 3**, which was also a factor for the low value of this set. Figure 5(b) represents the acidification curve for **Set 2** and **Set 3**. In the following figure it is seen that stationary phase is higher than that shown in its growth curve. It may be inferred that for this system production of acid was less than its growth.

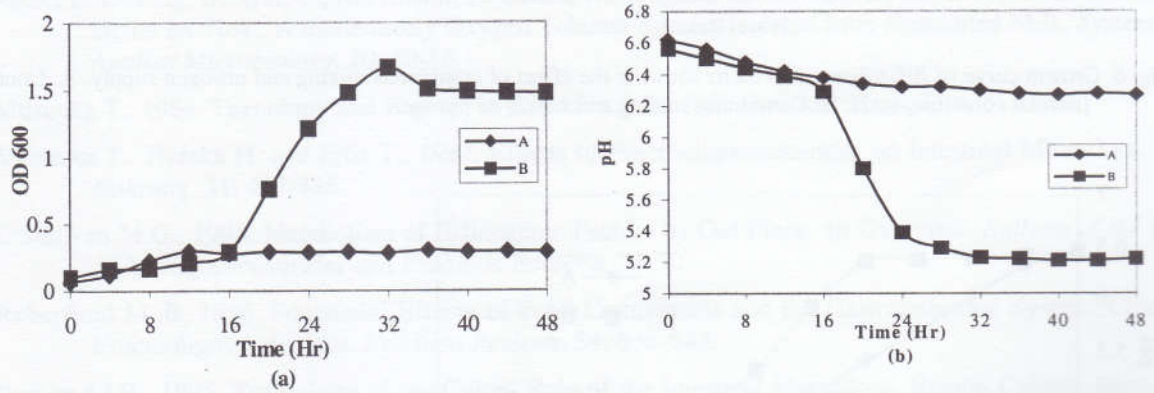

Fig. 5. Growth (a) and acidification (b) curves of *Bifidobacterium lactis* in MRS-IM media with same carbohydrate source but different inocula. A: control (3 steps pre- cultured in glucose and then inoculated in fermenter containing FOS; B: 3 steps pre- cultured in glucose and then one more step cultured in 2% FOS, then inoculated bioreactor containing 2% FOS media.

Effect of mixing in anaerobic condition: Mixing, which increases the mass transfer in the media is beneficial for growth. In literature (Willis, 1991) it is said that for strictly anaerobic organisms mixing is not good for the growth. It was thought like that, because of mixing, chance of oxygen diffusion might increase, which lowers the cell growth. To investigate this behavior, an experiment was done by using continuous nitrogen supply to make the environment anaerobic and then maintained continuous stirring. The results of this

Table 3. Summary results of cell growth in bioreactor containing MRS-IM media* and different fermentation conditions.

Substrate	*μ (hr-1)*	*Initial OD_{600}*	*Highest OD_{600}*	*Initial conc. (cfu/ml x 10^{-7})*	*Final conc. (cfu/ml x 10^{-7})*
Control (2% glucose)*	0.080	0.017	3.113	0.3025	38.266
2% FOS*	0.095	0.065	0.292	0.8912	3.675
2% FOS**	0.1097	0.101	1.665	1.3327	20.514

*Inocula of the bioreactor was grown in only glucose

**The same amount of glucose containing inocula was cultured in FOS media and then inoculated in bioreactor containing FOS (set3)

experiment are represented by figure 6 and 7 and in table 4 as summarized form. It is seen that for both the system cell growth was not different, but the growth rate had increased 2.5 times for continuous mixing. Lag phase was longer for continuous mixing. The reason is that, on that period continuous nitrogen supply could not remove all the air present in the system, which suppressed the cell growth. As soon as it became complete anaerobic, it took less time to reach the same concentration as of control.

Table 4. Summary results, showing the effects of continuous mixing.

Substrate	*μ (hr-1)*	*Initial OD_{600}*	*Highest OD_{600}*	*Initial conc. (cfu/ml x 10^{-7})*	*Final conc. (cfu/ml x 10^{-7})*
Control (no stirring)	0.080	0.017	3.113	0.3025	38.266
Continuous stirring	0.202	0.027	3.218	0.4257	39.554

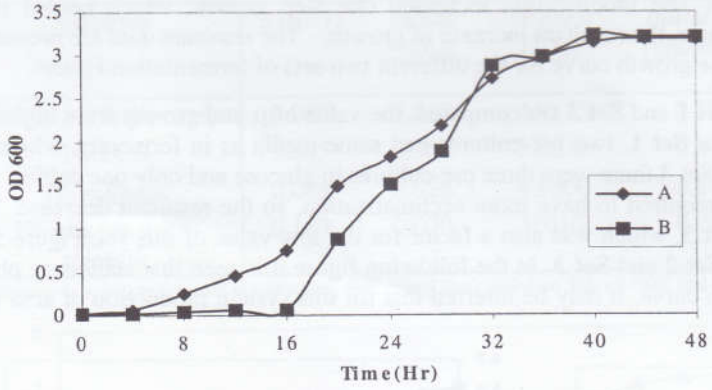

Fig. 6. Growth curve of *Bifidobacterium lactis* showing the effect of continuous mixing and nitrogen supply. A: Control (normal condition, set2); B: Continuous mixing and supply of nitrogen.

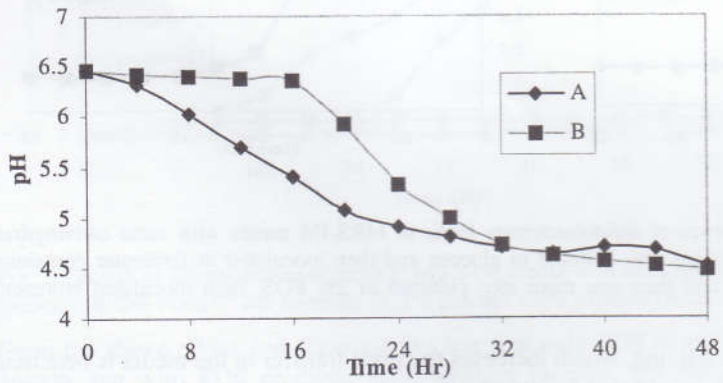

Fig. 7. Acidification curve of *Bifidobacterium lactis* showing the effect of continuous mixing and nitrogen supply. A: Control (normal condition, set2); B: Continuous mixing and supply of nitrogen.

From the various experiments it can be inferred that FOS, which is a well-known prebiotic, caused good growth of the cells. Along with FOS, soymilk, which is a cheap and easy source can also be used as important prebiotic. Stirring in presence of nitrogen to a growing culture of *Bifidobacterium lactis* was found to have a little beneficial effect on its growth rate.

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