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Cultivation of *Lactobacillus crispatus* KLB46 Isolated from Human Vagina

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Abstract Bacterial vaginosis can be treated by restoring the normal vaginal flora using lactobacilli. *Lactobacillus crispatus* KLB46 that was isolated from the human vagina has a strong antimicrobial activity and was grown in a batch and in a continuous fermentor. During batch cultivation, the maximum specific growth rate of *L. crispatus* KLB 46 was 0.63 h^{-1} and the highest viable cell count (1.9×10^9 CFU/mL) was obtained at pH 5.5. *L. crispatus* KLB 46 did not grow well at either pH 3.5 or 7.5. During continuous cultivation, the highest viable cell count (1.53×10^9 CFU/mL) was obtained at a dilution rate of 0.32 h^{-1}. However, the maximum productivity of viable cells was obtained at a dilution rate of 0.52 h^{-1}, and was 7.33×10^{11} CFU $L^{-1}h^{-1}$, that is approximately 5 times higher than that obtained from batch culture.

Keywords: *Lactobacillus crispatus*, bacterial vaginosis, cultivation, viable cell count

INTRODUCTION

Lactobacilli are considered to play an important role in the healthy human intestinal track, urinary track, and vagina. They secrete inhibitory substances, such as lactic acid, H_2O_2, and bacteriocin and protect humans against infection of other organisms [1,2]. In bacterial vaginosis, the normal microflora of the vagina is replaced by *Gardenerella vaginalis* and other anaerobic microorganisms. It has been suggested that the lack of protective vaginal lactobacilli gives rise to a condition, which predisposes women to bacterial vaginosis (BV) [3, 4]. The purpose of the therapy used for bacterial vaginosis is to eliminate overgrown BV-associated microorganisms and allow the resuscitation of a normal healthy vaginal flora, which is dominated by lactobacilli. The most commonly prescribed treatment for BV involves a variety of antibacterial medications (metronidazole) [5]. However, these attack not only the targeted anaerobic bacteria but also normal lactobacilli. As a result, they destroy the normal vaginal flora and increase susceptibility to infection. Recently, in order to overcome this secondary effect of antibiotics, various lactobacilli have been used to restore the normal vaginal flora or to prevent recurrent urinary tract infection [6-8].

Lactobacilli were isolated from Korean women and selected by antimicrobial activity testing. For probiotic use in the human vagina as a barrier to infections and as an alternative to the long-term use of antibiotics. The isolates were identified by PCR-RFLP, and one of the isolates that showed strong antimicrobial activity, *Lactobacillus* KLB 46 was identified as *Lactobacillus crispatus* by 16S rDNA sequencing [9].

Here, we describe the cultivation of *L. crispatus* KLB46 to investigate the effects of various cultivation conditions, which included changes in the concentration of the major carbon and nitrogen sources in the medium, and the pH and temperature of the culture broth, on the viable cell production. Cells were grown in batch and continuous culture modes to maximize the number of viable cells.

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MATERIALS AND METHODS

Microorganism, Culture Media, and Growth Conditions

The organisms used in the experiments were *Lactobacillus crispatus* KLB 46, which was isolated from the human vagina. The strain was maintained frozen at -70°C in 10% (v/v) glycerol containing MRS broth (de Man, Rogosa, Sharpe broth). It was propagated in MRS broth at 37°C for 24 h. One percent (v/v) inoculum was prepared from cultures in the logarithmic phase that usually occurred 12 h after inoculation [10]. Fermentation medium (modified MRS medium [11]) contained: 20 g/L glucose, 10 g/L proteose peptone No. 3 (Difco Lab.), 10 g/L beef extract (Difco Lab.), 5 g/L yeast extract (Difco Lab.), 1 g/L Tween 80, 2 g/L ammonium citrate dibasic, 5 g/L sodium acetate trihydrate, 2 g/L dipotassium phosphate, 0.1 g/L magnesium sulfate, and 0.05 g/L manganese sulfate.

Effects of Medium Compounds, Initial pH, and Temperature on the Viable Cell Count

As the concentrations of the carbon and nitrogen sources are important for cell growth, the concentrations of glucose and yeast extract in MRS broth were varied. Cells were grown at 37°C with 1% (v/v) inoculum. The effects of initial pH and temperature were investigated at various pHs such as 4.5, 5.5, 6.5, and 7.5 and various temperatures such as 35°C, 37°C, 39°C, and 41°C. The pH of the medium was adjusted with 5 N NaOH and 85% phosphoric acid [12]. Test tube culture experiments were carried out with 20 mL medium in 50 mL screw capped test tubes.

Cultivation Conditions

All batch and continuous cultivations were carried out in a 2.5-L jar fermentor (Korea Fermentor Co., Incheon, Korea) with a working volume of 1 L. Glucose was separately sterilized and added to the fermentation medium. The temperature of the fermentor was maintained at 37°C and the agitator speed was 150 rpm. The pH of the culture broth was controlled automatically with 50% (v/v) NH_4OH and 85% (v/v) phosphoric acid. The gas phase was replaced with N_2 gas before the initiation of batch cultivation and during sampling. Nitrogen gas was supplied continuously during the continuous culture [2,13].

Analytical Procedures

The measurement of viable cell counts was carried out by the spot counting method. One hundred microliters of the culture broth was diluted serially from 10 to 10^6 times with deionized water and 5 μL of the diluted solutions was spotted onto the surfaces of MRS agar (1.5%) plates. The plates were incubated anaerobically at 37°C to allow colonies to develop. The spot counting method needed less time and labor than the spreading method.

Cell density was measured by absorbance at 600 nm using a spectrophotometer (UV-1601, Shimadzu). Concentrations of glucose, lactic acid, and acetic acid were determined by HPLC using a HPX87H column (Bio-Rad). The concentration of glucose was measured using an RI detector (LC-10AD, Shimadzu) and the concentrations of lactic and acetic acid were analyzed using an UV detector (SPD-10A, Shimadzu) at 210 nm. The column was eluted with 0.008 N H_2SO_4 at a flow rate of 0.5 mL/min. The temperature of the column was maintained at 45°C [14,15].

RESULTS AND DISCUSSION

Effects of Glucose and Yeast Extract Concentration

To study the effects of glucose and yeast extract concentrations on the viable cell count of *Lactobacillus crispatus* KLB46, cells were grown in different concentrations of glucose and yeast extract. As shown in Fig. 1, a higher viable cell count (2.5×10^9 CFU/mL) was obtained in MRS broth containing 40 g/L glucose than in 20 g/L glucose. However, further increase in the glucose concentration did not result in an increased viable cell count. When the concentration of yeast extract was varied, the highest viable cell number (3.21×10^9 CFU/mL) was obtained in MRS broth containing 15 g yeast extract. When 20 g/L yeast extract was added in the medium, a lower viable cell number was obtained. In both experiments, it was found that the viable cell number did not increase proportionally with increased glucose or yeast extract concentration. The composition of MRS broth, especially in terms of the concentrations of glucose and yeast extract, was probably optimal for the growth of *L. crispatus* KLB 46.

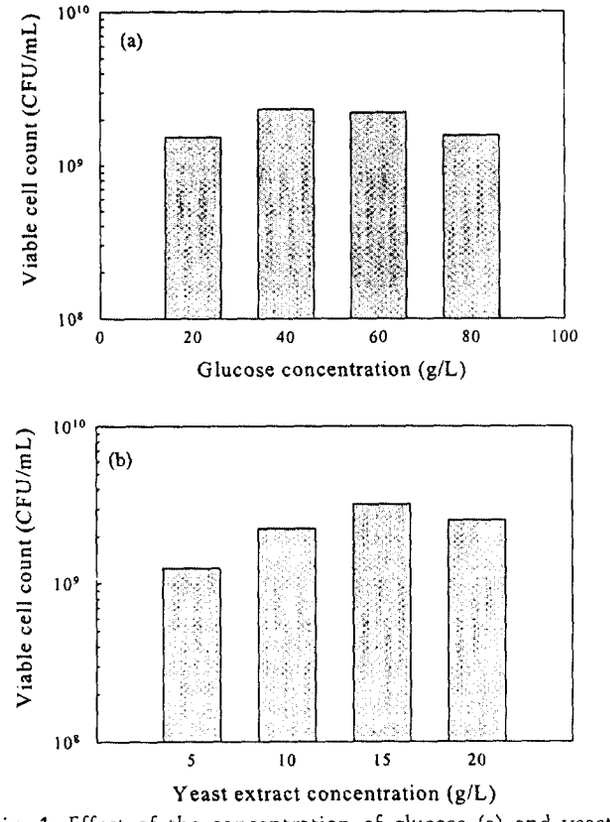

Fig. 1. Effect of the concentration of glucose (a) and yeast extract (b) on maximum viable cell count of KLB 46.

Effects of pH and Temperature

L. crispatus KLB 46 was cultured at various initial pHs and temperatures and maximum numbers of viable cells were determined (Fig. 2). After autoclaving the pH of the medium was adjusted to 4.5, 5.5, 6.5, or 7.5 with 5 N NaOH. Cells were grown at four different temperatures between 35 and 41°C. As shown in Fig. 2, the viable cell number was highest when cells were cultivated at a temperature of 37°C. Although the results obtained

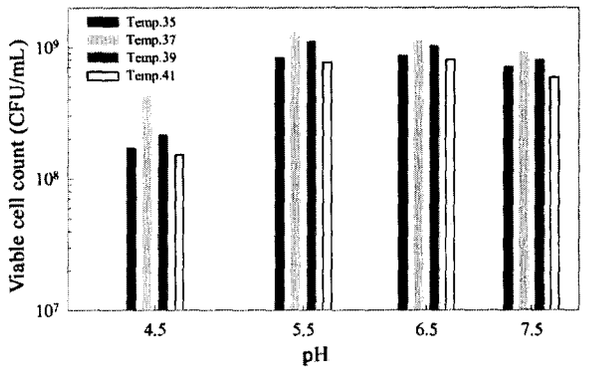

Fig. 2. Changes in maximum viable cell count of KLB 46 at various initial pHs and temperatures.

at pH 5.5-7.5 did not show much difference, the highest viable cell number was obtained at pH 5.5. As a result, the maximum viable number of *L. crispatus* KLB 46 was obtained at 37°C and at an initial medium pH of 5.5. At the end of the culture, the pH decreased to 3.9 regardless of the initial pH.

Batch Fermentation

In test tube culture it is difficult to control the medium pH accurately during cultivation. A set of pH-controlled batch cultivation experiments were conducted in a fermentor to obtain the optimum pH conditions for maximum viable cell number at pH levels between 3.5 and 7.5. The results of this batch fermentation of *L. crispatus* KLB 46 are shown in Fig. 3 (a), (b), and (c). *L. crispatus* KLB 46 did not grow well at pH's 3.5 or 7.5, however, high viable cell counts were obtained at pH's in the range between 4.5 and 6.5. This indicates that *L. crispatus* KLB 46 can grow well over a broad pH range. The highest viable cell count was obtained in the culture at pH 5.5 (1.9×10^9 CFU/mL). At pH 5.5, the maximum specific growth rate of *L. crispatus* KLB 46 was 0.63 h^{-1} and the maximum lactic acid concentration was 21 g/L (approximately 97% of the theoretical yield). The highest viable cell productivity and lactic acid productivity were 1.54×10^{11} CFU/mL and 1.61 g/L, respectively and these were obtained at pH 5.5.

After 15 h of fermentation, glucose in the medium had depleted completely and the lactic acid concentration reached its maximum. The concentration of acetic acid did not change significantly during fermentation. *L. crispatus* KLB 46 was found to be homofermentative lactic acid bacteria since obligately homofermentative lactobacilli ferment hexoses almost exclusively (> 85%) to lactic acid [16].

Continuous Fermentation

The effect of dilution rate on the maximum viable cell number from MRS medium by *L. crispatus* KLB46

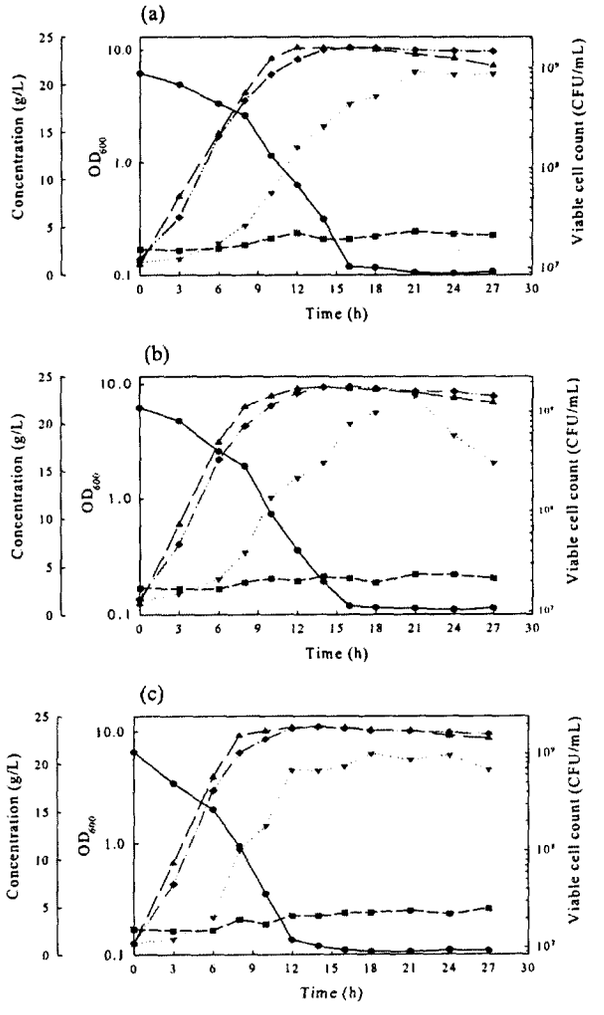

Fig. 3. Variations in optical density (◆), viable cell count (▲), glucose concentration (●), lactic acid concentration (▼), and acetic acid concentration (■) in batch culture at various medium pHs (a) 4.5, (b) 5.5, (c) 6.5.

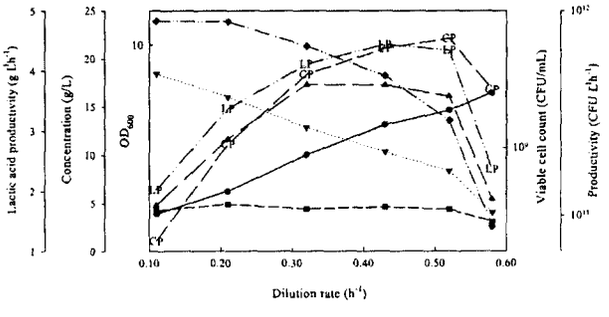

Fig. 4. Variations in optical density (◆), viable cell count (▲), glucose concentration (●), lactic acid concentration (▼), acetic acid concentration (■), viable cell productivity (CP), and lactic acid productivity(LP) in continuous culture at medium pH 5.5.

was investigated. Cells were grown at 5 different dilu-

tion rates between 0.11 and 0.58 h^{-1}. Fig. 4 shows the effect of dilution rate on viable cell count, lactic acid concentration, and their productivities. The highest viable cell count was obtained at a dilution rate of 0.32 h^{-1}, however, the maximum productivity of viable cells was obtained at a dilution rate of 0.52 h^{-1}, and this was 7.33×10^{11} CFU $L^{-1} h^{-1}$.

Although the optical density was high at a dilution rate lower than 0.21 h^{-1}, the viable cell count was much lower than those obtained at higher dilution rates. This indicates that much higher fraction of the cells is viable at higher dilution rates. As the dilution rate increased, the residual glucose concentration increased and the lactic acid concentration decreased. Some homofermentative lactic acid bacteria are known to produce other organic compounds produced by heterofermentative lactic acid bacteria depending on fermentation conditions [17,18]. Analysis of the concentrations of organic acid produced and glucose consumed (Fig. 4) showed that lactic acid was produced mainly from glucose in the medium. Along with the data obtained from batch fermentation (Fig. 3), this indicates that *L. crispatus* KLB46 is homofermentative. Consequently, *L. crispatus* KLB46 has an advantage not only as a viable cell product for the treatment of bacterial vaginosis but also as a lactic acid producer. The highest productivity of lactic acid, a major metabolite, was 4.44 g $L^{-1} h^{-1}$ and occurred at $D = 0.43\ h^{-1}$. As a result, much higher levels of viable cell (4.76 times) and lactic acid productivity (2.76 times) were obtained using continuous culture than batch culture.

CONCLUSION

From the test tube culture experiment it was found that the viable cell count of *Lactobacillus crispatus* KLB46 did not increase proportionally with increased glucose or yeast extract concentration and that the maximum viable cell count was obtained at 37°C and initial medium pH 5.5. During batch cultivation, the highest viable cell count was obtained at pH 5.5, and this was 1.9×10^9. *L. crispatus* KLB 46 did not grow well at pH 3.5 and 7.5, however, high viable cell counts were obtained over a broad pH range (between 4.5 and 6.5). In continuous culture, the maximum productivity of viable cell was 7.33×10^{11} CFU $L^{-1} h^{-1}$ that is 4.76 times higher than that obtained from batch culture.

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