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In Vitro Proliferation Model of *Helicobacter pylori* Required for Large-Scale Cultivation

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Abstract The composition of dissolved gases and nutrients in a liquid medium were determined for establishment of the optimum conditions for *in vitro* culture of *Helicobacter pylori*. A microaerobic condition favored by the organism was prepared by adjusting the partial pressure of the gas, agitation speed, and viscosity of the medium. The gaseous concentrations were controlled by utilizing CampyPak Plus that reduced oxygen while augmenting carbon dioxide. Agitation of the broth facilitated the oxygen transfer to the cells, yet inhibited the growth at high rates. An increase of viscosity in the medium repressed the culture although this variable was relatively insignificant. The chemical constituents of the liquid broth were examined to establish an economic model for *H. pylori* cultivation. The microbe required a neutral pH for optimum growth, and yet was also able to proliferate in an acidic condition, presumably by releasing the acidity-modulating enzyme, urease. Cyclodextrin and casamino acid were investigated as growth enhancers in place of serum, while yeast extract unexpectedly inhibited the cells. A low concentration of glucose, the unique carbon source for the organism, increased the cell density, yet high concentrations resulted in an adverse effect. Under optimally dissolved gas conditions, the cell concentration in brucella broth supplemented with serum substitutes and glucose reached 1.6×10^8 viable cells/ml which was approximately 50% higher than that obtained in the liquid medium added with only cyclodextrin or serum.

Key words: *H. pylori*, *In vitro* culture, optimization, oxygen tension, serum substitutes

A spiral microorganism found in the stomach tissues of patients with gastritis was first successfully cultivated *in vitro* in 1982 and turned out to be a species belonging to a new genus, *Helicobacter pylori* [5, 9]. The microbe showed a Gram-negative reaction and grew only under microaerobic conditions. Its spiral morphology facilitated an active movement in the mucosal layers of the tissues, yet tended to readily transform to a nonreproductive coccoid form that enabled the bacterium to survive in natural circumstances (e.g., water) for more than one year [9]. The dissemination route of *H. pylori* to humans is uncertain, however, the propagation seems to occur mainly via unsanitary drinking water and food preparation [5]. For such reasons, the infection rate is significantly higher, particularly among children (60 to 70%), in developing countries compared with developed nations.

Peptic ulcers associated with *H. pylori* appear in 10% of the world's population at least once in a lifetime, and the organism is typically detected in more than 80% of patients with duodenal ulcer [23]. It has also been reported that patients infected by the bacterium acquire one or more chronic gastritis symptoms almost without exception, and probabilities of transition to peptic ulcers and gastric cancers are 15 to 20% and 0.01 to 0.1%, respectively [10]. If ulcer patients are medicated with antibiotics, the *H. pylori* infection can be cured and recurrence of the disease is markedly lowered [17], suggesting a close relationship between peptic ulcers and *H. pylori* infection.

H. pylori proliferates (10^7 to 10^9 cells) in the gastric mucosa without adhesion to the surfaces and the infection provokes host responses. Because the inside of the stomach is strongly acidic (pH 2) [5, 17], commonly circulating bacteria such as *Escherichia coli* cannot survive in such an environment. However, *H. pylori* expresses the enzyme urease that neutralizes the local environment by raising the pH as a result of the catalytic conversion of urea to ammonia and carbon dioxide, and thus it prepares its own habitat for growth [5, 17]. The microbe under the stressed condition also produces a large amount of P-type ATPase necessary for the active transport of essential nutrients into the cell [29]. Such an organism residing in the mucosal layers encodes cytotoxin that causes formation of vacuoles on the tissues and eventually an inflammatory reaction of the host, i.e., gastritis [5, 15]. Meanwhile, the immune system is activated to produce antibodies that specifically bind to these foreign molecules. Although the binders

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cannot eliminate the bacterial cells, they are useful markers for a blood test to detect the invasion of *H. pylori* [5, 20].

To diminish risk factors causing ulcers and gastric cancers, the detection of *H. pylori* infection and its subsequent elimination are extremely important, as mentioned above [5, 7, 11, 14]. To these ends, an *in vitro* cultivation model of the microbe was prepared, which is economical and readily manageable by less skilled personnel. This model can be utilized for the production of bacterial antigens, preferably on a large-scale, as well as for routine tests of therapeutic drugs developed against the infection. Accordingly, this study presents the optimal compositions of gases and liquid media for the batch culture of *H. pylori*.

MATERIALS AND METHODS

Materials

The culture media, brucella agar and broth, casamino acid, and yeast extract were purchased from Difco (Detroit, U.S.A.). The (2,6-di-*O*-methyl)-β-cyclodextrin (CD), cefsulodin, vancomycin, trimethoprim, amphotericin B, glycerol, glucose, and methylcellulose were obtained from Sigma (St. Louis, U.S.A.). IsoVitalex and CampyPak Plus were purchased from BBL Microbiology Systems (Cockeyville, U.S.A.). The fetal calf serum and bacterium *H. pylori* (cell line 43504) were obtained from Gibco (Grand Island, U.S.A.) and ATCC (Rockville, U.S.A.), respectively.

Culture Medium

The basal liquid medium was prepared by dissolving 28 g/l brucella broth in deionized water, adding 10 ml/l IsoVitalex and 2 g/l CD, and then sterilizing the mixture in an autoclave at 121°C for 15 min. The solid agar medium was made of 43 g/l brucella agar dissolved in deionized water containing 10 ml/l IsoVitalex, and 50 ml/l calf serum was then added after sterilization. All media contained antibiotics, 6 mg/l cefsulodin, 5 mg/l vancomycin, 10 mg/l trimethoprim, and 5 mg/l amphotericin B, and were adjusted to pH 7.0 with 1 M NaOH.

Cell Stocks

H. pylori cells in lyophilized form were suspended in the brucella liquid broth, and the suspension was spread on the surface of the solid agar plate supplemented with 1 g/l CD. The cells were grown in an incubator (Model VS-9011C, Vision, Korea) maintaining 10% CO_2 at 37°C for 6 days. The cell colonies were collected, resuspended in the brucella liquid broth, respread on the solid medium, and then further cultivated for 5 days under the same condition. After harvesting, the cells were transferred into the brucella broth supplemented with 20% (v/v) glycerol, 50 ml/l calf serum, and 1 g/l CD. The suspension was then divided into 1 ml each aliquots and cryopreserved in a liquid nitrogen tank.

Preparation of Culture Inoculum

The cell stock was quickly thawed in a water bath maintained at 37°C, spread on the solid agar, and subsequently cultured under 10% CO_2 at 37°C for 4 days. The harvested cells were further grown in an Erlenmeyer flask (100 ml capacity) containing 20 ml of basal liquid medium. The flask was placed in a sealed chamber including CampyPak Plus to maintain a microaerobic condition [2, 19, 23], and incubated at 37°C in a shaker (Model SI-4000R, Jeiotech, Korea) with a speed of 100 rpm for 3 days. For the proliferation tests described below, 2 ml of the cell suspension was immediately inoculated into 18 ml of fresh broth, cultured at 37°C in triplicate, and the mean was used for plotting.

Concentration of Dissolved Oxygen

The dissolved oxygen tension in the liquid broth was varied by controlling the following three parameters: composition of the gas, agitation speed, and liquid viscosity. To examine the gaseous composition, the flask containing the basal broth inoculated with the cells was incubated under ambient air, an environment of 10% CO_2 balanced with air, or adjusted by CampyPak Plus that provided 5 to 15% O_2 and 5 to 12% CO_2 depending on the chamber volume. The effect of the agitation speed was tested by placing the culture flask in the chamber with CampyPak Plus and then shaking at different rates (0 to 150 rpm). To investigate the effect of viscosity, a selected modulator, methylcellulose (15 cP for 2% solution), at different concentrations (final 0

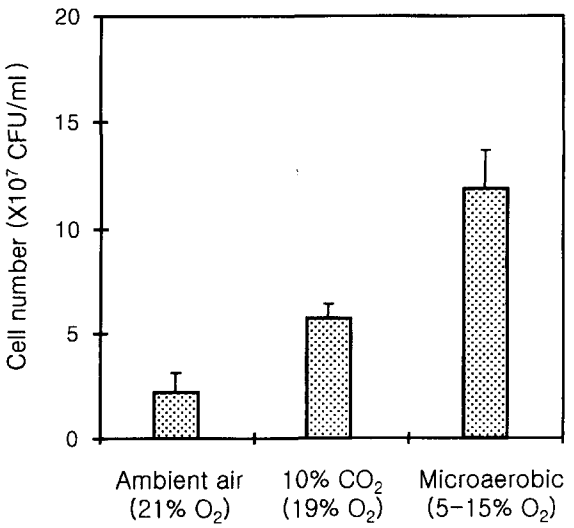

Fig. 1. Effect of gaseous composition on growth of *H. pylori*. When compared to air, the CO_2 concentration was increased to 10% by culturing the microbe in an incubator. The microaerobic condition was prepared by utilizing CampyPak Plus. Standard deviations about the mean of triplicate measurements under each condition are shown.

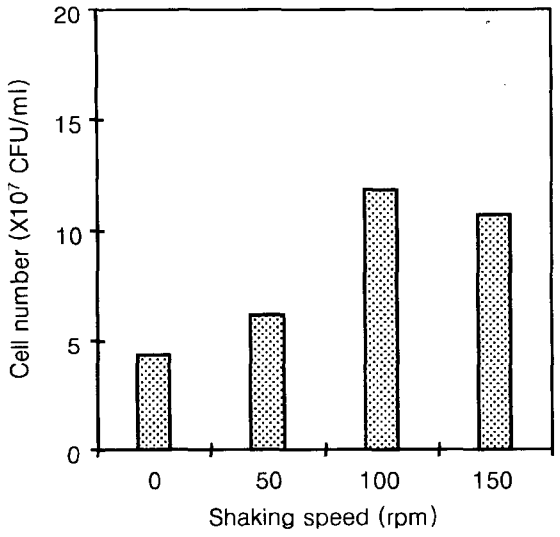

Fig. 2. Determination of the optimal shaking speed of incubator used for *H. pylori* cultivation.
The speed was measured in revolutions per min (rpm).

to 4%) was added into the basal broth, and the cells were cultured under optimal conditions for the other factors (i.e., in the presence of CampyPak Plus and agitation of 100 rpm; see Figs. 1 and 2).

For monitoring the bacterial growth, a method of colony counting was applied to each sample. The cells drawn from the flask were diluted with 10 mM phosphate buffer, pH 7.2, to a range of 10^2 to 10^3 cells/ml, and 100 µl of each suspension was then spread onto solid agar plates. The inoculated plates were incubated with 10% CO_2 at 37°C for 4 days, and the colonies formed were counted by microscopic examination of the cell morphology. *H. pylori* were further identified by means of Gram staining and an urease activity test.

Chemical Composition of Culture Medium

The pH and nutrients, such as growth factors and glucose as carbon source, were adjusted for a liquid broth with an optimal composition for *H. pylori* growth. The initial pH of the basal medium (the brucella broth containing IsoVitalex and CD) was varied within a range of 5 to 9 using 1 M HCl or 1 M NaOH. The inocula were then added to the respective broth and cultured at an agitation speed of 100 rpm under optimal oxygen tension in the liquid. For testing the neutralization ability of urease, the cells were initially grown under neutral pH for 1 day and the acidity was subsequently varied to a range of pH 3 to 7 by employing 1 M HCl. The cultures were continued for an another day after the addition of 30% sterilized urea (final 3 mmol/l). The pH change was monitored and the cell concentration was also quantified as mentioned above. In the absence of CD, fetal calf serum was added as a growth factor source in various concentrations (final 0 to 15%). The cells were then cultured under the same oxygenation conditions as those for the pH tests. As a substitute for serum, CD was supplemented in the broth at a final concentration of 2 g/l, i.e., the basal medium, and its effect on cell proliferation was compared with serum. Other potential alternatives, such as casamino acid and yeast extract, were also examined by separately adding them into the basal medium in different concentrations (0 to 10 g/l). Finally, the broth was supplemented with extra amounts of glucose (0 to 10 g/l) and the effect on the cell density was determined by the method of colony counting as described above.

Batch Growth Curves

To determine the time of termination and compare the growth curves of each culture, *H. pylori* was grown in defined broth conditions, and the cell densities were measured at regular intervals. The cells were inoculated into four different media: brucella broth containing 10 ml/l IsoVitalex, the same medium supplemented with 10% serum, brucella broth with 10 ml/l IsoVitalex and 2 g/l CD (the basal medium), and a modified basal medium with the addition of 2 g/l casamino acid and 4 g/l glucose. The cells were grown in a chamber with CampyPak Plus, and placed in an incubator maintained at 37°C with a shaking speed of 100 rpm. Fifty milliliters of each sample were removed from the culture flasks at predetermined times and analyzed for viable cells, i.e., colony counting, as mentioned earlier. The cell densities measured in each medium were plotted relative to the time course.

Results and Discussion

A successful preparation of an *in vitro* proliferation model for *H. pylori* would accelerate studies on its pathologic mechanism, vaccine production, the diagnostic monitoring of its spread, and development of therapeutic drugs. Unlike most microorganisms, the ulcer-associated bacterium requires highly specific breeding conditions that are similar to those for animal tissue cultivation. As one of the most notable features, it is susceptible to ambient oxygen and can only reside under a low oxygen tension [2, 5, 25], which reflects the environment of the primary habitat of *H. pylori*, i.e., the gastric mucosa of humans. Secondly, the microbe requires serum components as part of the nutrients needed for its survival and growth [1, 22, 25, 28]. Such a requirement is probably related to the fact that it proliferates in the mucosal layer of the human stomach and produces cytotoxins, thus irritating the surfaces. Other variables, for example, shear force of liquid agitation, may also change the effectiveness of the microbial culture.

Accordingly, the identification of the rate-limiting factors and determination of optimal growth conditions were systematically investigated to establish a *H. pylori* model

culture. Since the conditions may vary according to experimental settings, the model was analyzed in terms of two essential factors: dissolved oxygen tension and chemical composition of liquid medium.

Concentration of Dissolved Oxygen

Because the organism can only grow under microaerobic conditions, the dissolved oxygen (DO) concentration in the liquid medium should be adjusted. The major variables controlling DO at a constant temperature are a partial pressure of the oxygen in the gas phase, agitation speed of the medium, and its fluid properties such as viscosity [4]. The effects of all these variables on the cell growth were tested in a selecte culture flask.

Partial Pressure of Gas

In a homogeneous liquid culture, the DO concentration depends primarily on the partial pressure of the gaseous oxygen if the reactor vessels selected have a uniform geometry with a constant liquid head. The DO tension was varied by culturing the organism under ambient air (21% O_2), a 10% CO_2 environment (approximately, 19% O_2), or controlled conditions with CampyPak Plus to obtain reduced oxygen and increased CO_2 (5 to 15% O_2 and 5 to 12% CO_2 according to the information provided by the manufacturer) (Fig. 1). If the culture was exposed to the ambient atmosphere, the cells proliferated poorly as expected, and morphological transformation from spiral to cocci form occurred as an indication of degeneration [2]. In an incubator maintained at 10% CO_2, the growth was substantially improved. This effect probably resulted from the increase in the CO_2 concentration rather than the decrease in oxygen since there was only a minimal change in the gaseous composition of oxygen. An enhanced yield of cell production was achieved (about double) by providing both gas concentrations adequately adjusted using CampyPak Plus.

These results demonstrated that *H. pylori* can proliferate at a decent rate in a low oxygen tension as well as in a high CO_2. In fact, the natural habitat of the microbe, the gastric mucosa, is exposed to 5% oxygen [5]. Oxygen essentially acts as a terminal electron acceptor in the respiration of *H. pylori*, yet it becomes a poison at higher levels [25]. It should be noted that some microorganisms may demand CO_2 (in the form of bicarbonate) for their growth, and *H. pylori* also favors a CO_2-rich environment [25].

Agitation Speed

Oxygen dissolved at the gas-liquid interface diffuses to cells for consumption, and this transfer can be facilitated by agitation. To examine the effect of the oxygen supply rate, the shaking speed of the incubator with a constant gaseous composition using CampyPak Plus was increased until a growth inhibition was measured (Fig. 2). The cell density increased proportionally with the agitation velocity up to 100 rpm, compared to the cultivation at a stationary setting (no shaking) and thereafter the microbial growth deteriorated. Accordingly, stirring under oxygen deficiency accelerated its supply to cells under cultivation. However, when the supply exceeded the demand of the cells, continuous agitation appeared to result in DO accumulation and consequently cell growth inhibition. In this culture, the above-mentioned morphological transformation of the cells was observed before termination, which indicated that the stationary phase indeed came earlier. The degeneration may also have been caused by an increase in the stirring-invoked shear force that also suppresses the growth of the cells.

Viscosity of Liquid Medium

The viscosity of the culture medium can alter *H. pylori* cultivation, depending on two opposite effects: a high viscosity can either retard the molecular diffusion rate of the oxygen [8] or stimulate its mobile activity. Since it has been previously reported that a viscous medium, as in the mucosal layer of the stomach, produced an active movement of the microbe [12], the viscosity effects were tested by adding an inert modulator, methylcellulose [19], into the liquid medium (Fig. 3). The cell density in the culture decreased in response to even a slight increase of the viscosity, and only a minimal change in the density occurred as the viscosity further increased. In the viscous media, the morphological conversion of the organism to the coccal form recurred as an indication of deterioration. Therefore, a viscous medium seemed to limit the oxygen transfer rather than stimulate the cellular activity under the conditions used.

Based on the above results, *H. pylori* proliferation was found to be extremely sensitive to the gaseous oxygen

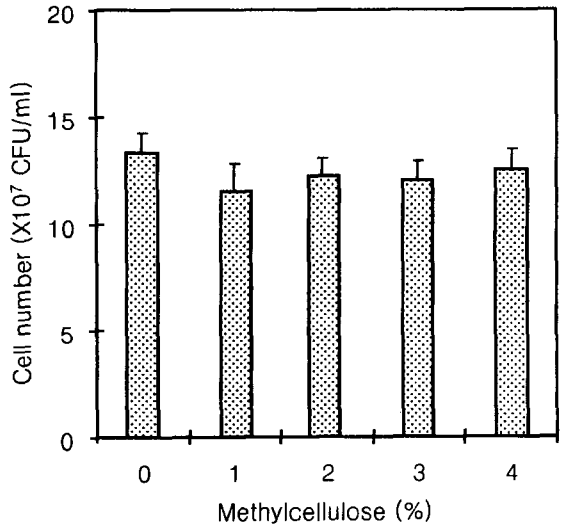

Fig. 3. Variation of medium viscosity to mimic the gastric mucosa where *H. pylori* naturally resides.
The viscosity was modulated by changing the concentration of methylcellulose.

concentration and agitation-induced mass transfer, yet relatively insensitive to the viscosity variation in the liquid medium. To determine the optimal conditions, the chemical composition of the medium was also varied to identify any additional rate-controlling variables and to examine potential substitutes for the complex nutrient (e.g., animal serum).

Chemical Composition of Culture Medium

It has been reported that *H. pylori* survives in the human stomach and requires animal serum for proliferation [1, 22, 25, 28]. Because the site that *H. pylori* populates is acidic, the organism must be able to either withstand an acidic environment or control the pH condition. Thus, it is important to determine the optimal pH for growth and also to clarify the survival mechanism in acid. Animal serum as a nutrient is undefined in terms of its supporting components and consequently may result in a variation in the cultures. It is also relatively expensive, and usually causes impurities in the products recovered and purified from cells after cultivation. To overcome such potential problems, culture conditions were defined to provide an *in vitro* model for *H. pylori* growth.

pH Condition

To determine the pH effect on the growth of *H. pylori*, the initial pH of the medium was varied by adding either acid or base (Fig. 4). When cultured for a constant period, the cell density attained a maximum at a neutral pH below which the curved-rod cell morphology was preserved during cultivation. Whenever the pH decreased or increased, the cellular concentrations significantly reduced in proportion to the degree of deviation from neutrality. Under such suboptimal conditions, the cell morphology transformed from a spiral shape to an inactive cocci form. These results indicate that the microbe cannot proliferate by itself under the stressed conditions imposed by acidity.

Table 1. Responses of *H. pylori* to pH variation during cultivation in the absence or presence of urea.

Culture conditions	Cell number ($\times 10^7$ cfu/ml),	Final pH
pH 3 no urea	9.7	4.33
3 mmol/l urea	9.3	5.32
pH 5 no urea	8.2	6.08
3 mmol/l urea	10.0	6.60
pH 7 no urea	11.8	7.32
3 mmol/l urea	9.5	7.38

The pH was shifted as indicated after growing the microbe at an optimal pH for 24 h.

H. pylori is a microorganism that naturally expresses the enzyme urease [18], and raises the pH as a result of the catalytic reaction on the substrate, urea [5, 25]. To examine the role of urease in an *in vitro* culture, the stomach conditions were mimicked by adding urea (approximately, 3 mmol/l) to the medium and shifting the pH to acidic conditions after cultivating the cells for a certain period under optimal conditions. The cell numbers obtained at various pH conditions did not show a correlation between the two variables regardless of the presence of urea (Table 1). However, the final pH of the acidic cultures containing urea was elevated by 0.5 to 1.0 unit, in proportion to the acidity, as compared to those without the substrate. Based on the results thus far obtained, it was concluded that *H. pylori* would survive in an acidic pH, probably either via the cellular transformation to a resting state (e.g.,

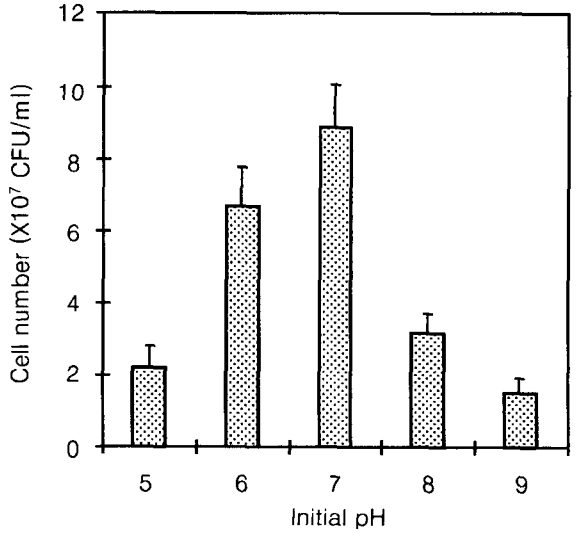

Fig. 4. Effect of initial pH of cultivation on growth of *H. pylori*. Triplicate determinations were carried out and the standard deviation from the average was evaluated.

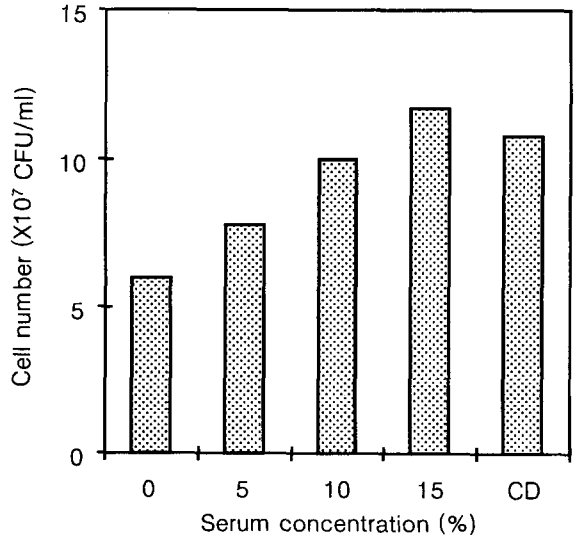

Fig. 5. Performance comparison between serum as nutritional component for growth of *H. pylori* and its substitute, cyclodextrin (CD, 2 g/l in medium).

coccal form) or by the release of the urease to maintain a neutral pH that is an optimal condition for the organism.

Serum Substitutes

Another notable characteristic in *H. pylori* cultivation is the requirement of animal serum for nutrient, similar to that of animal cells [1, 22, 25, 28]. Serum provides the components needed for cellular growth (e.g., carbon, nitrogen, lipid, growth factors) and also controls physico-chemical conditions (e.g., osmotic pressure, pH, protease activity) [6]. In spite of these significant effects on the microbe, serum is costly, and may also contain contaminants in products fractionated from the culture medium. Accordingly, efforts have been made to search for potential substitutes [13, 22].

CD was examined as a substitute for serum and its performance tested in *H. pylori* cultures (Fig. 5). In proportion to the concentration of serum as the control, the cell density increased within the range selected. When the nutrient in the medium was replaced by CD (0.2% CD in Fig. 5), the cell concentration was substantially higher than that without any supplements, and equivalent or better than the result obtained with 10% serum. Since CD is a cyclic oligosaccharide containing a hydrophobic pocket that can bind toxic molecules and also protects cells from mechanical stress induced by agitation [22], CD could be an effective alternative for serum, providing additional economic benefits in cultivating the cells, and possibly simplifying the recovery of the products.

A cell culture can be further improved by supplementing with the essential trace elements, i.e., growth factors such as vitamins, certain amino acids, fatty acids, and serum components [3]. Typical sources widely used for the cultivation of microbial cells are yeast extract and casamino acid, the acid hydrolysates of casein. These two materials were also tested as growth factors in the medium for growing *H. pylori* (Fig. 6). In the case of casamino acid, there was an optimal concentration range (2 to 4 g/l) yielding a maximum 30% increase in the cell density as compared to that without the growth factor (Fig. 6, left). In contrast, the inclusion of yeast extract in the medium inhibited the cell growth, the degree of which was directly proportional to the quantity added (Fig. 6, right).

The two growth factors used were quite different in the composition of some of their constituents (data provided by Sheffield Chemical Co. and Ohly, Inc.). In particular, casamino acid was comprised of high concentrations of aspartic acid and NaCl. According to our experimental results (data not shown), the cell density increased proportionally to the content of aspartic acid up to 4 g/l casamino acid although the effect was relatively inferior. In contrast, the density decreased in the presence of NaCl higher than the concentration equivalent to 2 g/l casamino acid, presumably due to osmotic cell lysis. It is thus conceivable that casamino acid containing aspartic acid and NaCl acted in opposite with each other on the micobial growth depending on its concentration in the medium (see Fig. 6, left). For the yeast extract, there was no apparent explanation for its inhibitory effect on the culture except for a possible inherent toxicity to the microorganism.

Glucose as Carbon Source

An analysis of the *H. pylori* genome suggests that glucose is its only source of carbohydrate as well as the main substance for substrate-level phosphorylation [26]. The substrate may also help the cells to retain improved abilities in controlling the pH of the medium and resisting against cell lysis [16]. To establish optimal culture conditions, the amount of glucose was increased beyond that present in the basal medium (1 g/l glucose; Fig. 7). The supplemented glucose in relatively low concentrations contributed a

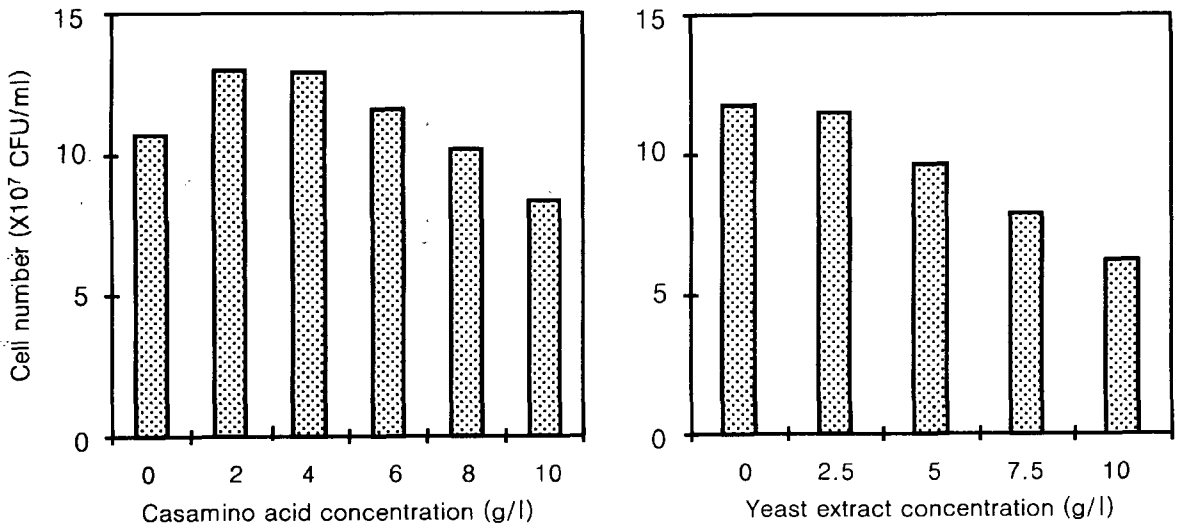

Fig. 6. Effects of casamino acid and yeast extract as growth factors on the proliferation of *H. pylori*.

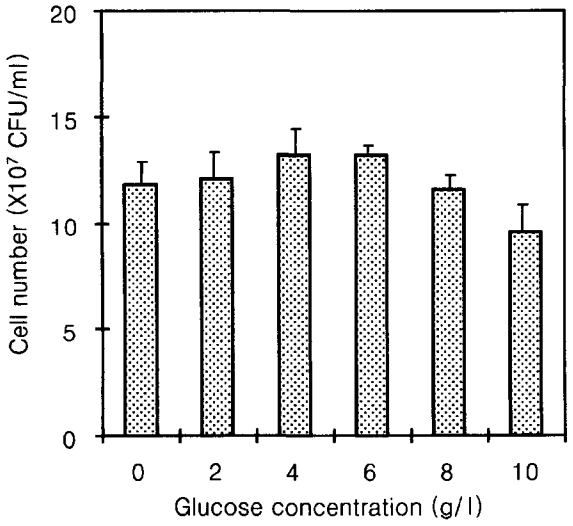

Fig. 7. Determination of the optimal concentration of glucose based on cell proliferation.

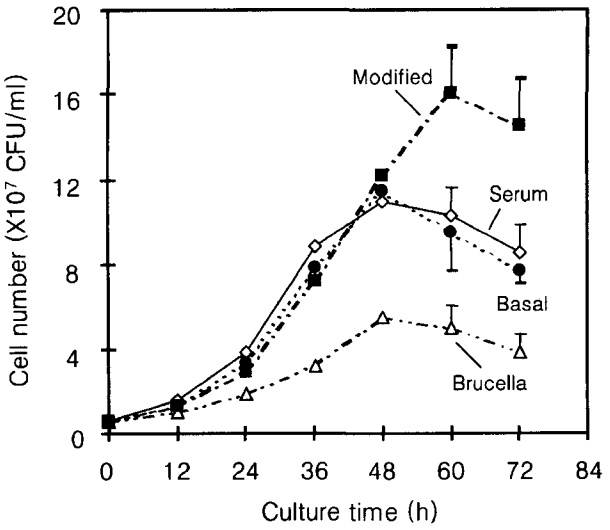

Fig. 8. Comparison of batch growth curves from *H. pylori* cultures under different medium conditions.
Standard deviation of triplicate measurements is indicated.

maximum 8% increase in the cell density. However, the culture abruptly declined with a higher concentration range of the nutritional source, which indicated an adverse effect of the substrate via catabolic repression [27]. Therefore, glucose should only be intermittently supplied to maintain a low concentration, if a prolonged culture is desired [16].

Based on the above results, it was concluded that *H. pylori* survives under acidic conditions by expressing an acidity-modulating enzyme, urease, rather than adapting itself to the environment. CD and casamino acid were feasible substitutes for animal serum, whereas yeast extract, normally used in many fermentations, produced an adverse effect under the conditions used. Glucose, as the sole carbon source for *H. pylori*, enhanced the cell growth at relatively low substrate concentrations, yet inhibited it at high concentrations.

Batch Growth Curve

H. pylori is a sensitive microbe that undergoes a rapid morphological transformation upon reaching the stationary phase, as previously mentioned [2]. Such degeneration causes a decrease in the amount of urease produced as well as a low survival rate in their subcultures. Thus, the cultivation in a batch mode should be terminated after an appropriate time period before a variation in the cellular shape predominates. To determine the optimal conditions, batch growth curves of the organism cultivated in four liquid media with different nutrient compositions were obtained (Fig. 8): brucella broth containing IsoVitalex (Brucella), the same medium supplemented with CD (Basal) or serum (Serum), and the basal medium containing casamino acid and glucose (Modified). The cell densities reached their maxima after 48-h cultivation except in the modified medium and immediately decreased thereafter. Such a phase change could be caused by a deficiency in the carbon source and/or the accumulation of ammonium ions released by cell lysis. When the highest values attained with each medium were compared, the addition of CD or animal serum resulted in about a 2-fold increase in the cell density. Under the modified culture condition, the growth continued for up to 60 h without deterioration and an approximately 50% extra cell proliferation was achieved by the supplementation of casamino acid and glucose in addition to CD. It was noted that the animal serum facilitated the cell growth only during the initial stage of the culture.

In conclusion, the *in vitro* proliferation conditions of *H. pylori* were determined by optimizing the oxygen tension and chemical constituents in the liquid medium, which potentially provides valuable data in designing and performing large-scale fermentation. First, the gas injected into the fermenter should contain a relatively low level of oxygen and high carbon dioxide compared with the ambient air that has been conventionally used for microbial growth. Second, since the organism possesses an acid-neutralizing ability, the control of the medium pH by the addition of base is not required. Third, the expensive and complex animal serum component can be replaced in the medium by defined substances such as CD and casamino acid. Finally, it is necessary to intermittently or continuously feed a low concentration of glucose, the sole carbon source, into the fermenter in order to sustain high cell culture density.

Acknowledgments

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REFERENCES

1. Albertson, N., I. Wenngren, and J. E. Sjostrom. 1998. Growth and survival of *Helicobacter pylori* in defined medium and susceptibility to Brij 78. *J. Clin. Microbiol.* **36:** 1232-1235.
2. Andersen, A. P., D. A. Elliott, M. Lawson, B. Peter, V. B. Hatcher, and E. G. Puszkin. 1997. Growth and morphological transformations of *Helicobacter pylori* in broth media. *J. Clin. Microbiol.* **35:** 2918-2922.
3. Atlas, R. M. 1993. *Handbook of Microbiological Media*, pp. 4-5. CRC press, Boca Raton, U.S.A.
4. Bailey, J. E. and D. F. Ollis. 1986. *Biochemical Engineering Fundamentals*, pp. 457-532. 2nd ed. McGraw-Hill Book Co., New York, U.S.A.
5. Blaser, M. J. 1996. The bacteria behind ulcers. *Sci. Amer.* **February:** 92-97.
6. Choi, T. B. 1989. Media design and development of serum-free media, pp. 1-30. *In* Dept. of Biotechnology, KAIST (ed.), *Principles of Animal Cell Reactor Engineering and Their Applications*, KAIST, Seoul, Korea.
7. Craanen, M. E., P. Blok, W. Dekker, and G. N. J. Tytgat. 1994. *Helicobacter pylori* and early gastric cancer. *Gut* **35:** 1372-1374.
8. Geankoplis, C. J. 1982. *Transport Process and Unit Operations*, pp. 371-414. 2nd ed. Allyn and Bacon Inc., Boston, U.S.A.
9. Goodwin, S. 1993. Historical and microbiological perspectives, pp. 1-10. *In* T. C. Northfield, M. Mendall, and P. M. Goggin (eds.), *Helicobacter pylori Infection*, Kluwer Academic Publishers, Lancaster, U.S.A.
10. Graham, D. Y., K. Kimura, T. Shimoyama, and T. Takemoto. 1994. *Helicobacter pylori* infection in Japan: current status and future options. *Eur. J. Gastroenterol. Hepatol.* **6:** S1-S4.
11. Harris, P. R., H. L. T. Mobley, G. I. Perez-Perez, M. J. Blaser, and P. D. Smith. 1996. *Helicobacter pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology* **111:** 419-425.
12. Hazell, S. L., A. Lee, L. Brady, and W. Hennessy. 1986. *Campylobacter pyloridis* and gastritis: Association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. *J. Infect. Dis.* **153:** 658-663.
13. Ki, M.-R., S.-K. Yun, W.-J. Lim, B.-S. Hong, and S.-Y. Hwang. 1999. Synergistic inhibition of membrane ATPase and cell growth of *Helicobacter pylori* by ATPase inhibitors. *J. Microbiol. Biotechnol.* **9:** 414-421.
14. Lee, A., J. Fox, and S. Hazell. 1993. Pathogenicity of *Helicobacter pylori*: A perspective. *Infect. Immun.* **61:** 1601-1610.
15. Leunk, R. D. 1991. Production of a cytotoxin by *Helicobacter pylori*. *Rev. Infect. Dis.* **13:** S683-S689.
16. Marchini, A., M. Paola, M. Roberto, and O. Roberto. 1994. Optimized conditions for the fermentation of *Helicobacter pylori* and production of vacuolating cytotoxin. *FEMS Microbiol. Lett.* **124:** 55-60.
17. McGowan, C. C., T. L. Cover, and M. J. Blaser. 1996. *Helicobacter pylori* and gastric acid: Biological and therapeutic implications. *Gastroenterology* **110:** 926-938.
18. Mobley, H. L. T. and P. A. Foxall. 1994. *H. pylori* urease, pp. 41-58. *In* R. H. Hunt and G. N. J. Tytgat (eds.), *Helicobacter pylori*, Kluwer Academic Publishers, Lancaster, U.S.A.
19. Myrvik, A. L., J. M. Whitaker, and R. E. Cannon. 1976. The use of cellulose products to reduce agar concentration in microbiological media. *Can. J. Microbiol.* **22:** 1002-1006.
20. Nedenskov, P. 1994. Nutritional requirements for growth of *Helicobacter pylori*. *Appl. Environ. Microbiol.* **60:** 3450-3453.
21. Newell, D. and A. Stacey. 1993. Serology, pp. 139-148. *In* T. C. Northfield, M. Mendall, and P. M. Goggin (eds.), *Helicobacter pylori Infection*, Kluwer Academic Publishers, Lancaster, U.S.A.
22. Olivieri, R., M. Bugnoli, D. Armellini, S. Bianciardi, R. Rappuoli, P. F. Bayeli, L. Abate, E. Esposito, L. D. Gregorio, J. Aziz, C. Basagni, and N. Fugura. 1993. Growth of *Helicobacter pylori* in media containing cyclodextrins. *J. Clin. Microbiol.* **31:** 160-162.
23. Ruws, E. A., W. Langenberg, H. J. Houthoff, H. C. Sanen, and G. N. Tytgat. 1988. *Campylobacter pyloridis* associated chronic active antral gastritis. A prospective study of its prevalence and the effects of antibacterial and antiulcer treatment. *Gastroenterology* **94:** 33-40.
24. Shahamat, M., U. E. H. Mai, C. Paszko-Kolva, H. Yamamoto, and R. R. Colwell. 1991. Evaluation of liquid media for growth of *Helicobacter pylori*. *J. Clin. Microbiol.* **29:** 2835-2837.
25. Stark, R. M., J. Greenman, and R. Millar. 1995. Physiology and biochemistry of *Helicobacter pylori*. *Br. J. Biomed. Sci.* **52:** 282-290.
26. Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388:** 539-547.
27. Wang, D. I. C., C. L. Cooney, A. L. Demain, P. Dunnill, A. E. Humphrey, and M. D. Lilly. 1979. *Fermentation and Enzyme Technology*, pp. 5-6. John Wiley and Sons, Inc., New York, U.S.A.
28. Westblom, T. U., S. Gudipati, and B. R. Midkiff. 1995. Enhanced growth of *Helicobacter pylori* using a liquid medium supplemented with human serum. *Eur. J. Clin. Microbiol. Infect. Dis.* **14:** 155-156.
29. Yun, S.-K. and S.-Y. Hwang. 1997. Expression of latent P-type ATPase and their presumptive roles in cell membrane of *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **7:** 378-385.