Korean J. Limnol. 35 (3): 145~151 (2002)

Effect of an Acid pH Shock on Physiological Changes of *Chlamydomonas acidophila* (Chlorophyta), UTCC 122

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pH 충격에 의한 *Chlamydomonas acidophila* (Chlorophyta), UTCC 122의 생리적 변화에 관한 연구. 이 경 · 기장서[1] · 김세화[2] · 한명수[1] · 최영길[1] · 유광일[1]* (가톨릭대학교 생물학과, [1]한양대학교 생명과학과, [2]용인대학교 생명과학과)

녹조류 *Chlamydomonas acidophia*, UTCC 122 균주를 이용하여 pH 변화에 따른 조류의 생리인 변화를 관찰하였다. 성장률(μ)은 pH 3.7～6.7의 배양에서 0.5～0.7 day^{-1}이었으며(ANOVA, p = 0.134), 점차 세포의 크기가 작아지는 경향을 보였다. pH 2.7의 배양에서는 성장하지 않았으며, 세포의 크기가 급격하게 증가하였다. 배양 1일 후 엽록소 *a*는 191～255 pg $cell^{-1}$이었으나, 5일 째는 pH 2.7 배지에서 210 pg $cell^{-1}$로 큰 변화가 없으며, 다른 pH의 배양에서는 60～103 pg $cell^{-1}$로 감소하였다. 단위세포에 대한 엽록소의 양은 세포 체적과 직접적으로 관련이 있다. Carbonic anhydrase (CA)의 활성도는 1.1～3.7×10^{-4} E.U. mm^{-2}이었으며, pH 2.7 과 pH 5.7배지를 제외하고 점차 증가하는 경향을 보였다. pH 농도 차에 의한 비교에서는 유사한 경향을 보였다. *C. acidophila*의 경우 CA 분자량은 29 kDa이었으며, pH 농도 구배에 의한 발현 차이는 없었다. 이것은 CO_2와 HCO_3^-를 조절하는 CA가 산성에서 수소이온조절에 직접 작용하지 않는 것을 의미한다. 단백질 발현양상은 41과 63 kDa은 pH가 낮은 배지에서 자랄수록 발현이 억제되었으며, 17 kDa 단백질은 점차 증가하였다. 본 연구를 통해, *C. acidophila*는 다른 생물과 달리 넓은 범위의 산성에서 잘 성장할 수 있으며, 낮은 산성에서 자랄수록 17 kDa의 단백질이 증가하는 것은 17 kDa 단백질이 산성에 적응하기 위한 기능을 하는 것으로 추정된다.

Key words : pH, acidophilic, *Clamydomonas acidophila*, physiological parameters, carbonic anhydrase

INTRODUCTION

Low pH and its effects on the biota have been studied in acidic environments, such as acid bogs, mining streams, and acidified lakes. The relationship between the chemical species of dissolved biological available carbon (BAC) and pH is well known. Variation in pH can affect metabolism and growth of algae in a number of ways, including altering the equilibrium of inorganic carbon (C) species, changing availability of nutrients, and directly affecting cell physiology. Most algae have pH optima for growth and photosynthesis in the neutral to alkaline pH range, but species may be found growing in acid conditions as low as pH 1.0 (Raven, 1990; Dorling *et al.*, 1997).

Clamydomonas acidophila occurs in low pH environments and is able to survive under highly acidic conditions. In many field surveys, *C. acidophila* was recorded as dominant species in acid-

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ic waters (Hargreaves *et al*., 1974; Hargreaves and Whitton, 1976; Pentecost, 1982; Sheath *et al*., 1982). A possibility to survive in extremely low pH might be possessed a special membrane structure and regulating proteins persisted efficiently from hydrogen attacks. Brock (1973) found eukaryotic algae, but not blue-green algae, in habitats below pH 4 or 5, thus implicating cell or plastid membrane function in withstanding H^+ ion. Tatsuzawa and Takizawa (1996) also demonstrated that a cell-surface barrier of *Chlamydomonas*, increasing the saturation of fatty acids in membrane lipids and decreasing membrane lipid fluidity, was extremely impermeable to protons, allowing them to live at low pH values.

The membrane structures have been well studied with acid-adaptation in acidophilic organisms, and acid shock proteins (ASP) in bacteria have been worked (Bradley *et al*., 1998; Browne and Dowds, 2002). However, with emphasis of proteins of algae less work has been done. Especially periplasmatic proteins, contacted with ambient high hydrogen ion might be have specialized properties to maintain their forms than that at other organells, since proteins do denature at acid pH. Evidently, Cassin (1974) reported that cell wall and cell membrane proteins of *C. acidophila* were resistant to H^+ at low pH. He suggested that membrane proteins of acid-tolerant organisms might perhaps lave a very low isoelectric point.

The studies on algal physiology and protein for acid adaptation were required with well-buffered medium for persisting in its original pH value during culture, because the pH change in the culture medium was frequently encountered in plant cell culture. For this reason, a few works have been done in the culture related with acid adaptation.

In this work, we studied the physiological parameters of *Clamydomonas acidophila* in acid environment, investigating growth rate, cell volume, pigments content, and protein profiles. Furthermore, as the formation of photosynthetic available carbon is influenced from hydrogen ions, carbon uptake-related protein, carbonic anhydrase (CA) suspected hydrogen ion regulating functions was studied.

MATERIALS AND METHODS

The medium used for experiments is AM (Cassin, 1974), adding to final concentration 10 mM of MES (M8250, Sigma) for pH buffer. Previously prepared chemical stocks but vitamins sterilized with 0.2 μm syringe filter were diluted final concentration, pH adjustments were made on a Orion, Model 920A equipped with a electrode, autoclaved for 20 min, 121°C, and added vitamins to them when they were cooled. The final volume of media in all experiments were 100 ml in 300 ml erlenmeyer flask. The flasks were sealed with silicon stopper for gas exchange and autoclaved for 20 min, 121°C. Growth condition is 20°C, light intensity of 30 μmol m^{-2} s^{-1}, and 12 : 12 (L : D).

Axenic cultures of *Chlamydomonas acidophila*, UTCC 122 was received from the University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC). Each three 300 ml erlenmeyer flasks containing 100 ml AM adjusted pH values to 2.7, 3.7, 4.7, 5.7, 6.7, and 7.7, respectively were prepared. The initial cell density in experimental flasks was calculated to be 4×10^4 cells ml^{-1} of exponential phase reaching cells.

Growth rate (μ) was determined from the increase of optical density at 550 nm and cell numbers calculated microscopically within 24 hr, using a spectrophotometer and a haemocytometer. Daily triplicate optical density measurement was performed on each culture and sometimes microscopic counts to compare each other.

Chlorophyll *a* content was determined with Jeffery and Humphry method (Jeffery and Humphry, 1975). Pigment extracts in 1 ml cultures added to 9 ml of 100% acetone. Store in 4°C dark room for 24 hr. Chlorophyll *a* was determined spectrophotometically.

Cell volume was measured under the light microscope. Approximately 20 cells were measured their size and calculated by applying cellular formula for solid geometric shape of prorate spheroid (Hillebrand *et al*., 1999).

The carbonic anhydrase (CA) activity was measured based on the rate of pH decrease following the addition of CO_2 into a cuvette containing buffer (Mercado *et al*., 1997). The time required for a drop of 0.4 pH units in the pH range from 8.4 to 7.4 was measured at 4°C, using a cuvette containing 3 ml of sample buffer. Identical number of cells were collected, washed and resuspended in the buffer (100 mM Tris, 5 mM Na-EDTA, and 25 mM ascorbic acid). The reaction was started by rapidly introducing 1 ml of ice-cold CO_2-saturated distilled water to 1 ml of the test material

and 1 ml of buffer. One unit of enzyme activity (E.U) was defined as $(t_o/t_c)-1$ where t_o and t_c are the time for pH change of the noncatalyzed and catalyzed reactions, respectively (Haglund *et al*., 1992). This unit relates the enzymetic activity to the spontaneous hydration of CO_2 and was recalculated on algal cell number. External (periplasmatic) CA activity was determined using intact cells washed in the same buffer, or we estimate external CA activity, based on the enhancement of CO_2 diffusive entry, when external CA activity is inhibited by AZ (acetazolamide).

Protein was determined using bovine serum albumin as a standard by the method of Bradford (1976). In one liter volumetric flask, dissolve 100 mg Coomassie brilliant blue G−250 (CBB) in 50 ml of 95% ethanol, and add 100 ml of 85% phosphoric acid, then bring to volume with water. Filter through Whatman No. 1 paper, store at 4°C. Add aliquots of 0.5 mg ml^{-1} (5, 10, 15, 20 μl) into microcentrifuge tube. Bring the volume in each tube to 100 μl with 0.15 M NaCl. Prepare a tube containing only 100 μl of 0.15 M NaCl. Add one ml CBB solution to each tube, vortex, and leave 2 min at room temperature. Measure the 595 using a 1 cm pathlength microcuvette (1 ml). Make a standard curve by plotting absorbance versus protein concentration. Measure 595 of wavelength and determine the protein concentration in the unknown from the BSA standard curve.

For protein preparation, after centrifugation (1,500 g, 5 min), then stored −20°C in deep freezer. In order to analyze, the cells were lysed hypo−osmotically by adding 200 μl (approximately 2 v/v) of extraction buffer (10 mM Tris−HCl pH 7.6, 10 mM phenylmethylsulphonylfluoride (PMSF), 20 mM DTT), sonicating for about 2 min. Protein was precipitated in 70% acetone at −20°C overnight. Proteins were then collected by centrifugation (12,000 g, 20 min). Mixing 100 μl 1 : 1 (v : v) of protein and with cooking buffer (62.5 mM Tris−HCl pH 6.8, 10% glycerol, 1% β−mercaptoethanol, 3% SDS, containing a little bromo phenol blue) in eppendorf tube. Heat sample for 5 min at 95°C in a "float" in a water bath (Golldack *et al*., 1995).

One−dimensional electrophoresis was performed in polyacrylamide gels (stacking gel 5% w/v, separating gel 7.5～15% w/v linear gradient) (Laemmli, 1970). Identical amounts of proteins were loaded in lane on the gels. After fixing, the gels were stained with CBB. Protein size makers were electrophorized for calculations of apparent molecular masses.

Proteins separated by SDS−PAGE were transferred to nitrocellulose paper. The membranes were blocked for 1 h with blocking buffer (80 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, 0.2% (v/v) NP40, 50 mM Tris−HCl pH 8.0, containing 5% (w/v) non−fat dry milk) at room temperature, then incubated with blocking buffer containing a monoclonal antibody against CA (1 : 500 dilution) for 1 hr at room temperature (*Coccomyxa* carbonic anhydrase antibodies were a generous gift from T. Hiltonen, Dept. of Plant Physiology, Umeå University, Sweden). After being washed with blocking buffer, the membranes were incubated with anti−rabbit immunoglobulin, peroxidase−linked species−specific antibody (1 : 1000 dilution) in blocking buffer for 1 hr at room temperature. CA was detected with the ECL detection system (RPN 2106, Amersham).

The effect of acid on the growth rate, cell volume, pigment content, and CA activity was tested using one−way ANOVA. When a significant effect was found, means were compared with Duncan post hoc test. Statistical analysis was performed using SPSS 10.0.7 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

AM medium (Cassin, 1974), adding to final concentration 10 mM of MES is suitable for acid buffer and growth of *Chlamydomonas acidophila* (data not shown).

Effects of pH on growth rate are shown in figure 1. Growth rate (μ) was 0.5～0.7 day^{-1} at pH 3.7～6.7, interestingly that of *C. acidophila* was falling to zero at pH 2.7. The significant difference of growth rate was not recorded between pH 3.7 and 6.7 (ANOVA, $p = 0.135$), the range of which was the pH optimum for growth. The optimal pH for growth at different pH was in agreement with previous work (Cassin, 1974), except pH 2.7 was not grown. For implicated reason, below pH 2.7 the defined medium is change of chemical formation influencing results because trace metal solubility and biological available carbon species depend on pH (Chuan *et al*., 1996).

Variations in cell volume of *Chlamydomonas acidophila* were illustrated in figure 2. Two distinct patterns were observed in each culture. Initi-

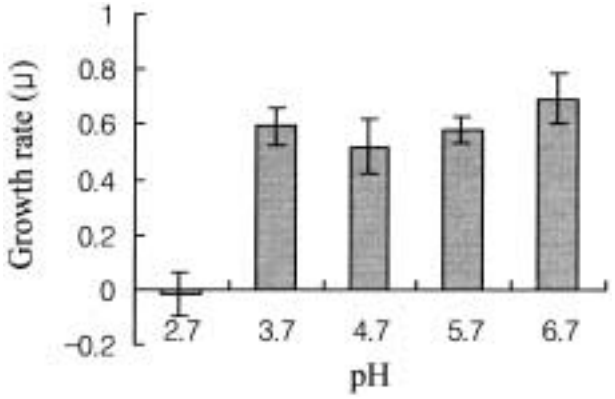

Fig. 1. Effect of pH on the growth rate (μ) of *Chlanydonmonas acidophila* cultured in each medium for 5 days. Error bar = S.D.

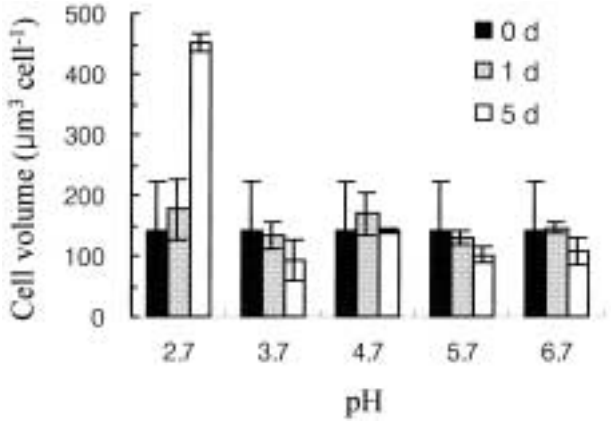

Fig. 2. Effect of pH on cell volume of *Chlanydonmonas acidophila* cultured in each medium for 5 days. Error bar = S.D.

al cell volume of 142.3 μm^3 $cell^{-1}$ increased to 451.1 at pH 2.7 during the culture. Cells cultured for 5 days at pH 2.7 were spherical shape, spore-like, and non-motile forms. In contrast, the cell volume slightly decreased to 92.6~141.7 μm^3 $cell^{-1}$ at pH 3.7 to 6.7, which was not shown any trend and significantly different (ANOVA, $p = 0.252$).

Chlorophyll *a* concentration was 255 ng $cell^{-1}$ as *C. acidophila* was inoculated each medium, after one day a significant change was not recorded in cultures. Chlorophyll contents of cells, however cultured for 5 days rapidly decreased to 29~103 ng $cell^{-1}$ at pH 3.7 to 6.7 (Fig. 3). Variations in chlorophyll content at pH 2.7 remained st-

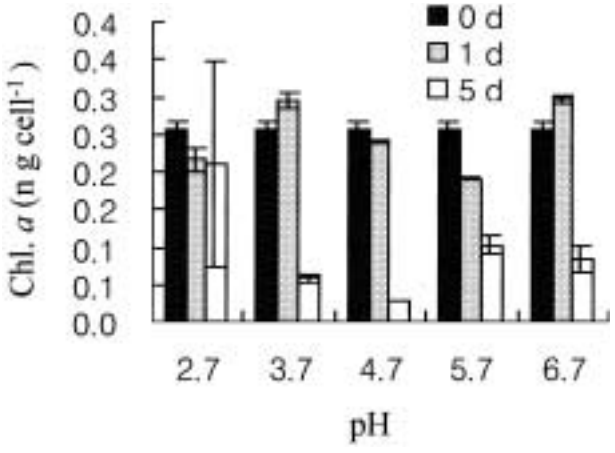

Fig. 3. Effect of pH on chlorophyll *a* concentration of single cell, which calculated with total chlorophyll *a* per cells, cultured in each medium for 5 days. Error bar = S.D.

able by being calculated with average measured value, whereas that in individual level was significantly different (ANOVA, $p < 0.001$). For this reason, Riemann (1989) demonstrated that chlorophyll content of the cultures was lower during nitrogen and phosphorus deficiency. During exponential growth of cultures, nutrients including nitrogen and phosphorus were rapidly consumed for their growth, consequently decreased chlorophyll contents. *C. acidophila* blow pH 2.7, however did not grow, nutrients deficiency might not be occurred.

External carbonic anhydrase (CA) activity was varied from 1.1 to 3.7×10^{-4} E.U. mm^{-2}, showing the gradual increase during culture, except at pH 2.7 and 5.7. The highest CA activity was observed at 5 days culture in pH 3.7 medium (Fig. 4). However there was not found any relationship among the pH gradient cultures. It was thought that the CA activity was less important at acid environment than neutral or alkaline conditions. In fact both *Dunaliella acidophia* and *Phaeocystis* under low inorganic carbon conditions caused a dramatic increase of CA activity, particularly the periplasmatic CA (Gimmler and Slovik, 1995; Elzenga *et al.*, 2000). The majority of the CA activity in *D. acidophila* was found to be localized in membranes. It was in agreement with this results, the increase of CA activity during culture might be caused by low CO_2 concentration. In acid condition, the CA of *Chlamydomo-*

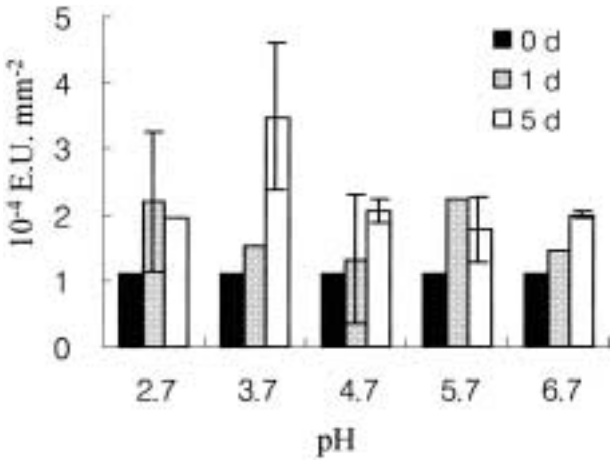

Fig. 4. Effect of pH on the external carbonic anhydrase activity per unit surface area of *Clamydomonas acidophila* cultured in each medium for 5 days. E.U. = Enzyme units. Error bar = S.D.

nas acidophila was not less important for acquiring CO_2, the exact function of the external CA, however remains unclear and it is necessary for further research to clarify their acclimation mechanism to survive in extremely low pH.

A comparison of the protein of total cells of *Chlamydomonas acidophila* in different pH medium during the culture after separation by one–dimensional SDS–PAGE could be seen: some proteins increased, others decreased at different pH. The apparent molecular masses of protein were 17, 41, and 63 kDa. The proteins of 41 and 63 kDa were not or very faintly expressed in low pH cultures, in contrast that of 17 kDa more expressed (Fig. 5). In bacteria, acid stress induced a pH homeostasis system that helped to maintain the internal pH at higher levels than a low external pH. In response to stress, a large set of protective and/ or damage repair proteins is induced (Foster, 1995). In *Salmonella typhimurium*, the survival of pH 3.3 requires the synthesis of acid shock proteins (ASPs), some of which are general stress proteins (Browne and Dowds, 2002). However, no one has identified specific genes required for acidophilic function (Harris, personal commun.), the proteins of algae are not clearly identified. It was suspected that *C. acidophila* expressed the unknown protein of 17 kDa, as their organisms came to acidic environments to survive.

A Western blot analysis with an antiserum raised against the CA, the CA molecular mass of

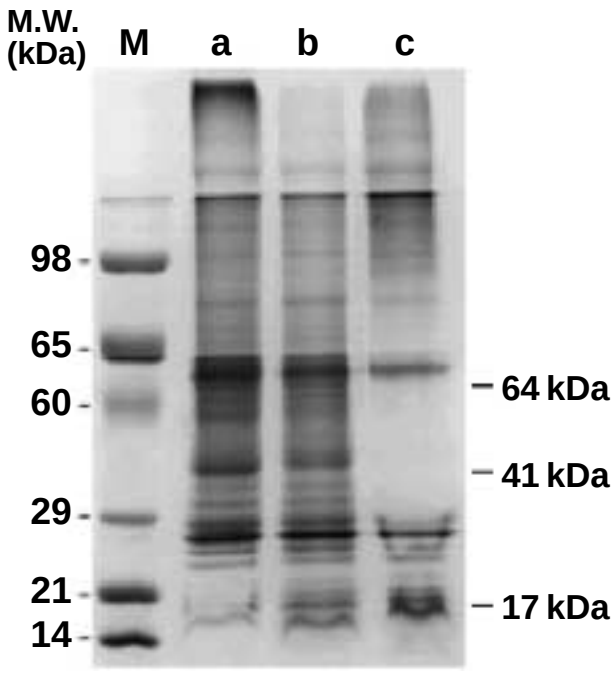

Fig. 5. Coomassie brilliant blue G–250 stained gels separated by SDS–PAGE. Band show total protein extracts from *Chlanydonmonas acidophila* cultured in each medium for 5 days. Lane a (pH 6.7), lane b (pH 4.7), and lane c (pH 2.7). 64 kDa, 41, and 17 kDa protein surprepressed or over–expressed. M = size marker. M.W. = molecular weight.

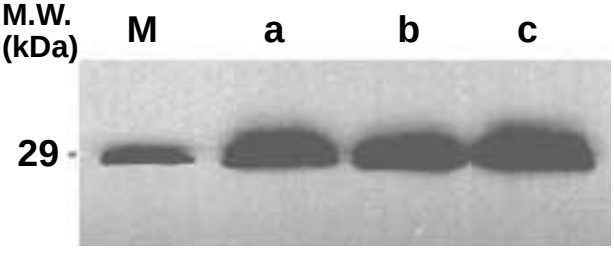

Fig. 6. Immuno–detection of the 29 kDa of carbonic anhydrase extracted cultures of *Chlamydomonas acidophila*. Lane a (pH 2.7), lane b (pH 4.7), and lane c (pH 6.7). M = size marker. M.W. = molecular weight.

Chlamydomonas acidophila was 29 kDa and the concentration of that was same in all acid cultures (Fig. 6). Although It was suggested that CA was hydrogen ion regulating enzyme for survival at extremely low pH, it was not measured any difference change during *C. acidophila* growing at different medium. The CA molecular mass of

C. acidophila was 29 kDa and the concentration of that was identical in all acid cultures. The molecular mass is slightly different compared with organisms, e.g. the surface CA of *Dunaliela parva* was a 32 kDa protein (Husic, 1990; Geib *et al*., 1996), glycosylated CA of *Chlamydomonas* molecular mass of 37 kDa, and higher plants cell express a CA of molecular mass 30 kDa (Roeske *et al*., 1990). The slightly different molecular mass may be due to the amount of glycosylation of CA (Golldack *et al*., 1995).

With a wide range of acid pH, *Chlamydomonas acidophila* can grow optimally, although most algae have pH optima for growth and photosynthesis in the neutral to alkaline pH range (Dorling *et al*., 1997). Interestingly, when *C. acidophila* grow well in low pH, it were not observed apparent variations among the cultures in physiological parameters (chl. *a*, cell volumes, and growth rates). Whereas 17 kDa of protein was over-expressed, which might be used to tolerate in low acid environments, their functions and mechanisms, however, remained unclear. Further studies should be centered on 17 kDa of a uncharacterized protein.

ACKNOWLEDGEMENT

We would like to thank Soo Youn Cho for measuring the carbonic anhydrase activity. We are very grateful to anonymous referees for reading and critical comments. This work was supported by the Institute of Basic Sciences of Hanyang university from the Korea Research Foundation Grant (KRF-1998-015-D00231).

ABSTRACTS

The effect of low pH on physiological changes was studied with the acidophilic green alga, *Clamydomonas acidophila*, UTCC 122. The growth rates (μ) were identical, 0.5 ~ 0.7 day^{-1}, at pH 3.7 ~ 6.7 and no significantly different (ANOVA, p = 0.134), showing cell volume reduced gradually as they were growing, whereas that at pH 2.7 was falling to zero and cell volume increased dramatically. Chlorophyll *a* concentration of the culturs incubated for one day was 191 ~ 255 pg $cell^{-1}$, after then it declined from 60 ~ 103 pg $cell^{-1}$ at pH 3.7 ~ 6.7 except 210 pg $cell^{-1}$ at pH 2.7, which was directly related with cell volume. External carbonic anhydrase (CA) activity was varied from 1.1 to 3.7×10^{-4} E.U. mm^{-2}, showing the gradual increase during culture, except at 2.7 and pH 5.7. However there was not found any relationship among the pH gradient cultures. CA molecular mass of *C. acidophila* was 29 kDa, and concentration of that was identical in all cultures. The proteins of 41 kDa and 63 were not or very faintly expressed in low pH cultures, in contrast that of 17 kDa more expressed. In this work, we found that *C. acidophila* could live optimally within a wide range of acidic pH, and 17 kDa of unidentified protein might be concerned with tolerating in low acid environment.

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(Received 5 Apr. 2002, Manuscript accepted 20 July 2002)