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Production of γ-Aminobutyric Acid (GABA) by *Lactobacillus buchneri* Isolated from *Kimchi* and its Neuroprotective Effect on Neuronal Cells

CHO, YU RAN, JI YOON CHANG, AND HAE CHOON CHANG*

Department of Food and Nutrition, Chosun University, Gwangju 501-759, Korea

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Abstract Lactic acid bacteria that accumulated γ-aminobutyric acid (GABA) in culture medium were screened to identify strains with high GABA-producing ability. One strain, MS, which was isolated from *kimchi*, showed the highest GABA-producing ability among the screened strains. MS was identified as *Lactobacillus buchneri* based on Gram-staining, metabolic characteristics, and 16S rDNA sequence determination. Optimum culture conditions for GABA production were determined: MRS broth containing 5% MSG, 1% NaCl, and 1% glucose, at an initial pH of 5.0, the incubation temperature at 30°C for 36 h. Under these conditions, MS produced GABA at a concentration of 251 mM with a 94% GABA conversion rate. Moreover, culture extracts of *Lb. buchneri* MS partially or completely protected neuronal cells against neurotoxicant-induced cell death.

Key words: GABA, *Lb. buchneri*, neuroprotective effect

γ-Aminobutyric acid (GABA) is a nonprotein amino acid that is widely distributed in nature [20]. GABA is produced through the α-decarboxylation of L-glutamic acid in a reaction catalyzed by glutamate decarboxylase (GAD, EC 4.1.1.15). GAD has been found in bacteria, animals, and higher plants [28]. Unlike GABA production in animals and microorganisms, GABA production in plants is increased by the binding of calmodulin to Ca^{++}, which activates GAD [28]. In bacteria, GABA is functionally involved in the germination of *Bacillus megaterium* spores [6] and confers resistance to acidic pH in *E. coli* and *Lactococcus lactis* [2, 24].

GABA has several physiological functions; *e.g.*, it has hypotensive, diuretic, and tranquilizing effects [7, 15]. A recent study also showed that GABA strongly induces insulin secretion from the pancreas [1] and effectively prevents diabetic conditions [8]. In addition, GABA has been implicated in the regulation of neurological disorders. Moreover, when the concentration of GABA in the brain diminishes to below a certain threshold, various neurological disorders that include epilepsy, seizures, convulsions, Huntington's disease, and Parkinsonism may occur.

Because of its physiological functions, the development of functional foods containing GABA at high concentrations has been actively studied. GABA enrichment has been achieved in anaerobic incubated tea (gabaron tea) [27] and in rice germ soaked in water [23]. Various microorganisms (bacteria, yeast, fungi) have been reported to produce GABA [11, 18, 21], and interest has been focused on the utilization and mass production of GABA as a bioactive food component. Recent research undertaken to increase GABA-producing lactic acid bacteria (LAB) in food include the use of *Streptococcus thermophilus* and *Lactobacillus delbreukii*, isolated from commercial yoghurt, and *Lb. hilgardi*, *Lb. sakei*, *Lb. brevis*, *Lb. reuteri*, *Lb. confusus*, *Lb. casei*, *Lb. acidophilus*, *Lb. bulgaricus*, *Lb. plantarum*, *Leuconostoc mesenteroides* isolated from *kimchi* or *jotgal* [5, 9, 12, 16, 17].

In this study, we isolated a new GABA-producing microorganism from *kimchi*. The isolated strain *Lb. buchneri* MS has high GABA-producing ability and the GABA produced by the isolate was found to have a neuroprotective function. We also established optimal conditions for producing GABA using *Lb. buchneri* MS.

Materials and Methods

Isolation of GABA-Producing LAB

GABA-producing LAB were isolated from *kimchi*. The isolated strains, which formed a clear zone on MRS plates containing 2% (w/v) $CaCO_3$ at 30°C for 24 h, were considered as tentative LAB. The isolated LAB were cultured in MRS containing 5% monosodium glutamic

*Corresponding author
Phone: 82-62-230-7345; Fax: 82-62-222-8086;
E-mail: hcchang@mail.chosun.ac.kr

acid (MSG) at 30°C for 48 h. Culture broth was centrifuged at 1,500 ×*g* for 15 min, and the supernatant was analyzed for determination of GABA by using thin-layer chromatography (TLC). The isolated GABA-producing strain was identified by morphological observations under a microscope, by Gram staining, and from its biochemical properties using an API kit (50CHL, bioMériux Co., France), and by 16S rDNA sequence determination [3].

Assay of GABA

Levels of GABA were determined qualitatively by TLC with aluminum TLC plate (Sigma-Aldrich Co., Germany) [13]. Culture broth was centrifuged at 1,500 ×*g* for 15 min, and 4 μl of supernatant was then spotted onto TLC plates. TLC was conducted using an acetic acid:1-butanol:distilled water (1:4:5) solvent mixture, and plates were subsequently immerged into 0.5% (w/v) ninhydrin solution and then heated.

GABA was quantitatively analyzed using a previously described spectrophotometric assay [30]. Culture supernatant (1 μl) was added to 400 μl of methanol, and then incubated at 25°C in a water bath for 10 min. The reaction mixture was then dried using a speed vacuum concentrator (Centra-vac, vs-802, Vision Co., Korea) and 1 ml of 70 mM $LaCl_3$ was added. Samples were then shaken for 15 min, centrifuged at 13,000 ×*g* for 5 min, and 800-μl aliquots of supernatant were removed and placed in eppendorf tubes. Then, 160 μl of 1 M KOH was added to the supernatant and shaken for 5 min, and centrifuged at 13,000 ×*g* for 5 min.

The 1-ml assay system contained 550 μl of the prepared supernatant, 200 μl of 0.5 M K^+pyrophosphate buffer (pH 8.6), 150 μl of 4 mM $NADP^+$, 50 μl of 2.5 units GABASE per ml, and 50 μl of 20 mM α-ketoglutarate. The initial absorbance was read at 340 nm before adding α-ketoglutarate, and the final absorbance was read after 60 min. The difference in A_{340} values was used to calculate GABA content in the culture supernatant. The equation of the GABA standard curve was Abs=0.0030×X mM+0.0069 (R=0.999).

High-Performance Liquid Chromatography (HPLC)

The GABA produced by *Lb. buchneri* MS was confirmed by HPLC. One ml of culture broth was collected and centrifuged at 1,500 ×*g* for 15 min. The supernatant was derivatized with PITC (phenylisothiocyanate) and then filtered through a 0.45-μm filter, and analyzed by HPLC (Hewlett Packard 1100 series). GABA analysis was performed using a HPLC equipped with a Waters symmetry C_{18} column (4.6×250 mm, 5 μm), a HP 1100 series binary pump, a HP 1100 series autosampler, column oven (46°C), and a HP 1100 series UV detector (254 nm). The elution solvent system was comprised of (A) 1.4 mM NaHAc, 0.1% TEA, and 6% CH_3CN (pH 6.1), and (B) 60% CH_3CN. The column was eluted for 50 min with a linear gradient of 0–100% at 1.0 ml/min with (B). Twenty-one amino acid standards (cysteine, asparagine, glutamic acid, asparagine, serine, glutamine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cystine, isoleucine, leucine, phenylalanine, tryptophan, and lysine) and authentic GABA were used as controls.

Cell Growth and pH

Lb. buchneri MS was cultivated in MRS broth with or without 5% (v/v) MSG (Yakuri Pure Chemicals Co., Japan) containing 1% (v/v) inoculum at 30°C for 48 h. Growth of the isolate was determined by measuring culture turbidity at 550 nm every 4 h, and culture pH was also measured at this time (the same time intervals).

Effect of Initial pH on GABA Production

Media containing 5% MSG at the initial pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 were used for cultivation of *Lb. buchneri* MS to study the effect of initial pH on the GABA production.

Effect of NaCl on GABA Production

To determine the effect of NaCl on GABA production and cell growth, NaCl was added at 0, 1, 3, 5, or 7% (w/v) to MRS broth containing 5% MSG.

Effect of Carbon Source on GABA Production

To compare the GABA productivity for different carbon sources, MRS media containing 5% MSG were prepared with maltose at 1.0 or 2.0% and with arabinose, fructose, galactose, glucose, lactose at 0.5, 1.0, or 2.0%, respectively.

Measurement of Cell Proliferation

MTT assays are based on the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), by actively growing cells. PC12 cells (a cell line derived from a transplantable rat pheochromocytoma and which responds reversibly to nerve growth factor by inducing the neuronal phenotype) were maintained in 0.5% serum media (RPMI 1640 media containing 0.5% horse serum and 1% penicillin/streptomycin) at 37°C in a humidified incubator under 5% CO_2. Cells were initially inoculated into a 96-well plate (at 1.6×10^4 cells per well). After incubation for 24 h, the test chemicals [H_2O_2 (50 μM, 100 μM, 200vM), rotenone (1 μM, 10 μM, 100 μM), SNP (sodium nitroprusside) (100 μM, 500 μM, 1 μM), paraquat (10 μM, 1 μM, 2 μM), dieldrin (10 μM, 20 μM, 40 μM), or $MnCl_2$ (100 μM, 250 μM, 500 μM)] were added. Cell extracts of *Lb. buchneri* MS (at a final concentration of 10 μg/ml or 100 μg/ml) were subsequently added to the PC12 cells with the chemicals, and then incubated for 24 h.

Culture extracts were prepared as follows; *Lb. buchneri* MS was cultured in MRS broth containing 5% MSG at 30°C for 48 h. Culture broth was centrifuged at 1,500 ×*g* for 15 min, and supernatant was filtered through a 0.45-μm

filter and the filtrate was freeze-dried. This freeze-dried extract was then dissolved in distilled water to a final concentration of 10 mg/ml and used as a stock solution.

Control wells were treated with 0.5% serum media alone. The other control wells were treated with 0.5% serum media and *Lb. buchneri* MS culture extracts.

Thereafter, MTT (at a final concentration of 0.5 mg/ml) was added to each well and incubated for an additional 4 h. Culture media were discarded, 200 μl of DMSO (dimethyl sulfoxide) was added and vibrated for 20 min. Absorbance was measured at 540 nm using an ELISA reader (BIO-Tek, PowerWave^{TM}XS MQS200R). Cell viabilities were calculated using the following formula: cell viability (%)=(absorbance of treated sample)/(absorbance of control)×100.

Results and Discussion

Screening and Identification of GABA-Producing LAB

GABA-producing LAB were isolated from *kimchi*. The isolated strains, which formed a clear zone on MRS plates containing 2% (w/v) $CaCO_3$ at 30°C for 24 h, were considered as tentative LAB. One isolate among the screened LAB showed high GABA production by TLC [the produced GABA was confirmed by HPLC (Fig. 1)]. The isolate was a Gram-positive strain of the rod cell type and formed creamy, opaque, and circular colonies on MRS plates. The isolate was identified as *Lactobacillus buchneri* by examining its metabolic characteristics. The 16S rDNA sequence (1,384 bp) of the isolate showed 99% homology with that of *Lb. buchneri* AB205055, and thus the strain was designated as *Lb. buchneri* MS.

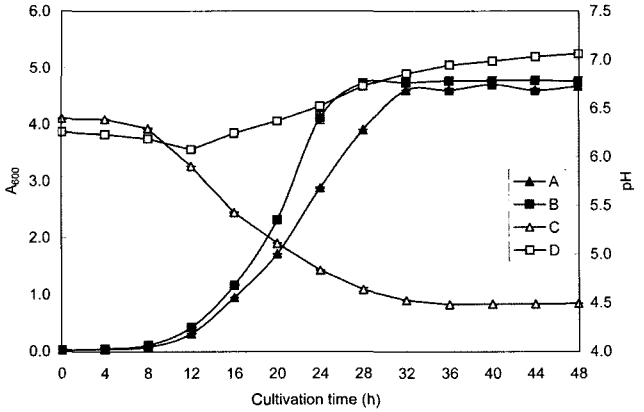
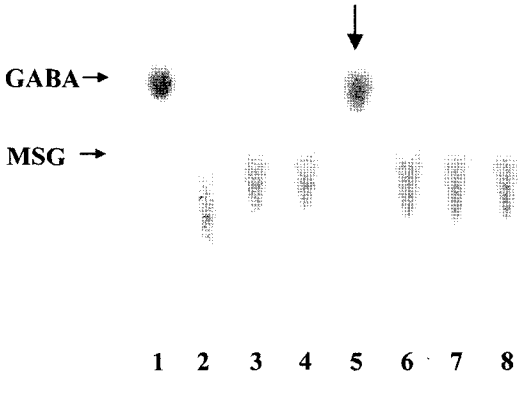


Fig. 2. Growth and pH changes of *Lb. buchneri* MS in culture.
Lb. buchneri MS was cultivated for 48 h at 30°C. A: cell growth in MRS broth; B: cell growth in MRS broth with 5% MSG; C: culture pH in MRS broth; D: culture pH in MRS broth with 5% MSG. All values are means±SE (n=3).

Growth and pH

Fig. 2 shows the cell growth achieved and pH changes for *Lb. buchneri* MS cultured in MRS with or without 5% MSG. Its growth reached at stationary phase after 32 h of cultivation in MRS medium without 5% MSG (pH 4.5), but growth reached the stationary phase after 24 h. The pH of the culture medium increased gradually to pH 7.06 at 48 h.

According to Small and Waterman [26], cytoplasmic decarboxylation results in the consumption of an intracellular proton after the uptake of glutamate by its specific transporter. The reaction product GABA is exported from cells by an antiporter, and the net result is an increase in the pH of the



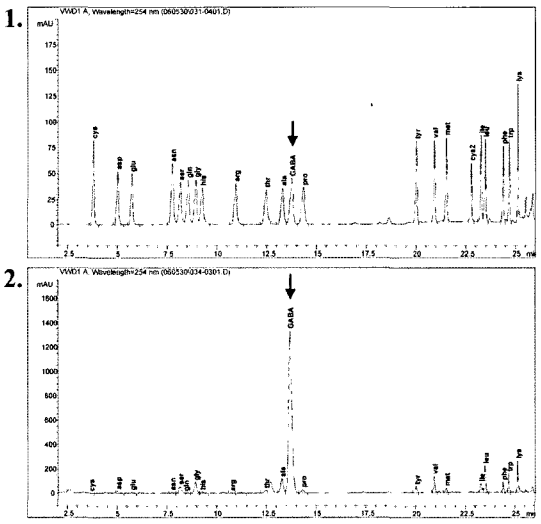


Fig. 1. GABA production by the isolated lactic acid bacteria.
Left: TLC chromatogram of GABA production. Lanes 1, GABA standard; 2, MSG in MRS (control); 3, isolate AF1; 4, isolate Y3; 5, isolate MS; 6, isolate S3; 7, isolate S4; 8, isolate S5. Right: HPLC chromatogram of GABA production by isolate MS. **1**. Amino acid standards with authentic GABA. **2**. GABA produced by the isolate MS. The arrow indicates GABA (RT=13.75 min).

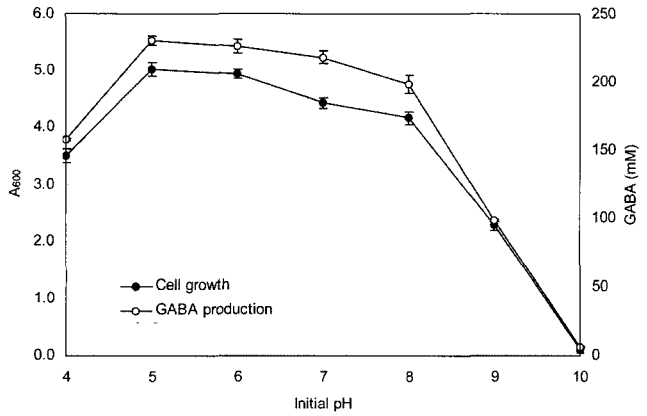


Fig. 3. Effects of initial media pH on growth and GABA production.
Lb. buchneri MS was cultivated for 48 h at 30°C in MRS broth containing 5% MSG at different initial pH values. All values indicated are means±SE (n=3).

cytoplasm, due to the removal of hydrogen ions, and a slight increase in the extracellular pH, due to the exchange of extracellular glutamate for the more alkaline GABA.

Effect of Initial pH and NaCl on GABA Production

The optimal initial pH for GABA production was found to be pH 5.0. High GABA production (above 200 mM) was maintained at pH range 5.0–8.0. GABA production rapidly decreased at pH values below 4.0 or above pH 8.0 (Fig. 3).

The optimal NaCl concentration for GABA production was found to be 1% (w/v). High cell growth and GABA production were maintained at a NaCl concentration of 0–5%, but cell growth was not observed at a NaCl concentration of 7%. High cell growth invariably resulted in high GABA production (Fig. 4).

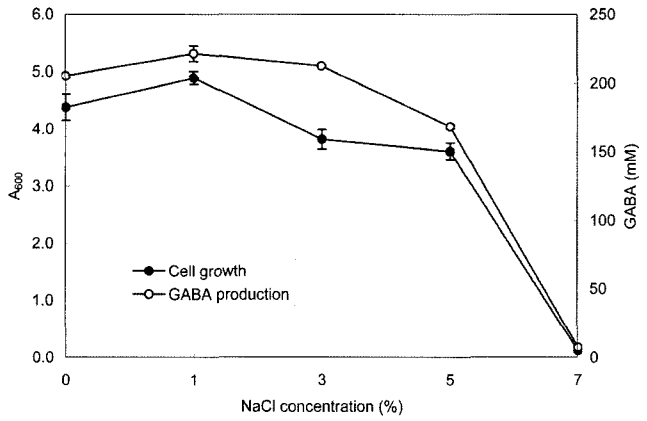


Fig. 4. Effects of NaCl on growth and GABA production.
Lb. buchneri MS was cultivated for 48 h at 30°C in MRS broth containing 5% MSG at different concentrations of NaCl. All values are means±SE (n=3).

Table 1. Production of GABA in MRS media containing 5% MSG and various carbon sources.

Sugars		A_{600}	GABA production (mM)	GABA conversion yield (%)
Compound	Conc. (%)			
Control	0.0	4.32	212.62±1.80	79.6
Arabinose	0.5	6.41	227.59±3.25	85.2
	1.0	6.42	131.14±7.43	49.1
	2.0	7.26	9.48±3.34	3.5
Fructose	1.0	5.20	222.92±5.32	83.5
	2.0	5.54	212.28±2.32	79.5
Galactose	1.0	5.40	226.09±6.21	84.7
	2.0	6.64	232.20±5.69	87.0
Glucose	1.0	5.56	234.81±3.76	87.9
	2.0	6.70	234.61±3.74	87.9
Lactose	1.0	4.51	219.55±4.00	82.2
	2.0	4.25	220.70±7.19	82.7
Maltose	1.0	5.78	228.37±3.79	85.5
	2.0	7.04	233.03±5.01	87.2

Effect of Carbon Source on GABA Production

The effects of various carbon sources on the production of GABA by *Lb. buchneri* MS were investigated. As shown in Table 1, cell growth and GABA production were $A_{600}\simeq$ 4.32 and ≃212 mM, respectively, in MRS media containing 5% MSG after culture at 30°C for 48 h (control). The addition of carbon sources increased growth, especially the addition of 2.0% arabinose ($\simeq A_{600}$ 7.26). However, this high cell density did not result in increased GABA production; the addition of 2% arabinose caused the highest cell number ($A_{600}\simeq$7.26), but the GABA concentration reached only 9.48 mM (3.5% conversion yield). The addition of over 1% of arabinose to culture media rapidly reduced the GABA conversion yield. However, GABA concentrations reached over 200 mM after adding fructose, galactose, glucose, lactose, or maltose (0.5–2.0%). The optimal carbon source was found to be glucose (at 1%), which resulted in a *ca.* 230 mM of GABA production (a 87.9% conversion yield).

GABA Production Under Optimal Culture Conditions

To determine the optimal culture conditions required for GABA production, *Lb. buchneri* MS was cultured in MRS medium containing 5% MSG, 1% NaCl, and 1% glucose, at an initial pH 5.0 for 120 h at 30°C. In the control experiment, we used an MRS medium containing 5% MSG (initial pH 7.0) at 30°C for 120 h. Highest GABA production was achieved by culturing *Lb. buchneri* MS under optimal conditions for 48 h, and the highest cell growth for 36 h. Under optimal culture conditions (5% MSG, 1% NaCl, and 1% glucose in MRS at an initial pH 5.0), GABA production was significantly enhanced, and reached 251 mM (GABA conversion rate: 94%) at 48 h of cultivation. Under control conditions in MRS with 5% MSG, the concentration of GABA was 212 mM (a 79% conversion rate) (Fig. 5).

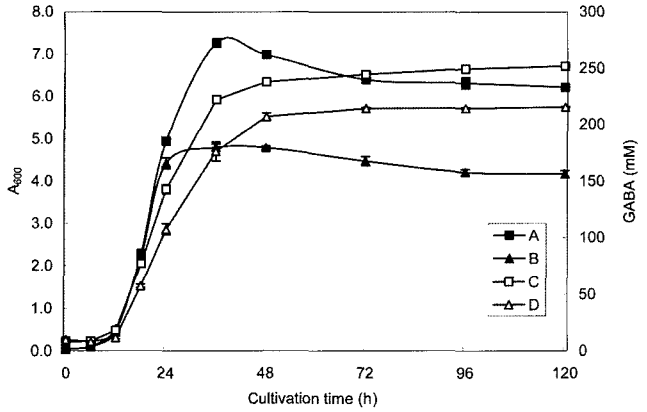


Fig. 5. Cell growth and GABA production under optimal culture conditions.

Lb. buchneri MS was cultivated for 120 h at 30°C. A. Cell growth under optimal culture conditions; B. Cell growth in MRS broth containing 5% MSG; C. GABA production under optimal culture conditions; D. GABA production in MRS broth containing 5% MSG. All values are means±SE (n=3).

Neuroprotective Effect of *Lb. buchneri* MS Culture

PC12 cells were exposed to various chemicals (H_2O_2, retonone, SNP, paraquat, dieldrin, or $MnCl_2$) that can induce neuronal cell death (Fig. 6). Proliferation of PC12 cells treated with *Lb. buchneri* MS culture medium increased depending on the culture medium concentration (at 10 μg/ml 120% and at 100 μg/ml 140% cell viability; $C_{1\text{-}a}$ and $C_{1\text{-}b}$ in Fig. 6). H_2O_2, which causes neuronal cell death, was administered to PC12 cells, and increasing H_2O_2 concentrations (50–200 μM) gradually increased cell death. However, cells treated with bacterial culture medium at a final concentration of 100 μg/ml retained 100% cell viability. *Lb. buchneri* MS culture had no neuroprotective effect against rotenone, which inhibits complex I in, the mitochondrial electron transfer system, or SNP, a NO donor. Treatment of PC12 neuronal cells with *Lb. buchneri* MS culture medium completely protected them from paraquat (10–200 μM) and $MnCl_2$ (100–500 μM). In the case of dieldrin treatment (<40 μM), which can cause Parkinson's disease, *Lb. buchneri* MS culture had a partial or complete neuroprotective effect. These results indicated that the culture extract of *Lb. buchneri* MS (final concentration 10 μg/ml or 100 μg/ml) had a partial or complete neuroprotective effect against various chemicals that induce PC12 cell death, and that it increased the proliferation of PC12 neuronal cells (Fig. 6). LAB are known to have various beneficial effects on health; *i.e.*, they lower cholesterol, prevent cancer, enhance immunomodulation, protect liver injury, improve lactose intolerance, and ameliorate atopic dermatitis and constipation [5, 10, 14, 19, 22, 25].

Here, we reported the neuroprotective effect of *Lb. buchneri* MS against the neuronal cell death induced by chemicals. The GABA-producing ability of *Lb. buchneri* MS isolated from *kimchi* was apparently higher than that of *Lb. brevis* IFO12005 [29]. To our knowledge, this is the first report of GABA production by a *Lb. buchneri* strain. Consequently, this study indicates that the production of GABA by *Lb. buchneri* MS may be of great interest to the food industry.

Acknowledgment

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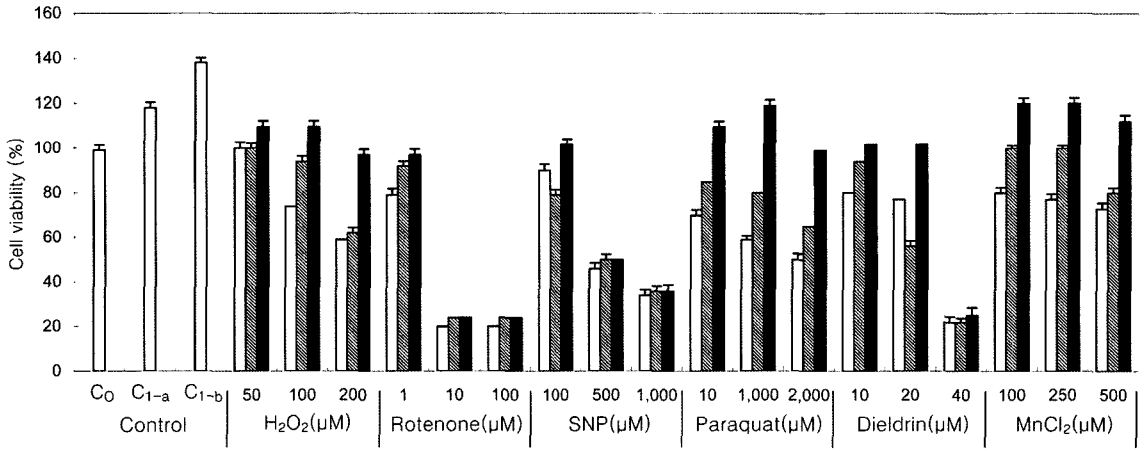

Fig. 6. Neuroprotective effect of *Lb. buchneri* MS culture medium on neurotoxicant-induced neuronal cell death.

C_0: PC12 cells without bacterial culture medium; $C_{1\text{-}a}$: PC12 cells with bacterial culture medium at 10 μg/ml; $C_{1\text{-}b}$: PC12 cells cultured in the presence of 100 μg/ml of bacterial culture medium. All chemicals were treated for 24 h. Bars represent the mean values of triplicate determinations with standard errors (□: PC12 cells+chemical; ▨: $C_{1\text{-}a}$+chemical; ■: $C_{1\text{-}b}$+ chemical).

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