Use of Sulfite and Hydrogen Peroxide To Control Bacterial Contamination in Ethanol Fermentation

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Lactic acid bacteria isolated from an industrial-scale ethanol fermentation process were used to evaluate sulfite as a bacterial-contamination control agent in a cell-recycled continuous ethanol fermentation process. The viabilities of bacteria were decreased by sulfite at concentrations of 100 to 400 mg liter^{-1}, while sulfite at the same concentrations did not change the viability of the *Saccharomyces cerevisiae* strain used in this process. Sulfite was effective only in the presence of oxygen. Bacteria showed differences in their susceptibilities to sulfite. Facultatively heterofermentative *Lactobacillus casei* 4-3 was more susceptible than was obligatory heterofermentative *Lactobacillus fermentum* 7-1. The former showed higher enzyme activities involved in the production and consumption of hydrogen peroxide than did the latter. The viability of *L. fermentum* 7-1 could be selectively controlled by hydrogen peroxide at concentrations of 1 to 10 mM. Based on these findings, it is hypothesized that the sulfur trioxide radical anions formed by peroxidase in the presence of hydrogen peroxide are responsible for the control of contaminating bacteria. Sulfite did not kill the yeast strain, which has catalase to degrade hydrogen peroxide. A cell-recycled continuous ethanol fermentation process was run successfully with sulfite treatments.

Most industrial-scale ethanol production processes are operated in the presence of measurable numbers of bacterial contaminants. Bacterial contamination causes a reduction in ethanol yield and an inhibition of yeast growth (6, 29). Lactic acid bacteria are the major bacterial contaminants in ethanol fermentation. They ferment carbohydrate to lactic acid, reducing the ethanol yield, and yeast fermentation is inhibited by lactic acid (20, 29).

Few processes have been developed to control bacterial contaminations during ethanol fermentation. One of the most widely used processes is acid washing (32, 33). Cells are collected from the fermentation broth, and sulfuric acid is used to adjust the pH of the cell paste to 2.0, which is kept for 2 h before being returned to the fermentor. This method can be successfully applied to a batch fermentation process but is not satisfactory in a cell-recycled continuous process (19). Other methods involve antibiotics such as β-lactam antibiotics, but such an approach is very expensive (2, 9).

Although sulfite has long been recognized as an antimicrobial agent and been used in wine making, its antibacterial mechanism has not been known (1, 11, 24, 30). The oxidation of sulfite to sulfate is known to involve free radical formations (11, 14, 22, 28). Peroxidase is known to catalyze the oxidation of sulfite to sulfur trioxide free radicals (25–27). Sulfur trioxide free radicals ($HSO_3^-\cdot$ and $SO_3^-\cdot$) produced as a result of oxidation are thought to be involved in a number of reactions of biological significance (3, 11, 13, 21, 31, 35).

Hydrogen peroxide is a product of some flavoprotein oxidases of lactic acid bacteria with oxygen, and it may accumulate under aerobic conditions. Accumulation of hydrogen peroxide results from a greater capacity to form hydrogen peroxide than to break it down, because bacteria do not possess the catalase enzyme (7).

The aim of the present study was to investigate the use of sulfite and hydrogen peroxide to control bacterial contamination in a cell-recycled ethanol fermentation.

MATERIALS AND METHODS

Organisms. An industrial strain of *Saccharomyces cerevisiae* was obtained from Jin-Ro Fermentation (Ansan, Korea). *Lactobacillus casei* 4-3 and *Lactobacillus fermentum* 7-1 were isolated from a commercial ethanol plant where tapioca and barley were used as raw materials (6).

Media and culture conditions. Yeast cells were cultivated at 30°C in peptone-yeast extract-malt extract (PYM) broth containing the following (in grams per liter): glucose · H_2O, 200.0; peptone, 20.0; yeast extract, 12.0; and malt extract, 12.0. pH was adjusted to 4.5 before autoclaving. Continuous fermentation was run with a medium containing the following (in grams per liter): glucose · H_2O, 200.0; yeast extract, 12.0; NH_4Cl, 8.0; $Na_2HPO_4 \cdot 7H_2O$, 3.9; KH_2PO_4, 0.5; $MgSO_4$, 0.5; citric acid monohydrate, 4.3; trisodium citric acid dihydrate, 1.25; and $CaCl_2$, 0.28. $CaCl_2$ was added to the medium after autoclaving. The pH was the same as that of PYM broth. The inoculum was prepared by growing cells aerobically in PYM broth at 30°C for 24 h with shaking (150 rpm). Fermentation was initiated with a 5% (vol/vol) inoculum. Inocula of lactic acid bacteria were made anaerobically in lactobacillus MRS broth (Difco Laboratories, Detroit, Mich.) by growing the culture at 30°C for 24 h.

Cell-recycled continuous ethanol fermentation. The experimental setup for cell-recycled continuous ethanol fermentation is shown in Fig. 1. A fermentor (LH 500 series; LH Fermentation Ltd., Bells Hills, United Kingdom) with a 2-liter vessel and a 1-liter working volume was used; a membrane module was connected to it. The membrane module (Millipore Tangential-Flow system; Millipore Corporation, Bedford, Mass.) contained four membranes (hydrophilic Durapore membrane; Millipore Corporation). The area of each membrane sheet was 60 cm^2 with a pore size of 0.45 μm. They were sterilized with 100 to 200 ppm of NaClO solution for 2 h and washed with 10 liters of sterilized water before use.

Continuous operation was started by the activation of the medium pump after the initial batch culture. The dilution rate was 0.1 h^{-1} during the whole operation. The pH was controlled to 4.5 with 1 N NaOH, and the agitation speed was 150 rpm. The temperature and air flow rate were 30°C and 0.09 vol/vol/min, respectively. The broth from the fermentor was fed to the membrane module at the rate of 340 ml min^{-1}, where cells were concentrated. The cell paste was returned to the fermentor through a 170-ml sulfite reactor for sulfite treatment as needed. When the permeate flow exceeded the fresh medium feed rate (100 ml h^{-1}), the pressurizing pump to the membrane module was inactivated, and excess cell paste was discarded from the sulfite reactor when the permeate was less than the feed. During operation, the flux of filtrate was decreased due to membrane fouling. Intermittently, a part of the permeate was flushed back through the membranes to recover filtration capacity.

The specific growth rate of the yeast strain was around 0.3 h^{-1} in a batch culture. Since the system was run at a dilution rate that was lower than the

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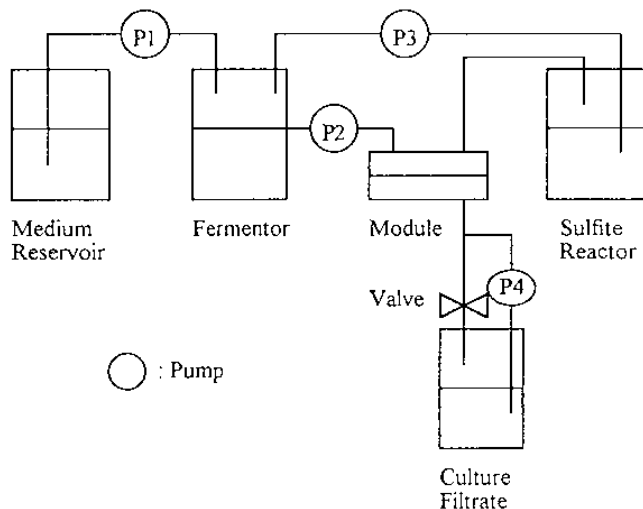
FIG. 1. Schematic diagram of cell-recycled fermentation and contaminant control system.

FIG. 2. Effects of sulfite concentrations on the viabilities of yeast and bacteria strains. Symbols: ○, *L. fermentum* 7-1; ●, *L. casei* 4-3; △, *S. cerevisiae*.

growth rate and cells were recycled, complete substrate utilization was achieved. The conditions were set to test the effects of bacterial contamination on substrate utilization and product productivity and the effects of sulfite.

The contaminants were inoculated into the contamination-free continuous fermentation when complete substrate consumption was achieved.

Preparation of sulfite and hydrogen peroxide solution. Sodium *meta*-bisulfite ($Na_2S_2O_5$; Sigma Chemical Co., St. Louis, Mo.) was used to make sulfite solution. It was prepared anaerobically to prevent oxidation by molecular oxygen. Distilled water was gassed with oxygen-free nitrogen gas before a stock solution of 1 g per liter was made. The stock solution was autoclaved immediately before use. Reagent-grade hydrogen peroxide solution (35%) was diluted by 10-fold serially with sterile distilled water. This solution was not sterilized.

Sulfite and hydrogen peroxide treatment. Cells harvested at the end of the log phase were resuspended in air-saturated fresh media, and 5 ml of the suspension was placed in anaerobic sterilized pressure tubes (Bellco Glass, Inc., Vineland, N.J.) with butyl rubber stoppers and aluminum caps. Stock solutions of sulfite or hydrogen peroxide were added to the tubes by using syringes before they were incubated at 30°C for 2 h. When cell suspensions were treated in a different manner, the details are given in the text. The chemistry of sulfite is very complex, especially in a biological system. The total sulfite concentration was used throughout (17).

Viable-cell count. Viable bacteria and yeast cells were counted by using MRS agar supplemented with 10 mg of cycloheximide liter^{-1} and PYM agar supplemented with 100 mg of penicillin G liter^{-1}, respectively (20). For bacterial counts, plates were incubated at 30°C in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, Mich.) because *L. fermentum* 7-1 was found to be sensitive to molecular oxygen (see below).

Determinations of LC$_{50}$s and decimal reduction times. The viable-cell count was used to calculate mortality as a percentage before being converted to a probit scale (23), which was then plotted against the sulfite concentration to determine the 50% lethal concentration (LC$_{50}$) of sulfite. Mortality was calculated by the following equation: Mortality (%) = [(CFU/ml of control − CFU/ml of treatment)/(CFU/ml of control)] × 100. The decimal reduction time (*D*) was determined by reading the time (in hours) required for a 10-fold decrease in viable-cell numbers under given conditions (4).

Analyses. The cell concentration was determined by measuring the optical density with a spectrophotometer (UVIDEC-610; Jasco, Tokyo, Japan) at 525 nm. Ethanol was quantified with a gas chromatograph (model 3300; Varian, San Fernando, Calif.) equipped with a packed column (0.2 by 200 cm) of Super Q (Alltech Associates Inc., Deerfield, Ill.) and a flame ionization detector. The temperatures of the injector and detector were 220 and 240°C, respectively. The oven temperature was programmed from 180 to 200°C at a gradient of 5°C per min. Nitrogen was used as a carrier gas at the flow rate of 25 ml per min. Glucose was quantified by the glucose oxidase-peroxidase method using an enzyme kit (BC 103-E; Young-Dong Pharmaceuticals, Seoul, Korea). Protein was quantitatively analyzed by using Coomassie brilliant blue G with bovine serum albumin as a standard (8).

Preparation of cell extract. To obtain cell extract, cells were harvested and washed with the buffer used in enzyme activity assays. The cell suspension was passed through a French press (SLM Instruments Inc., Urbana, Ill.) at 20,000 lb/in^2 gauge. Cell debris was removed by centrifugation at 10,000 × *g* for 30 min. The supernatant was used as the enzyme source. The entire procedure was
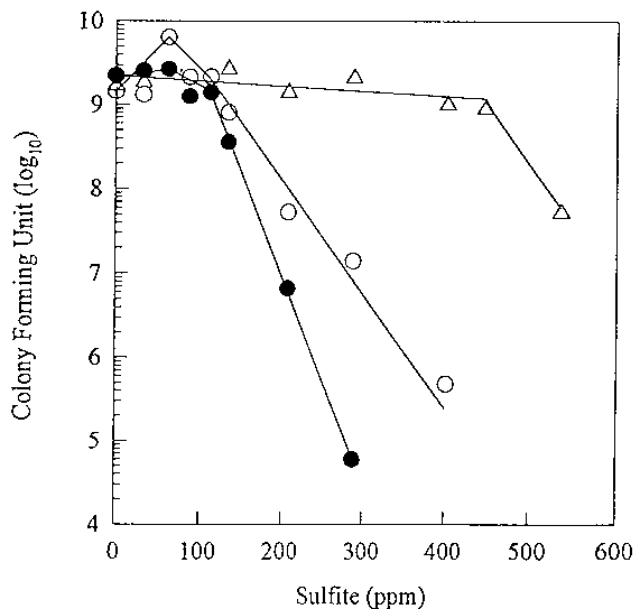
carried out at 4°C. The cell extract not used immediately was stored at 4°C for no longer than 48 h.

Enzyme assays. For NADH oxidase (NADH:H_2O_2 oxidase and NADH:H_2O oxidase) and NADH peroxidase (EC 1.11.1.1) (15), NADH oxidase activity was assayed at 30°C by monitoring the oxidation of NADH (ε_{340} = 6.22 μmol^{-1} cm^{-1}) spectrophotometrically at 340 nm. The reaction mixture (1 ml) contained air-saturated 40 mM potassium phosphate buffer (pH 7.2), 0.2 mM EDTA, and 0.17 mM NADH with (NADH:H_2O_2 oxidase) or without (NADH:H_2O oxidase) 0.02 mM flavin adenine dinucleotide (FAD). H_2O_2-forming oxidase required FAD for full activity, but H_2O-forming oxidase did not. The reaction was initiated by adding cell extract. NADH peroxidase activity was measured at 30°C by monitoring the oxidation of NADH by H_2O_2 in an anaerobic cuvette. The reaction mixture (1 ml) contained 40 mM potassium phosphate buffer (pH 7.2), 0.2 mM EDTA, 0.17 mM NADH, 0.02 mM FAD, and 1.3 mM H_2O_2. The reaction mixture was prepared in an anaerobic glove box (Coy Laboratory Products, Inc.). The reaction was initiated by adding H_2O_2. One unit of NADH oxidase and peroxidase was defined as the amount of enzyme (in milligrams of protein) which catalyzed the oxidation of 1.0 μmol of NADH per min.

For pyruvate oxidase (EC 1.2.3.3) (12), lactate oxidase (EC 1.1.3.2) (10), and L-α-glycerophosphate oxidase (EC 1.1.3.21) (16), oxidase activities were assayed at 30°C by measuring the H_2O_2 formed by using peroxidase with quinonediimine dye (ε_{565} [pH 6.7] = 23.56 μmol^{-1} cm^{-1} for pyruvate oxidase; ε_{565} [pH 6.5] = 35.33 μmol^{-1} cm^{-1} for lactate oxidase; ε_{500} [pH 8.2] = 13.3 μmol^{-1} cm^{-1} for L-α-glycerophosphate oxidase). The reaction mixture (1 ml) contained 1.5 mM 4-aminoantipyrine, 5 U of peroxidase ml^{-1}, and 0.04% (vol/vol) *N,N*-dimethyl-aniline. For pyruvate oxidase, 0.2 M phosphate buffer (pH 6.7) was used with 0.02 mM FAD, 0.2 mM thiamine pyrophosphate, 10 mM MgCl$_2$, and 100 mM potassium pyruvate. Lactate oxidase assays employed 3,3-dimethylglutarate-NaOH buffer (pH 6.5) with 50 mM DL-lactate, and 40 mM Tris-HCl buffer (pH 8.2) was used with 0.2% (wt/vol) phenol, 0.05% (wt/vol) Triton X-100, and 100 mM disodium DL-glycero-3-phosphate for L-α-glycerophosphate oxidase. One unit of oxidase was defined as the amount of enzyme (in milligrams of protein) which generated 1.0 μmol of H_2O_2 per min. The activities presented are the means of three separate assays using a different cell extract for each assay. The presence of catalase activity was determined by gas bubbles formed after 1 drop of hydrogen peroxide solution was placed on a colony of fresh cultures (34).

RESULTS

Changes in the viabilities of cultures treated with sulfite. Yeast and bacterial strains were grown and resuspended in air-saturated fresh media in pressure tubes before treatment with various concentrations of sulfite for 2 h. The viability of yeast cells was not affected by treatment with sulfite up to 400 ppm, but the CFU of bacterial strains decreased exponentially as the sulfite concentration increased above 100 ppm (Fig. 2).
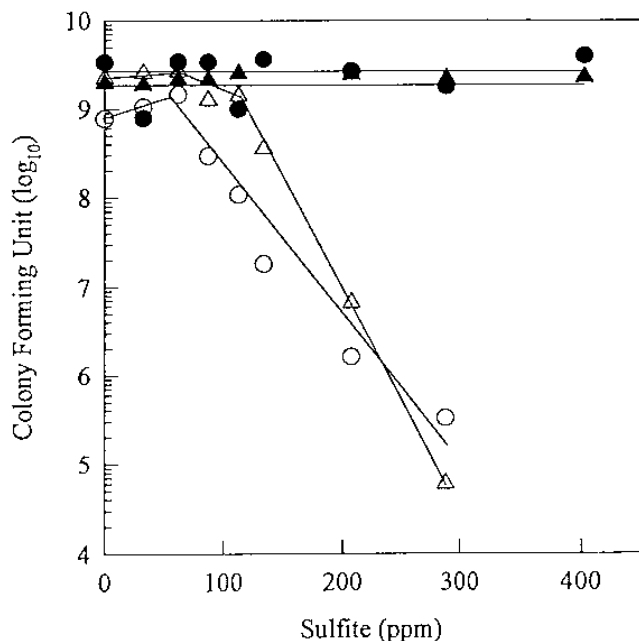
FIG. 3. Effects of sulfite treatment on bacterial viability under aerobic (open) and anaerobic (closed) conditions. Symbols: circles, *L. fermentum* 7-1; triangles, *L. casei* 4-3.

FIG. 4. Effects of the oxygen concentration in the reactor headspace on sulfite treatment. Symbols: ○, *L. fermentum* 7-1; ●, *L. casei* 4-3.

L. casei 4-3 cells were more sensitive to sulfite than were *L. fermentum* 7-1 cells. The LC_{50}s of sulfite were about 130 and 100 ppm for *L. fermentum* 7-1 and *L. casei* 4-3, respectively. The CFU of the *L. fermentum* culture treated with 87-ppm sulfite were more numerous than those of the control culture. This might be due to the reactivity of sulfite with molecular oxygen or its metabolite, which might be lethal to the bacterium.

The effects of molecular oxygen on the bactericidal activity of sulfite. Bacterial cell suspensions were made with an air-saturated medium in flasks or a medium without dissolved oxygen in pressure tubes before sulfite treatment. Figure 3 shows viable-cell counts after sulfite treatment for 2 h. Cells were killed by 100-ppm sulfite treatment in the presence of dissolved oxygen but were unaffected by 400-ppm sulfite treatment in the absence of dissolved oxygen. These results suggest that bacterial cells become susceptible to sulfite through molecular oxygen or that bacterial strains possess a system that produces a toxic compound from molecular oxygen and sulfite.

In comparison to *L. casei*, *L. fermentum* showed higher susceptibility to a low sulfite concentration. Figures 2 and 3 exhibit variations in the effects of sulfite at a low concentration on *L. fermentum*. These differences could be due to differences in the exposure time to air before sulfite treatment and suggest that *L. fermentum* is more susceptible to molecular oxygen than is *L. casei*.

In order to determine the effects of oxygen concentration on the enhancement of sulfite treatment, bacterial suspensions in anaerobic fresh medium were transferred to anaerobic tubes with 400-ppm sulfite. Nitrogen gas in the tubes was replaced by known amounts of oxygen, and tubes were incubated for 2 h before viable cells were counted (Fig. 4). As in the previous experiments, the bactericidal effect of sulfite was observed only in the presence of molecular oxygen. A higher molecular oxygen concentration in the headspace (e.g., 20%) resulted in a greater bactericidal effect of sulfite on *L. fermentum*. In con-
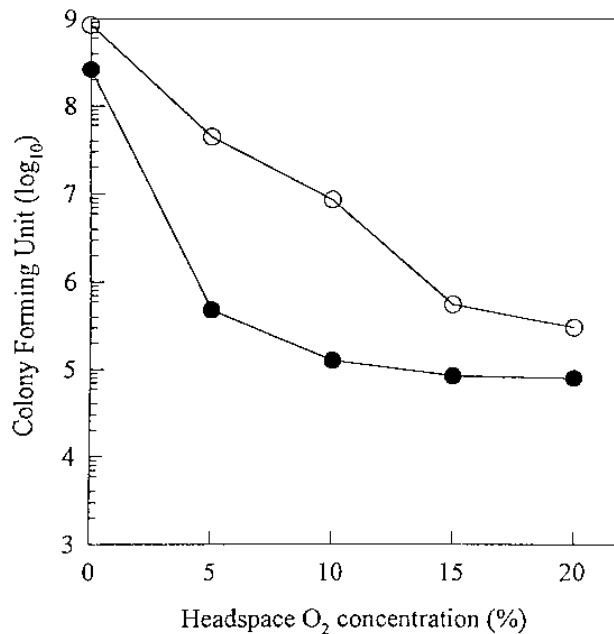
trast, even 10% molecular oxygen had a bactericidal effect similar to that of 20% on *L. casei*.

Effects of time and temperature on bactericidal activity of sulfite. Bacterial strains were treated with various sulfite concentrations (up to 287 ppm), and viable cells were counted every hour up to 5 h. As shown in Fig. 5, both bacterial strains were susceptible to sulfite at concentrations of over 134 ppm. The viable-cell counts decreased exponentially during incubation with sulfite at concentrations of over 134 ppm. The *D* values of *L. fermentum* and *L. casei* were 0.96 and 0.79 h in the presence of 134-ppm sulfite, whereas the respective *D* values for 287-ppm sulfite treatments were 0.90 and 0.51 h, respectively.

The bacterial and yeast strains were incubated with 400-ppm sulfite at different temperatures for 2 h, and viable cells were counted. The CFU of bacteria were unchanged by sulfite treatment at 4°C, but the bactericidal activity of sulfite increased exponentially as the temperature increased up to 40°C. The viability of yeast cells was not affected by sulfite at the temperatures tested (data not shown). These results can be used to design the sulfite treatment unit to be used in a cell-recycled continuous ethanol fermentation process.

Effects of hydrogen peroxide. It was hypothesized that *L. fermentum* is susceptible to molecular oxygen or its metabolites since when the culture was exposed to the air, more colonies formed in the presence of sulfite at low concentrations (Fig. 2, 3, and 5). To test this hypothesis, bacterial and yeast strains were incubated with various concentrations of hydrogen peroxide at 30°C for 2 h before viable cells were counted (Fig. 6). The viability of yeast cells did not change with hydrogen peroxide up to 40 mM, and the viability of *L. casei* did not change with hydrogen peroxide up to 10 mM. However, the viability of *L. fermentum* was reduced by hydrogen peroxide even at a concentration of 0.5 mM.

Effects of sulfite and hydrogen peroxide treatments on the viabilities of mixed cultures. A previous study has shown that the heterofermentative *L. fermentum* deteriorates ethanol fer-
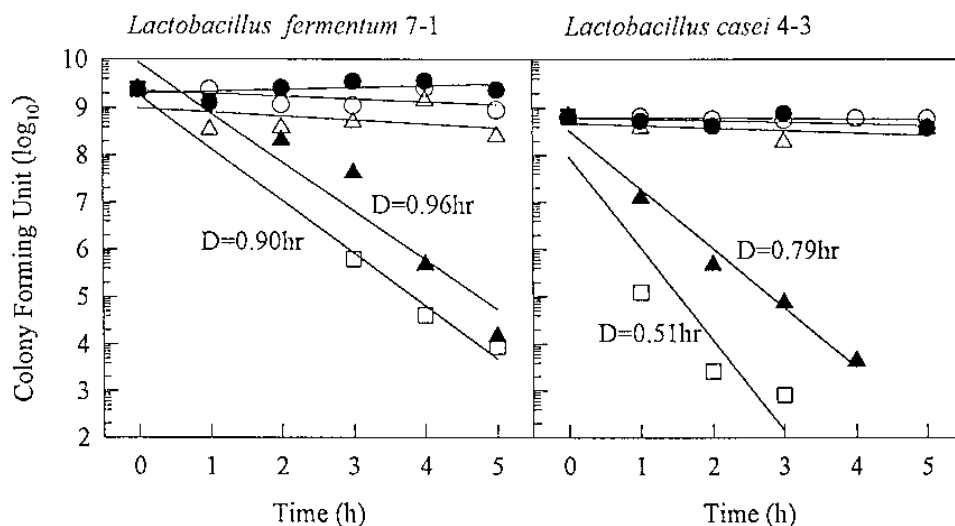
FIG. 5. Survival of *Lactobacillus* species in relation to sulfite treatment time. Symbols: ○, 0 ppm; ●, 32 ppm; △, 87 ppm; ▲, 134 ppm; □, 287 ppm.

mentation more than *L. casei* does (6). Mixed cultures of *S. cerevisiae* and *L. fermentum* were used to test the effects of sulfite and hydrogen peroxide on their viabilities. Cultures of *S. cerevisiae* and *L. fermentum* were resuspended in fresh PYM broth and incubated at 30°C for 2 h after the addition of sulfite or hydrogen peroxide to a final concentration of 400 ppm or 200 mM, respectively. The viable-cell counts of this bacterium were selectively reduced by both sulfite and hydrogen peroxide treatments, but the yeast strain was less susceptible (Table 1). The bactericidal effects of these chemicals were less significant on the mixed culture with yeast cells than they were on a pure culture. This might be the result of the decomposition of these chemicals by yeast cells.

Enzyme activities related to hydrogen peroxide of bacterial contaminants. Our results showed that molecular oxygen is needed for the bactericidal effects of sulfite. Enzyme activities related to molecular oxygen were determined by using cell extracts of bacterial strains (Table 2). The data shown in Table 2 are the means of three separate assays using a different cell extract for each assay. Catalase activity was not detected in either *L. casei* 4-3 or *L. fermentum* 7-1. *L. casei* showed high-level activities of enzymes producing (oxidases) and consuming (peroxidase) hydrogen peroxide, while much lower levels of enzyme activities were found in the *L. fermentum* extract. The susceptibility of *L. fermentum* to hydrogen peroxide can be explained by the low peroxidase activity.

Control of bacterial contamination by sulfite in a cell-recycled ethanol fermentation. A cell-recycled ethanol fermentation was run for several days to achieve complete glucose consumption. At this stage, the yeast cell concentration was 2.5×10^8 cells per ml with an ethanol productivity of 9.0 g liter^{-1} h^{-1} (Table 3).

L. fermentum 7-1 was inoculated into the fermentor (inoculum size, 5%) to test the effects of bacterial contamination on fermentation. The cell-recycled ethanol fermentation was run for another 3 days before the fermentation parameters were determined (Table 3). Bacterial contamination resulted in reductions for all the parameters analyzed, ethanol productivity,
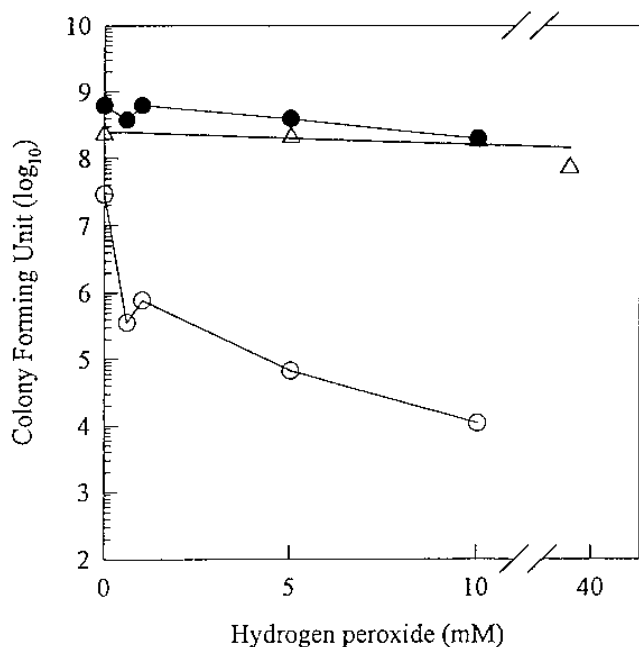

FIG. 6. Viability changes of bacteria and yeast cells treated with hydrogen peroxide. Symbols: ○, *L. fermentum* 7-1; ●, *L. casei* 4-3; △, *S. cerevisiae*.

TABLE 1. Sulfite and hydrogen peroxide treatments of mixed cultures of *S. cerevisiae* and *L. fermentum* 7-1

Treatment[a]	CFU/ml	
	S. cerevisiae	*L. fermentum* 7-1
Sulfite		
0	3.9×10^7	3.0×10^8
400	3.2×10^7	6.7×10^6
Hydrogen peroxide		
0	1.4×10^8	1.4×10^9
40	1.4×10^8	4.1×10^8
100	1.0×10^8	5.4×10^7
200	8.4×10^7	9.7×10^5

[a] Sulfite treatments are expressed in parts per million, and hydrogen peroxide treatments are expressed as millimolar concentrations.

TABLE 2. Specific activities of enzymes related to hydrogen peroxide

Enzyme	Sp act (mU/mg of protein)	
	L. fermentum 7-1	*L. casei* 4-3
Pyruvate oxidase	0.50 ± 0.09	4.83 ± 0.74
Lactate oxidase	1.88 ± 0.58	0.13 ± 0.03
L-α-Glycerophosphate oxidase	0.98 ± 0.15	0.45 ± 0.05
NADH:H_2O_2 oxidase	8.99 ± 0.78	920 ± 37.0
NADH:H_2O oxidase	6.22 ± 0.38	239 ± 20.5
NADH:peroxidase	2.66 ± 0.25	179 ± 25.5
Catalase	ND[a]	ND

[a] ND, not detected.

substrate utilization, and yeast cell concentration. The bacterial cell concentration was 3.1×10^8 cells per ml. These results are consistent with previous reports that lactic acid inhibits ethanol yield and yeast fermentation (20, 29).

Sulfite was added to a final concentration of 400 ppm to the cell paste concentrated by the membrane module. The cell paste was aerated at the rate of 1 volume min^{-1} (volume of sulfite reactor, 170 ml). The fermentation parameters were determined after sulfite treatment for 2 days (Table 3). After sulfite treatment, the viable-cell count of *L. fermentum* 7-1 decreased from 3.1×10^8 to 1.9×10^7 cells per ml. The ethanol concentration increased from 57.2 to 78.1 g $liter^{-1}$. It is believed that yeast fermentation was recovered due to the dilution of toxic fermentation products of bacterial contaminant. These results indicate that sulfite can be used to control bacterial contamination in this system.

A separate cell-recycled fermentation using an industrial fermentation broth to inoculate the fermentor containing fresh medium was run. The system was run for 3 days before the fermentation parameters were determined. The yeast and bacterial cell counts were lower than those from the fermentation contaminated deliberately with *L. fermentum* 7-1, and the glucose concentration in the effluent was also lower. The lower values of the fermentation parameters were due to the shorter run of the cell-recycled operation than in the previous experiment. The ethanol concentration and productivity were higher than in the previous run, 60.4 g $liter^{-1}$ and 6.04 g $liter^{-1}$ h^{-1}, respectively.

Sulfite treatment was carried out as described above, and the fermentation parameters were determined. The bacterial viable-cell number decreased from 5.3×10^7 to 1.5×10^7 cells per ml, and the ethanol concentration increased from 60.4 to 74.6 g

$liter^{-1}$ (Table 3). These results show that sulfite treatment is effective in controlling bacteria contamination in an industrial fermentor.

DISCUSSION

Bacterial contamination is unavoidable in an industrial ethanol fermentation process using starchy materials as the substrate. Even a successful batch fermentation on an industrial scale had contaminants at 3.0×10^8 bacterial cells per ml, many of which were lactic acid bacteria (6, 20, 29).

Productivity is one of the major factors that affect the economics of ethanol production. Cell-recycled continuous processes have been developed to obtain higher ethanol productivities. The application of these processes has been hindered by the problems associated with bacterial contamination (19). It has been shown here that sulfite kills lactic acid bacteria selectively (Fig. 2). These results have been applied to control bacterial contamination in a laboratory-scale cell-recycled continuous ethanol fermentation (Table 3).

When lactic acid bacteria were mixed with yeast cells (Table 1), the bactericidal effect of sulfite was not as good as it was on pure cultures (Fig. 2 and 3). This might be due to rapid oxidation of sulfite by yeast cells, lowering the concentration of sulfite in the reactor, as shown in *Escherichia coli* cells exposed to peroxidogenic streptococci (18). However, the ethanol fermentation parameters were improved significantly by sulfite treatment. The ethanol productivity increased from 6.0 to 7.5 g $liter^{-1}$ h^{-1} with sulfite treatment. As mentioned in the introduction, an industrial ethanol fermentation operates with a measurable number of bacterial contaminants. In this sense, sulfite treatment would be a good way to control bacterial contamination in a cell-recycled continuous ethanol fermentation process.

Because sulfite is used in diluted concentrations in the cell paste, this process might be more economical than is acid washing, where large amounts of sulfuric acid are used, or antibiotics, which are used directly in the fermentor.

Sulfite is known to arrest acetaldehyde during sugar fermentation by yeast cells, forcing yeast cells to produce glycerol. In this case, yeast cells metabolize sugar actively to produce glycerol in the presence of about 40 g of sodium sulfite $liter^{-1}$ (5). It is well documented that sulfite at a low concentration does not affect the metabolism of yeast cells (1). Because the cell paste is treated with very diluted sulfite and a large part of the sulfite is believed to be oxidized during treatment, the amount of acetaldehyde arrested by sulfite would not affect ethanol production.

TABLE 3. Effects of sulfite treatment on control of bacterial contamination during a cell-recycled ethanol fermentation

Culture	D	Ethanol (concn g/liter)	Glucose (concn g/liter)	Ethanol productivity (g/liter/h)	Yield of ethanol production (g/g)	$Y_{p/s}$ (g/g)[a]	η (%)[b]	10^7 CFU/ml	
								Yeast	Bacteria
Pure	0.1	90.0		9.00	0.50	0.50	97.8	25.0	
Contaminated by *L. fermentum* 7-1	0.1	57.2	40.0	5.72	0.32	0.41	62.2	5.3	31.0
Sulfite treatment of *L. fermentum* 7-1	0.1	78.1	4.8	7.81	0.43	0.45	84.9	12.0	1.9
Industrial fermentation broth as inoculum[c]	0.1	60.4	35.9	6.04	0.34	0.42	65.7	3.0	5.3
Sulfite treatment fermentation broth[c]	0.1	76.4	21.2	7.46	0.41	0.47	81.1	13.0	1.5

[a] $Y_{p/s}$, ethanol yield coefficient.
[b] η, theoretical ethanol yield.
[c] The culture was inoculated with fermentation broth collected from the industrial fermentor operated by Jin-Ro Fermentation with tapioca as the substrate.

Sulfite showed bactericidal activity only in the presence of molecular oxygen (Fig. 3) and was more effective in killing facultative *L. casei*, which possesses high activities of hydrogen peroxide-related enzymes, including peroxidase (Table 2). *L. fermentum*, with low peroxidase activity, was less susceptible to sulfite but very susceptible to hydrogen peroxide (Fig. 6). Sulfite conversion to a very reactive sulfur trioxide radical by horseradish peroxidase in the presence of hydrogen peroxide has been shown previously (27). It is plausible that sulfite is oxidized to the sulfur trioxide radical by peroxidase of lactic acid bacteria, killing the host, but yeast cells are not killed because hydrogen peroxide is removed by catalase in yeast cells.

In wine making, sulfite is used as an antimicrobial agent in the absence of molecular oxygen (1, 30), but this study has shown that sulfite is active only in the presence of molecular oxygen. The differences are believed to be due to the differences in the length of treatment time. In wine making, a certain concentration of sulfite should be maintained in the system for several days, while in a cell-recycled continuous ethanol fermentation, the cell paste cannot be kept for longer than several hours.

Although laboratory-scale experiments have shown that a cell-recycled continuous ethanol fermentation can be maintained successfully by controlling bacterial contamination with sulfite and hydrogen peroxide, to apply this finding to commercial-scale operations, a pilot scaleup experiment is needed. Because the concentrations of sulfite and hydrogen peroxide might be decreased by oxidation and decomposition, in the pilot plant the quantities of added sulfite and hydrogen peroxide must be controlled to maintain optimum conditions. Determinations of redox potential can be suggested as a way to measure their concentrations during treatment.

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