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Bacterial Contamination and Its Effects on Ethanol Fermentation

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Samples were collected from a commercial ethanol production plant to enumerate the bacterial contamination in each step of a starch based ethanol production process. Though the slurry of raw material used in the process carried bacteria with various colony morphology in the order of 10^4 per ml, only the colonies of white and circular form survived and propagated through the processes to the order of 10^8 per ml at the end of fermentation. Almost all of the bacterial isolates from the fermentation broth were lactic acid bacteria. Heterofermentative *Lactobacillus fermentum* and *L. salivarius*, and a facultatively heterofermentative *L. casei* were major bacteria of an ethanol fermentation. In a batch fermentation *L. fermentum* was more detrimental than *L. casei* to ethanol fermentation. In a cell-recycled fermentation, ethanol productivity of 5.72 g l^{-1} h^{-1} was obtained when the culture was contaminated by *L. fermentum*, whilst that of the pure culture was 9.00 g l^{-1} h^{-1}. Similar effects were observed in a cell-recycled ethanol fermentation inoculated by fermentation broth collected from an industrial plant, which showed a bacterial contamination at the level of 10^8 cells per ml.

Ethanol is produced by large scale fermentation. Due to its scale and unit processes where enzymes are used to hydrolyze starchy materials it is very difficult to maintain an ethanol fermentation process under aseptic conditions. Starchy materials can be sterilized during the cooking process, but a low temperature cooking process is preferred to save energy and this results in increased chances of bacterial contamination. Starchy materials are heated to 90°C with α-amylase in the low temperature cooking process, before being cooled to 60-63°C for saccharification with glucoamylase. Saccharified mash is not sterilized because glucoamylase activity is needed during fermentation for complete starch hydrolysis (8).

Most of the 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 (2, 6, 11, 12). Losses of ethanol yield due to contamination were determined quantitatively during whisky fermentation. The study showed a loss of 1-3% ethanol in the fermentation with a bacterial count of 10^6-10^7 cells per ml, and 3-5% ethanol loss with a bacterial count 10^7-10^8 cells per ml (5). Makanjuola *et al.* reported a 17% reduction of ethanol with 4.5×10^8 contaminants per ml in batch fermentation for 30 hours (11). Continuous or cell-recycled continuous processes are preferred to batch process to increase productivity (10, 18), and this is one of the factors that determine the economics of ethanol fermentation. However, bacterial contamination is more serious in those processes. It was reported that about 75% of the continuous ethanol fermentation plants constructed in the 80's in the USA failed due to the difficulties of controlling bacterial contaminations (8). The present study was undertaken to evaluate bacterial contamination during a commercial ethanol fermentation process and assess its effects on ethanol fermentation.

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Key words: bacterial contamination, ethanol fermentation

MATERIALS AND METHODS

Organisms

An industrial strain of *Saccharomyces cerevisiae* was obtained from Jin-Ro Fermentation (Ansan, Korea). Bacterial strains were isolated from the commercial ethanol plant of the same company where tapioca and barley were used as raw materials.

Isolation of Bacterial Contaminants

Samples collected from the industrial ethanol plant were serially diluted and incubated on MRS agar plates in an anaerobic glove box (Coy Lab. Co., U.S.A.). All colonies were isolated from the plates with the highest dilution of fermentation broth.

Media and Culture Conditions

Batch fermentation at 30°C was performed using PYM broth with the following constituents in each gram per liter: glucose·H_2O, 200; peptone, 20; yeast extract, 12; malt extract, 12, and pH was then adjusted to 4.5. Continuous fermentation was performed using a medium containing the following constituents in each gram per liter: glucose·H_2O, 200; yeast extract, 12; NH_4Cl, 8; $Na_2HPO_4 \cdot 7H_2O$, 3.9; KH_2PO_4, 0.5; $MgSO_4$, 0.5; citric acid monohydrate, 4.3; trisodium citric acid dihydrate, 1.25; $CaCl_2$, 0.28. $CaCl_2$ was autoclaved separately. pH was the same as PYM broth. The inoculum was prepared by growing cells aerobically in PYM broth at 30°C for 24 hours on a shaking incubator (150 rpm). Fermentation was initiated by a 5% (vol/vol) inoculum. The inocula of lactic acid bacteria were made anaerobically in Lactobacilli MRS broth (Difco Laboratories, Detroit, Michigan, U. S. A.) for 24 hours at 30°C. Phosphate buffered basal medium (PBBM) with 2% glucose was used for the analysis of bacterial fermentation products (4).

Cell-Recycled Continuous Ethanol Fermentation

The experimental set-up for cell-recycled continuous ethanol fermentation is shown in Fig. 1. A fermentor (LH 500 series, LH fermentation Ltd. Bells Hills, U.K.) with a 2 liter vessel was used with a 1 liter working volume, to which a membrane module was connected. The membrane module (Millipore Tangential-Flow system, Millipore Corporation, Bedford, U.S.A.) contained 4 membranes (hydrophilic durapore membrane, Millipore Corporation, Bedford, U. S.A.). The area of the membrane sheet was 60 cm^2 with a pore size of 0.45 μm. They were sterilized using 100-200 ppm of NaClO solution for 2 hours and washed with 10 liters of sterilized water before use. The dilution rate was 0.1 h^{-1} during the whole operation. The broth from the fermentor was fed to the membrane module at the rate of 340 ml/min, where cells were concentrated. Cell paste was returned to the fermentor. During the operation the flux of filtrate was decreased due to the membrane fouling. Intermittently a part of the permeate was flushed back through the membranes to recover filtration capacity. Continuous operation was started by the activation of the medium pump after the initial batch culture. The contaminants were inoculated to the contamination-free continuous fermentation when complete substrate consumption was achieved. pH was controlled to 4.5 with 1 N NaOH and agitation speed was 150 rpm. Temperature and air flow rate were 30°C and 0.09 vvm, respectively.

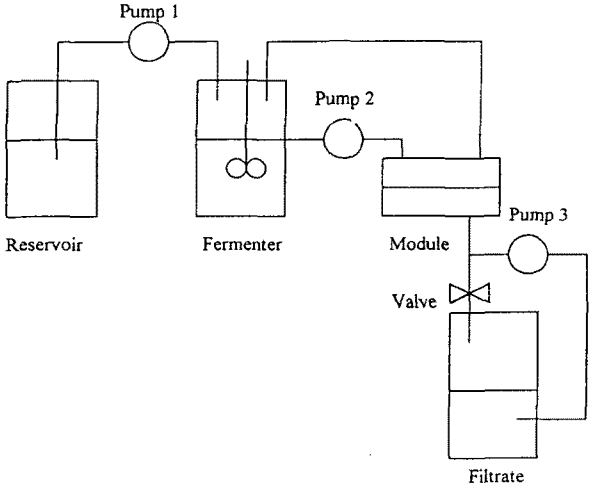

Fig. 1. Schematic diagram of cell-recycled ethanol fermentation.

Analyses

Cell concentration was determined by measuring optical density using a spectrophotometer (Jasco model UVIDEC-610, Tokyo, Japan) at 525 nm. Ethanol was quantified by gas chromatography (Varian model 3300, CA, U.S.A.) equipped with a packed column (0.2×200 cm, Super Q (Alltech, IL, U.S.A.)) and a flame ionization detector. The temperature of the injector and detector were 220, 240°C, respectively. Oven temperature was programmed from 180 to 200°C at a gradient of 5°C per minute. Nitrogen was used as carrier gas at the flow rate of 25 ml per minute. Lactate and acetate were measured by a HPLC (Young In model 910, Seoul, Korea) equipped with a Aminex HPX-87H column (Bio-rad, CA, U.S.A.) at 210 nm. The mobile phase used was 0.005 N H_2SO_4 at the flow rate of 0.6 ml/min.

Glucose was quantified by a glucose oxidase-peroxidase method using an enzyme kit (BC 103-E, Young-Dong Pharm., Seoul, Korea).

Viable cells of the yeast and the bacteria were counted using solid media containing selective inhibitors. MRS agar supplemented with cyclohexamide (10 mg per liter) was used for bacterial counts, and PYM agar supplemented with penicillin G (100 mg per liter) for yeast (12).

Identification of the Bacterial Isolates

The characteristics of the isolates were determined according to standard methods (15), and compared with those of the type cultures (7).

RESULTS

Bacterial Count During Ethanol Fermentation

Samples collected from each step of an ethanol production process were plated on MRS agar plate and

incubated under strictly anaerobic conditions to avoid the growth of yeast before the colonies were counted (Table 1). The slurry of the raw materials before liquefaction showed the CFU of about 5×10^4 per ml. Based on their colony morphologies they were grouped into yellow and irregular, white and circular and white and irregular. Only the white and irregular colonies survived during liquefaction and cooking processes, suggesting that they are spore formers. All forms of bacteria reappeared during the saccharification process, but only the white and circular forms propagated during the fermentation process.

Isolation of Bacteria and Their Identification

In order to isolate bacterial strains which contaminate ethanol fermentation, all colonies from the plates of highest dilution of the fermentation broth were selected and identified (Table 2). *Lactobacillus fermentum* and *L. casei* were two dominant contaminants of the ethanol fermentation process when tapioca was used as the raw material, whilst barley fermentation was contaminated mostly by *L. fermentum* and *L. salivarius*. A facultatively heterofermentative, *L. casei* 4-3 and a heterofermentative, *L. fermentum* 7-1 were selected among the bacterial isolates to test the effects of bacterial contamination on ethanol fermentation. The characteristics of these strains were compared with the type strains (Table 3). All the tested characteristics of the isolates were very close to the type strains.

Cultural Characteristics of Selected Strains

The selected strains were cultured on PBBM containing 20 g of glucose per liter. At the end of fermentation, the products were found to be lactate,

Table 1. Bacterial contaminants from a commercial ethanol fermentation process based on tapioca.

Stage	Colony morphology and numbers*		
	Yellow Irregular	White Circular	White Irregular
Before liquefaction	1.5×10^4	1.4×10^4	1.9×10^4
After liquefaction	1.5×10^2	1.4×10^4	1.9×10^3
After cooking	-	-	1.9×10^2
Before saccharification	4.0×10	1.1×10	1.0×10
After saccharification	4.0×10	5.0×10	1.0×10
After fermentation	-	$>3.0\times10^8$	-
Storage	-	$>3.0\times10^8$	-

*Colony numbers were counted colony forming unit (CFU/ml).

Table 2. Biochemical characteristics of bacterial contaminants from ethanol fermentation of tapioca and barley used as carbon source.

Tapioca		Barley	
Strain No.	Identification	Strain No.	Identification
4-3	*Lactobacillus casei*	S-1	*Lactobacillus* sp.
4-4	*Lactobacillus* sp.	S-3	*Lactobacillus* sp.
4-5	*Lactobacillus* sp.	S-5	*Lactobacillus salivarius*
4-6	*Lactobacillus casei*	S-61	*Lactobacillus* sp.
4-7	*Lactobacillus casei*	S-7	*Lactobacillus salivarius*
5-3	*Lactobacillus casei*	K-1	*Lactobacillus fermentum*
5-4	*Lactobacillus casei*	K-2	*Lactobacillus fermentum*
5-5	*Lactobacillus* sp.	A-1	*Lactobacillus salivarius*
6-1	*Lactobacillus fermentum*	A-2	*Lactobacillus salivarius*
6-2	*Lactobacillus fermentum*	A-9	*Lactobacillus* sp.
6-3	*Lactobacillus fermentum*	A-13	*Lactobacillus salivarius*
6-4	*Lactobacillus fermentum*	A-21	*Lactobacillus fermentum*
6-6	*Lactobacillus fermentum*	A-23	*Lactobacillus fermentum*
7-1	*Lactobacillus fermentum*		
7-2	*Lactobacillus fermentum*		
7-3	*Lactobacillus fermentum*		
7-4	*Lactobacillus fermentum*		

Table 3. Physiological and biological characteristics of the isolated and type* strains.

Strains	7-1	*L. fermentum*	4-3	*L. casei*
Arabinose	-	d	-	-
Xylose	+	d	-	-
Rhamnose	-	-	-	-
Sorbose	-		-	
Ribose	+	+	+	+
Glucose	+	+	+	+
Mannose	+	+(w)	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Sucrose	+	+	w	+
Maltose	+	+	+	+
Cellobiose	-	d	-(w)	+
Lactose	-	+	-	d
Trehalose	+	d	+	+
Raffinose	+	+	-	-
Melezitose	-	-	+	+
Starch	-	-	-	-
Glycogen	-		-	
Inulin	-		-	
Glycerol	-	-	-(w)	
Mannitol	-	-	+	+
Sorbitol	-	-	+	+
Erythritol	-		-	
Inositol	-		w	
Dulcitol	-		-	
Esculin	-	-	+	+
Salicin	-	-	+	+
Amygdalin	-	-	+	+
Starch hydrolysis	-	-	-	-
Esculin hydrolysis	-	-	+	+
H_2S production	-	-	-	-
Indol production	-	-	-	-
Nitrate reduction	+		-	

Symbols: +, 90% or more positive, -, 90% or more negative, d, 11-89% positive, w, weak reactions, +w, positive weak reactions, -w, negative weak reactions. *The data of type strains taken from Bergey's Manual of Systematic Bacteriology vol. 2.

ethanol and acetate (Table 4). Both strains produced lactate and acetate and ethanol was produced only by isolate 7-1. These results further substantiate the identification of *L. fermentum* 7-1 and *L. casei* 4-3. Among the selected strains *L. fermentum* 7-1 produced more acetate, which is more inhibitory than other products to yeast ethanol fermentation.

The Effect of Bacterial Contaminations on Batch Fermentation

PYM was inoculated by yeast alone, and with *L. fermentum* 7-1 or *L. casei* 4-3 and the ethanol production was monitored (Fig. 2). The initial colony forming unit (CFU) of yeast was about 10^4 cells per ml and those of the bacterial isolates were about 10^6 cells per ml. Few differences were observed in the ethanol production at the beginning of fermentation. But the final ethanol concentration was about 10% less in the fermentation made with *L. fermentum* 7-1 than in the control. The ethanol yield in the fermentation with *L. casei* 4-3 was reduced by about 6%.

Cell-Recycled Fermentation

A cell-recycled ethanol fermentation was run for several days to achieve complete glucose consumption. At this stage cell concentration was 2.5×10^8 cells per ml with ethanol productivity of 9.0 g l^{-1} h^{-1} (Table 5). *L. fermentum* 7-1 was inoculated to the fermentor to test the effect of bacterial contamination on the fermentation. The cell-recycled ethanol fermentation was run for 3 days after bacterial inoculation, before the fermentation parameters were determined (Table 5). The bacterial contamination resulted in the reduction of all the fermentation parameters analyzed including ethanol productivity, substrate utilization and yeast concentration. The bacterial concentration was 3.1×10^8 cells per ml.

Cell-Recycled Fermentation Inoculated by Fermentation Broth Collected from an Industrial ethanol Plant

A separate cell-recycled fermentation was run. The fermentation broth collected from a commercial ethanol plant was used to inoculate the fermentor. The system was run for 3 days before the fermentation parameters were determined. The results are shown at the bottom of Table 5. The yeast and bacterial counts were lower than that of fermentation contaminated deliberately by *L. fermentum* 7-1, and the glucose concentration in the effluent was also lower. The ethanol concentration and productivity were 60.4 g l^{-1} and 6.04 g l^{-1} h^{-1}, respectively. These figures are much lower than the control, and substantially higher than that contaminated by *L. fermentum* 7-1.

DISCUSSION

Bacterial contamination which decreases ethanol productivity is unavoidable in an industrial ethanol fermentation process. This fact was confirmed in this study. Even a successful batch fermentation showed

Table 4. Fermentation products profiles of selected bacterial contaminants.

Strain	Products (mM)		
	Lactate	Ethanol	Acetate
Lactobacillus fermentum 7-1	51.9	25.2	6.60
Lactobacillus casei 4-3	220	-	5.80

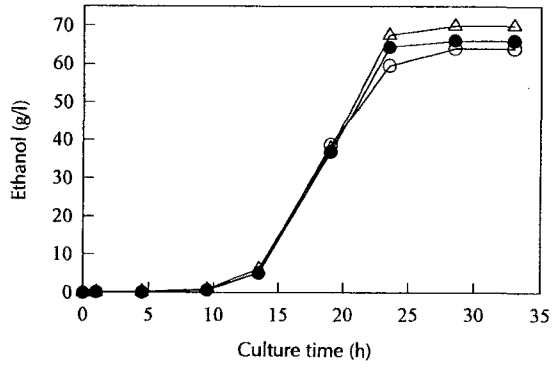

Fig. 2. The effect of bacterial contamination on batch ethanol fermentation.
○: mixed culture with *Lactobacillus fermentum* 7-1, ●: mixed culture with *Lactobacillus casei* 4-3, △: pure culture.

Table 5. The effects of bacterial contamination on cell-recycled ethanol fermentation.

Culture	D	P	S	PD	Y	Yp/s	η	Cell No.	
								Yeast	Bacteria
Pure	0.1	90.0	-	9.00	0.50	0.50	97.8	25	-
Contaminated by									
L. fermentum 7-1	0.1	57.2	40.0	5.72	0.32	0.41	62.2	5.3	31
fermentation broth*	0.1	60.4	35.9	6.04	0.34	0.42	65.7	3.0	5.3

D: Dilution rate (h^{-1}), P: Ethanol concentration (g/l), S: Glucose concentration (g/l), PD: Ethanol productivity (g/l h), Y: Yield of ethanol production (g/g), Yp/s: Ethanol yield coefficient (g/g), η: Theoretical ethanol yield (%), Cell No.: Viable cell number ($\times 10^7$ CFU/ml).
*: The culture was inoculated by fermentation broth collected from the industrial fermentor operated by Jin-Ro using tapioca as the substrate.

bacterial counts of over 3×10^8 (Table 1). Ethanol productivity is one of the major factors affecting the economics of ethanol production. Cell-recycled continuous processes have been developed to obtain higher ethanol productivities, but bacterial contaminations are much more serious in these processes than in a batch process (8). The ethanol productivity decrease of about 10% was illustrated by bacterial contamination of batch fermentation (Fig. 2), whilst over a 30% reduction in productivity was observed in a cell-recycled continuous process (Table 5). These results show that the contaminating bacteria can grow faster than the yeast in continuous process systems.

The raw materials for ethanol production contain various endogenous bacteria, but only lactic acid bacteria were able to propagate during the ethanol fermentation (Table 1). From these results it can be said that ethanol fermentation conditions are not suitable for the growth of common soil bacteria and enteric bacteria possibly due to the toxic effects of ethanol and high osmotic pressure. Ethanol oxidizing acetic acid bacteria were not considered in the study, because they cannot survive at a low oxygen tension such as those found under ethanol fermentation conditions.

All the bacterial strains isolated from the ethanol fermentation broth were lactic acid bacteria. This finding support earlier reports (11, 12). The predominating bacteria were *Lactobacillus fermentum*, *L. casei* and *L. salivarius*. They are either obligate or facultative heterofermentative. It is not clear if the heterofermentative bacteria are predominant due to their ability to produce acetate, which is more toxic to the yeast than lactate (9). It was shown that *L. fermentum* produced more acetate (Table 4) and caused more deterioration to the yeast than *L. casei*.

Few processes have been developed to control bacterial contaminations during ethanol fermentation. One of the most widely used processes is acid pickling (14, 16). Cells are collected from the fermentation broth and sulfuric acid is used to adjust the pH of the cell paste to 2.0, and kept for 2 hours at this pH before being returned to the fermentor. This method can be successfully applied to a batch fermentation but is not satisfactory in a cell-recycled continuous process. Other methods are the uses of antibiotics (1, 3). β-lactam antibiotics can be used to control the contaminants, but it is a very expensive method. A novel process can be developed to control bacterial contaminations in ethanol fermentation based on the physiological differences between the yeast and the heterofermentative lactic acid bacteria.

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