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NOTE

Prevention of bacterial contamination using acetate-tolerant *Schizosaccharomyces pombe* during bioethanol production from molasses

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Bacterial contamination causes yield reduction during ethanol production from molasses. To prevent contamination, construction of a fermentation system using acetate-tolerant yeast under an acetate-containing condition was attempted. *Schizosaccharomyces pombe* was screened as an acetate-tolerant strain. Bacterial contamination was significantly prevented by the combined use of *Sc. pombe* and acetate.

[**Key words:** Acetate-tolerant yeast; Bacterial contamination; Bioethanol; Molasses; Co-cultivation; *Schizosaccharomyces pombe*]

Bioethanol is one of the most important renewable fuels contributing to the reduction of the global warming effect and negative environmental impact generated by the worldwide utilization of fossil fuels. Bioethanol production generally utilizes derivatives from food crops such as corn grain and sugar cane. In Southeast Asian nations such as Thailand, sugar cane molasses is mainly used as feedstock for bioethanol production. This study focuses on improvement of the bioethanol production process from molasses.

Bacterial contamination is known to be a major cause of reduction in ethanol yield during ethanol production from molasses because of sugar consumption by bacteria (1–4). Such bacteria also produce a by-product which inhibits yeast growth (2). *Lactobacillus* sp. and *Bacillus* sp. may be the most harmful of the bacteria that contaminate molasses because of their rapid growth abilities (3). Because *Lactobacillus* sp. are tolerant to high temperature and low pH, it is especially difficult to prevent *Lactobacillus* sp. from growing (5).

It has been reported that various agents, including antiseptics such as sulfite, hydrogen peroxide, 3, 4, 4-trichlorocarbanilide, and urea hydrogen peroxide (1, 4, 6) and antibiotics such as penicillin, tetracycline, monensin, and virginiamycin (7, 8) are effective in preventing bacterial contamination. Penicillin and virginiamycin are currently used commercially to prevent contamination in the bioethanol production process (7, 8), and some facilities use these antibiotics prophylactically. *Bacillus* sp. and *Lactobacillus* sp. isolated from Brazilian industrial fermentation units were shown to be susceptible to penicillin and the ionophore antibiotic monensin (8). However, antibiotics remaining in the waste, which should be recycled, can lead to the emergence and spread of tolerant mutants to the antibiotics in the environment and consequently create a direct impact on food, animals and human health. It is particularly important to prevent bacterial contaminants during bioethanol production without using antibiotics. Previously, we proposed that a fermentation system using the lactate-tolerant yeast *Candida glabrata* under a lactate-containing condition was effective for preventing *Lactobacillus* sp. contamination (9). However, *C. glabrata* cannot be used for ethanol production from molasses because it does not have the ability to assimilate sucrose.

In this study, we focused on acetate as an agent for the prevention of bacterial contamination during ethanol production from molasses because acetate has anti-bacterial activity at low concentrations (10), and is also inexpensive compared with other organic acids. To design a bioethanol production process under acetate-containing conditions, acetate-tolerant yeast is needed because acetate reduces the fermentation ability and growth of typical yeast used for ethanol production, such as *Saccharomyces cerevisiae*. Phowchinda et al. observed a 75% reduction in the maximum specific growth rate of *S. cerevisiae* when 6 g/l of acetate was added to the medium (11). To construct the bioethanol production process under acetate-containing conditions, the screening of acetate-tolerant yeast is considered necessary. Acetate-tolerant yeasts were previously isolated for baking and galactose fermentation (12, 13). These strains showed acetate tolerance at a concentration of 0.8% (w/v). However, there have been no reports on the isolation of acetate-tolerant yeasts suitable for producing ethanol from molasses. In this study, we show the results of screening of acetate-tolerant yeast and higher abilities of ethanol production from molasses under acetate-containing conditions. We also show that the screened strain is suitable for constructing a molasses fermentation system which eliminates bacterial contamination.

To determine the possibility of designing an ethanol production process which eliminates bacterial contamination, the effects of acetate addition on bacterial growth were analyzed. We selected *Bacillus subtilis* and *Lactobacillus* sp. including *Lactobacillus plantarum*,

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TABLE 1. Genetic and phenotypic characteristics of strain NFRI 3807.

Parameter or test	Characteristic
Homology of 26s rDNA partial sequence	99.6% identity with *Sc pombe* NRRL Y-12796^T
Spore formation	Oval or round ascospores
Assimilable sugars	D-glucose and d-saccharose
Non-assimilable sugars	Glycerol, calcium 2-keto-gluconate, L-arabinose, d-xylose, adonitol, xylitol, D-galactose, inositol, D-sorbitol, d-lactose, methyl-α-D-glucopyranoside, *N*-acetyl-glucosamine, d-maltose, d-treharose, l-melezitose and d-raffinose

NFRI: National Food Research Institute, Tsukuba, Japan.
NRRL: National Center for Agricultural Utilization Research, Peoria, IL, USA.

L. paracasei subsp. *paracasei* as models of contamination bacteria, because previous studies suggested that such strains were dominant contaminants in molasses during the ethanol production process (1, 3). To estimate the sensitivity of model bacteria to acetate, the growth in molasses medium containing 0–1% acetate was monitored. Molasses medium (2% of sugar cane molasses, 0.225% of urea and 0.046% of KH_2PO_4) was prepared according to Nishida et al. (14) with modification. *B. subtilis* IFO 3007 or *Lactobacillus* sp. containing *L. plantarum* ATCC 8041 and *L. paracasei* subsp. *paracasei* JCM 1181 were pre-cultured at 30 °C for 24 h using TSB medium (Difco Laboratory, Detroit, USA) or MRS medium (Difco Laboratory), respectively. Portions of the pre-culture were transferred to molasses medium containing 0–1% acetate at the cell density of 3×10^6 cell ml^{-1}. The growth of the strains was monitored as the optical density at 600 nm (OD_{600}). All bacterial strains tested grew rapidly and showed maximum growth after 12 h cultivation in acetate-free molasses medium (data not shown). However, the growth of the bacterial strains was completely inhibited in molasses medium containing 0.7% acetate or higher (data not shown). We also found that the bacterial strains could not grow, although they could survive, under the acetate-containing condition determined by viable counts assay (data not shown). It is suggested that the addition of 0.7% acetate may be effective for inhibiting bacterial growth in molasses medium, although disinfection of such bacteria is not possible.

To obtain acetate-tolerant yeasts that could grow in molasses medium containing elevated levels of acetate, we first assessed the growth of approximately 1700 yeast strains obtained from the Microbiological Bank of the National Food Research Institute (NFRI) and from nature. The yeast strains from the NFRI Microbiological Bank were grown in microtiter plates (Corning Inc., Corning, NY, USA) containing 100 μl of YPD medium (1% of yeast extract [Difco Laboratory], 2% of peptone [Difco Laboratory], 2% of glucose) at 30 °C for 48 h (pre-culture). Portions (1.2 μl) of the pre-culture were transferred into molasses medium containing 0.5–1.0% (v/v) of acetate and incubated at 30 °C for 48 h. OD_{630} of the cultures was measured by a microtiter plate reader (Elx800; Bio Tek Instruments, Winooski, VT, USA). We found three strains (NFRI 3807, NFRI 3815 and NFRI 3820) which grew rapidly, and had turbidities exceeding $OD_{630} > 0.75$ at 48 h cultivation (data not shown). The ethanol contents of the cultures were measured by the method described previously (9). Among the screened strains, NFRI 3807 produced ethanol in the molasses medium containing acetate up to 1.0% for 48 h. Therefore, strain NFRI 3807 was selected for further studies.

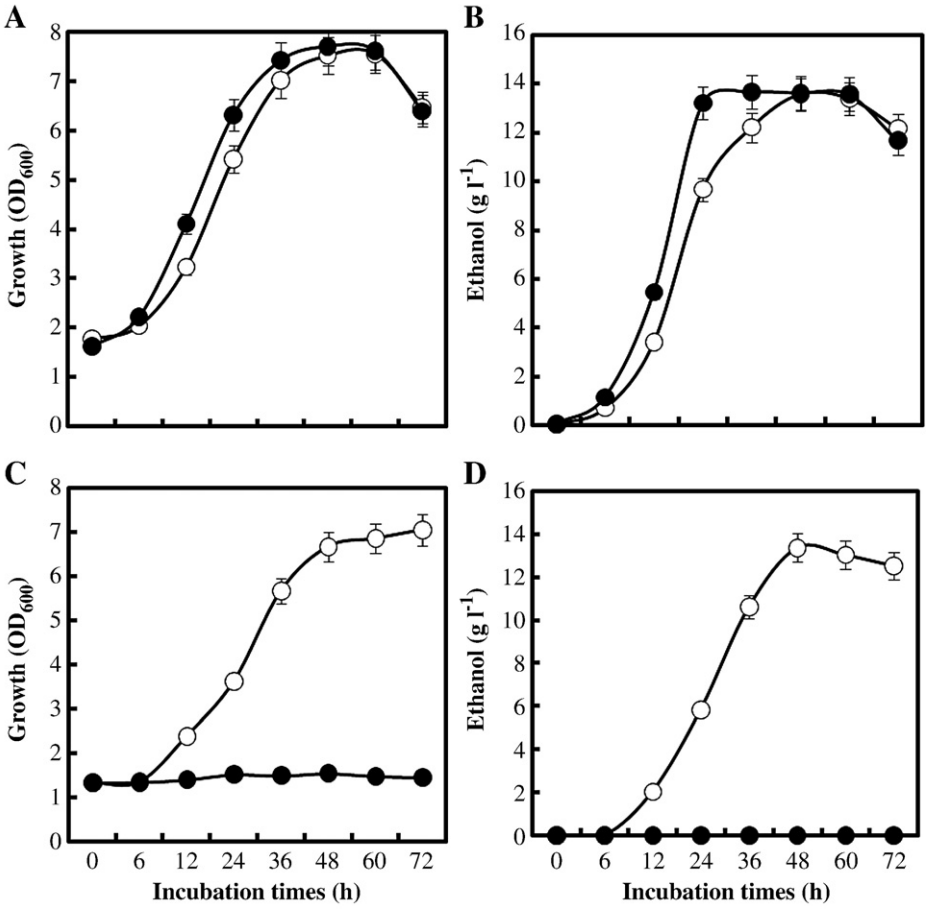

FIG. 1. The effects of acetate addition on the growth (A, C) and ethanol production (B, D) of *S. cerevisiae* NBRC 0224 (closed circles) and *Sc. pombe* NFRI 3807 (open circles). The growth rate and ethanol production in acetate-free molasses medium (A, B), and in molasses medium containing 1% acetate (C, D) were monitored. Data shown are mean ± SD.