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Hilary, Zakpaa D.
Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

Tanaka, Kenji
Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

Ishizaki, Ayaaki
Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

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Preliminary investigation of the effect of the use of pineapple juice and the waste on ethanol production by *Zymomonas mobilis*

Zakpaa D. Hilary, Kenji Tanaka and Ayaaki Ishizaki

Laboratory of Microbial Technology, Faculty of Agriculture,
Kyushu University, Fukuoka, 812–8581 Japan
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The effect of the use of the juice of rotten–or discharged pineapple fruits and the wastes derived from the processing of pineapple juice on ethanol production by *Z. mobilis* was investigated. *Z. mobilis* ATCC 10988 produced 59.0 gl^{-1} of ethanol in the undiluted pineapple juice although the supplementation of any nutritions and the controlling of pH were not carried out. Unhydrolyzed waste and the enzymatically hydrolyzed waste were also converted to ethanol at the relatively high yield coefficients. These results suggest that pineapple juice and the waste can be useful for ethanol production by *Z. mobilis*. Further, it was shown that the use of expensive organic–nitrogen complex such as yeast extract and the controlling of pH can be omitted from the fermentation process using pineapple juice and the waste.

INTRODUCTION

The crop of pineapple fruit (*Ananas comosus*) is increasing in tropical regions of the world and 12,769,960 tons of pineapple were produced over the world in 1997. Most of pineapple fruits are consumed as fresh product or processed fruit (mainly canned) but very high quality fruits are only selected for the processing and shipments. Low quality fruits is therefore left to rot on the farms due to the lack of markets. A large proportion of pineapple fruits are processed into juice, leaving a large amount of unusable pulp as waste material. This pulpy waste still contains 10% (w/w) of sugars (mainly sucrose), in addition starch and hemicellulose. On the other hand, pineapple juice contains a lots of organic nitrogen compounds and minerals. Then, it is expected that the juice from these discarded fruit as well as the waste can be useful as the low cost substrates for the fermentative production of biofuel ethanol.

The aim of this study is to investigate the effect of the use of feasibility of the juice from discharged pineapple fruit and the waste on the ethanol fermentation by *Z. mobilis* ATCC 10988.

MATERIALS AND METHODS

Microorganism

Z. mobilis ATCC 10988, was used throughout this study.

Sample preparation

Pineapples were purchased from a supermarket and left to fully ripen at room

temperature for 10 days. The pineapples were peeled, and blended in a house-hold juice maker for 3 min. The juice was then extracted by squeezing through a double-fold of gauze cotton cloth, and particulate matters were removed by centrifuging at 10,000×g for 10 min at 4 °C. The undiluted juice, containing approximately 125.0 gl^{-1} sucrose and the waste material obtained by the extraction of juice were separately stored at 20 °C.

Enzymatic hydrolysis of pineapple waste

Meicelase, a commercial cellulase provided by Meiji Seika Co. Ltd. (Tokyo), was used for the enzymatic hydrolysis of the pineapple wastes. A 60% (v/v) suspension of pineapple waste was first prepared and the pH was adjusted to 4.8 with 5.0 M NaOH. Enzymatic hydrolysis of the waste was carried out at 50 °C and for 24 hours. The enzyme was used at a protein concentration of 0.3 mg/ml with a specific activity of 1.82 (units/mg) in filter paper assay. The reaction was terminated by heating for 10 min in boiling water.

Preparation of culture media

When unhydrolyzed pineapple waste was used as the substrate for ethanol fermentation by *Z. mobilis*, the waste was appropriately diluted with distilled water before use. pH was adjusted to 5.5 with 5.0 M NaOH then the diluted waste was autoclaved at 121 °C for 20 min. When enzymatically hydrolyzed waste was used as substrate, the hydrolyzate was diluted with distilled water to give 40%, 30% and 15% (v/v) aliquots. Two hundred milliliter of each diluted hydrolyzate were transferred into 300 ml flasks with cotton-stoppers. Three sets of flasks were prepared for each solution. The first set of flasks were not supplemented with any organic nutrients or mineral salts. The second set of flasks were supplemented with 5.0 gl^{-1} yeast extract. The third set of flasks were supplemented with 0.5 gl^{-1} $MgSO_4 7H_2O$. Control cultures were also carried out by using a natural culture media containing sucrose as carbon source or glucose. The natural medium was composed of 10 gl^{-1} yeast extract; 1 gl^{-1} KH_2PO_4; 1 gl^{-1} $(NH_4)_2SO_4$ and 0.5 gl^{-1} $MgSO_4 7H_2O$, and was autoclaved at 121 °C for 20 min before use. The pHs of all culture media were adjusted to 5.5 with 5 M NaOH, then they were autoclaved at 121 °C for 20 min.

Culture conditions

Z. mobilis was refreshed by inoculating 0.5 ml of the stock culture to 10 ml of YM liquid medium (Difco) in a test tube then incubating at 30 °C for 18 h without shaking. Ten milliliter of the broth was transferred to 40 ml of YM medium and incubated for 18 h at 30 °C. Cells were harvested by centrifugation at 10,000×g for 10 min at 4 °C, then rinsed with 50 ml of autoclaved distilled water and centrifuged again. The cells were suspended in 50 ml of sterilized distilled water and used for flask culture and batch culture. Batch culture was carried out using a glass jar fermenter with a total volume of 1 l. The working volume was 500 ml and the agitation speed was 200 rpm. The temperature was kept at 30 °C. In some cases, pH was automatically maintained at 5.5 by the addition of 2.0 M NaOH with a pH controller (PHC-2201, Biott Co., Ltd., Tokyo, Japan). Investigations using pineapple waste were carried out using 300 ml conical flasks with cotton stopper at 30 °C for 24 h.

Analytical methods

Ethanol concentration was determined by gas chromatography (GC 8 APE; Shimadzu Co. Ltd., Kyoto, Japan) equipped with a PEG column (80/100 mesh). The temperatures in the column oven and the injection room were set to 70 °C and 90 °C, respectively. Glucose concentration was determined by a glucose analyzer (Model 23 A, Yellow Spring Instrument Co. Ltd., Ohio, USA). Sucrose concentration was determined by using β -fructosidase (Boehringer Mannheim, Germany). Supernatant of culture broth was diluted with 2.0 M acetate buffer (pH 4.6) including the enzyme then the solution was incubated at 56 °C for 90 min. Concentration of liberated glucose was determined by the glucose analyzer.

RESULTS

Ethanol production with undiluted pineapple juice

Ethanol production by *Z. mobilis* ATCC 10988 using undiluted pineapple juice was first investigated by batch culture. The control culture was also carried out using the natural culture medium containing 110 $g l^{-1}$ sucrose. The controlling of pH during cultivation was not carried out. Figure 1 shows the time courses of ethanol production. The final concentration of ethanol was 59.0 $g l^{-1}$ while that in the culture with 110 $g l^{-1}$ sucrose medium was 36.9 $g l^{-1}$. Figure 2 shows the change in residual concentrations of

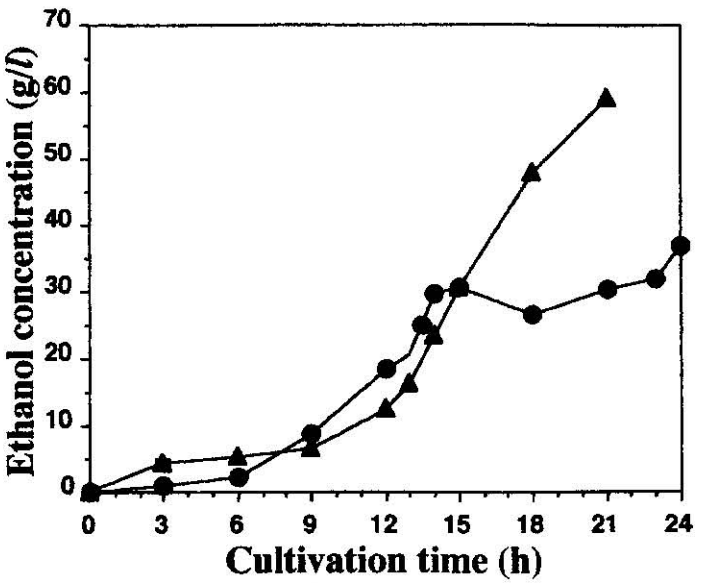

Fig. 1. Time course of ethanol production in batch cultures of *Z. mobilis* ATCC 10988 using undiluted pineapple juice and natural culture medium without controlling pH. Symbols: undiluted pineapple juice; (▲), natural medium containing 110 $g l^{-1}$ sucrose; (●).

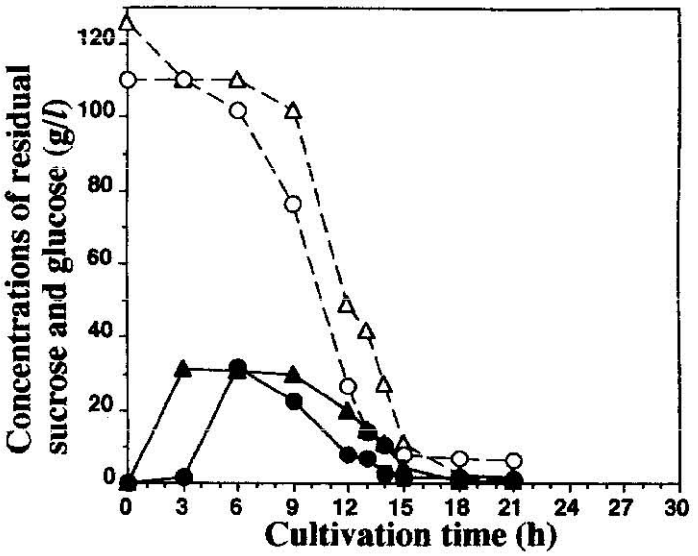

Fig. 2. The changes in residual sugar concentration in batch cultures of *Z. mobilis* ATCC 10988 using undiluted pineapple juice and natural culture medium without controlling pH. Symbols: undiluted pineapple juice; (▲), natural medium containing 110 $g l^{-1}$ sucrose; (●). Solid lines indicate glucose concentration and broken lines indicate sucrose concentration.

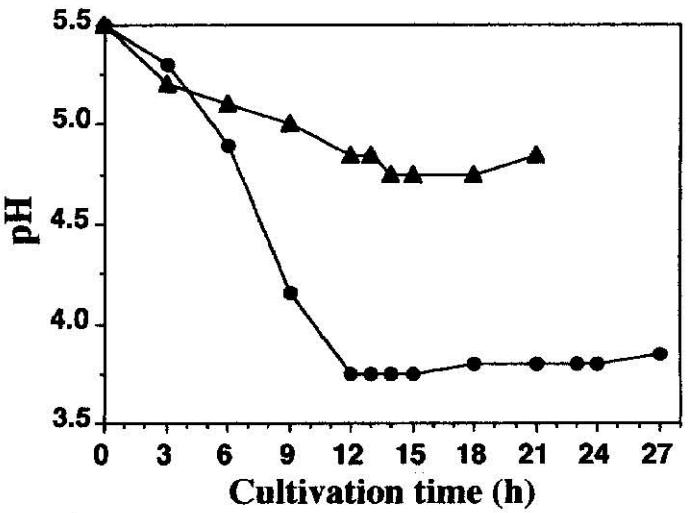

Fig. 3. The changes in pH in batch cultures of *Z. mobilis* ATCC 10988 using undiluted pineapple juice and natural culture medium without controlling pH. Symbols: undiluted pineapple juice; (▲), natural medium containing 110 $g l^{-1}$ sucrose; (●).

Table 1. The results of ethanol fermentation by *Z. mobilis* ATCC 10988 using pineapple juices supplemented with 5.0 g/l yeast extract or 0.5 g/l $MgSO_4 7H_2O$

Nutrients supplemented to prineapple juice	Fermentation time (h)	Ethanol yield referred to theoretical yield (%)
5.0 g/l yeast extract	21	97.7
5.0 g/l $MgSO_4 7H_2O$	21	78.3

glucose and/or sucrose in each cultivation. Sucrose was hydrolyzed to glucose and fructose then utilized by *Z. mobilis* ATCC 10988. The yield of ethanol in the batch culture with pineapple juice was 92.4% of theoretical yield while that with 110 $g l^{-1}$ sucrose medium was only 65.6%. These fermentation results suggest that pineapple juice contains adequate amounts of organic nitrogen compounds which are essential for the sufficient cell growth of *Z. mobilis*. Filtered pineapple juice contains 17.1 $g l^{-1}$ protein; 3.3 $g l^{-1}$ total sugars; 2.6 $g l^{-1}$ citric acid; 0.7 $g l^{-1}$ ascorbic acid; and 0.6 $g l^{-1}$ phenolics (Lozano-de-Gonzalez et al., 1993). Therefore, it was expected that the juice extracted from rotten or discarded pineapples can be fermented by *Z. mobilis* without supplementing with yeast extract. Figure 3 shows the change in pH during batch culture. In the batch culture using the natural medium with 110 $g l^{-1}$ sucrose, pH decreased to 3.8 after 12 h of cultivation. In the culture using pineapple juice, the decrease in pH was very slow and pH was always higher than 4.8 until the fermentation finished. It was thought that pineapple juice has strong buffering action against pH change, which resulted in the reduction in fermentation time in the culture with pineapple juice than in the culture with sucrose medium. The effect of the addition of 5.0 $g l^{-1}$ yeast extract or 0.5 $g l^{-1}$ $MgSO_4 7H_2O$ into pineapple juice on the ethanol production by *Z. mobilis* was investigated by batch culture without controlling pH. The fermentation results are shown in Table. 1. The use of yeast extract slightly increased ethanol yield from pineapple juice however the use of $MgSO_4 7H_2O$ decreased ethanol yield.

Ethanol production with pineapple waste by *Z. mobilis*

The bulk of the waste discharged from the pineapple juice-manufacturing process is pulp of the fruit. This bulky waste is rich in fiber, carbohydrates and unextracted juice. Therefore, ethanol fermentation using the pineapple waste was also investigated. Figure 4 shows the concentrations of ethanol after 24 h of cultivation when various dilutions of unhydrolyzed pineapple waste were used. More than 3.5 $g l^{-1}$ ethanol was produced in the culture using the 15% (v/v) dilution of unhydrolyzed waste. In the control cultures using 5 $g l^{-1}$ sucrose and 5 $g l^{-1}$ glucose media, ethanol production were 1.3 $g l^{-1}$ and 2.3 $g l^{-1}$, respectively. The ethanol production by *Z. mobilis* with the hydrolyzed waste was also investigated and the result is shown in Fig. 5. When the 15%, 30% and 40% (v/v) dilutions of the hydrolyzed waste were used, the yields of ethanol were 5.0 $g l^{-1}$, 7.6 $g l^{-1}$, and 9.3 $g l^{-1}$, respectively. The addition of 5.0 $g l^{-1}$ yeast extract to hydrolyzed waste didn't

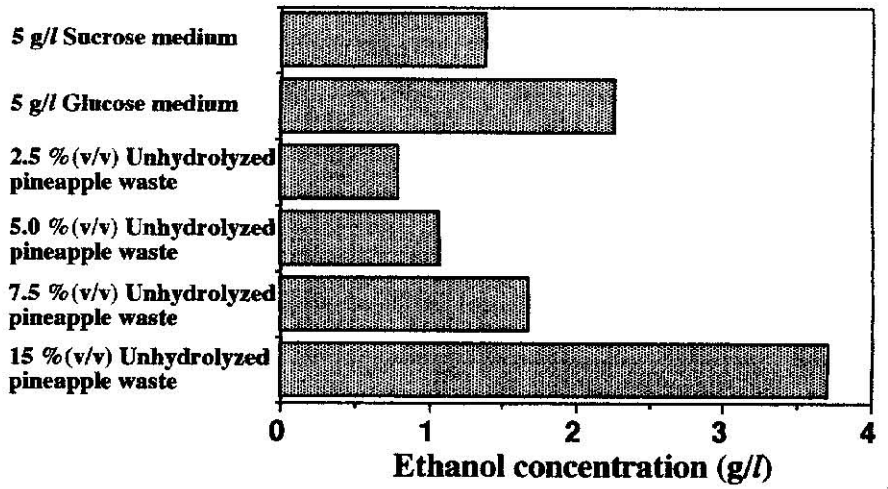

Fig. 4. Ethanol production in flask cultures of *Z. mobilis* ATCC 10988 using various dilutions of unhydrolyzed pineapple waste.

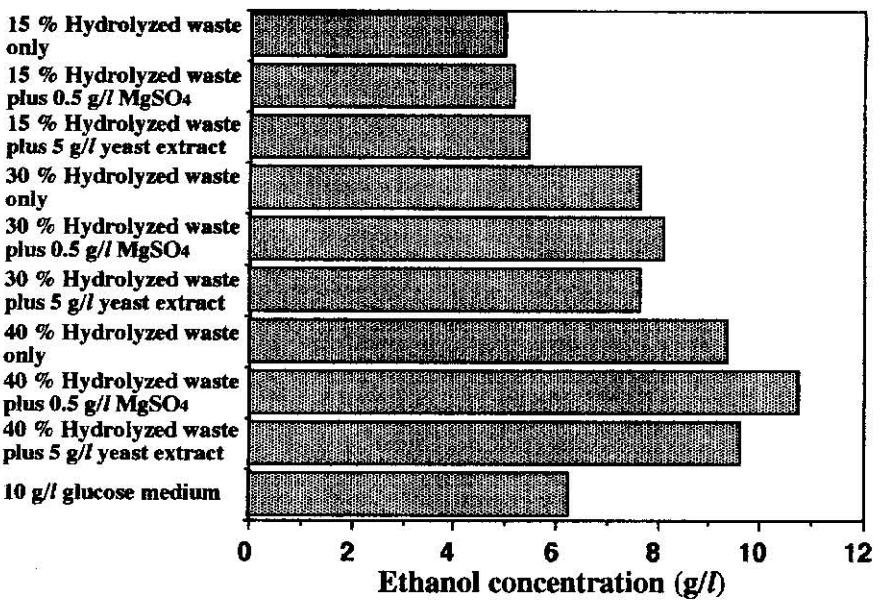

Fig. 5. Ethanol production in flask cultures of *Z. mobilis* ATCC 10988 using various dilutions of hydrolyzed pineapple waste.

promote ethanol production by *Z. mobilis*. On the other hand, the addition of 0.5 g l^{-1} $MgSO_4 7H_2O$ into the hydrolyzed waste solutions slightly increased ethanol production.

DISCUSSION

Alain et al. reported the ethanol production from pineapple juice by the best-preselected yeast strains (Alan, 1987). When *Saccharomyces cerevisiae* var. sake was used, the maximum yield of ethanol (89% of theoretical yield) was obtained but the productivity was 1.44 g $l^{-1}h^{-1}$. When *S. cerevisiae* CM1 was used, the maximum ethanol productivity (2.22 g $l^{-1}h^{-1}$) was obtained but the ethanol yield was 77%. In our study employing *Z. mobilis* ATCC 10988, the ethanol yield from pineapple juice was 92.4% of theoretical yield and the productivity was 2.68 g $l^{-1}h^{-1}$. On the other hand, in the culture with the pineapple wastes, the yield coefficient of ethanol was not determined completely. The pulpy waste (press cake) of the pineapple fruits consisted of about 80% (w/w) of water, 10% (w/w) of saccharides and 10% (w/w) of other components. Therefore, theoretical yield of ethanol from the waste was estimated to be about 0.51 g-ethanol/g-waste. When a 60% (v/v) suspension of pineapple waste was hydrolyzed and 30% (v/v) dilution of the hydrolyzate was used for fermentation, 7.6 g l^{-1} of ethanol was produced. Then, in the case of using 30% (v/v) dilution of hydrolyzed waste, the yield of ethanol in the culture was estimated to be 82.6% of the theoretical yield. Further, it was shown that the pineapple juice and the waste contain a lots of organic nitrogen compounds which are essential for the growth of this microorganism, and the controlling of pH by the addition of alkali can be omitted. These results suggest that the juice from discharged pineapple fruits and the waste can be useful as low cost substrate for the production of biofuel ethanol by *Z. mobilis*.

The addition of magnesium sulfate into hydrolyzed waste slightly promoted ethanol production by *Z. mobilis*. It is known that magnesium ions are cofactors for a variety of enzymes of the Entner-Doudoroff pathway (Osman and Ingram, 1985). Further, magnesium ions have the ability to restore the stability of outer membrane permeability of the cell, therefore the addition of magnesium ions is useful for the restore of the ethanol-damaged *Z. mobilis* cells and for the preventing the cells from the leakage of intermediates. This is particularly important when ethanol concentration in culture system increased. However, the reason why ethanol yield decreased in the culture using pineapple juice which was supplemented with 0.5 g l^{-1} $MgSO_4$ is not clear at present.

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