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Microbiological and Chemical Changes in Indonesian Tea Cider Fermentation

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Abstract Twelve yeast strains were isolated from traditionally fermented Indonesian tea cider. Seven of them were high alcohol producers and were identified as *Candida variabilis, C. famata, C. guilliermondii* and *C. fennica. Acetobacter* sp. was also found among 35 strains of bacterial isolates. This bacterium and 7 selected yeast strains were used in various combinations to study their ability to produce good quality and acceptable tea cider. The changes of sugar, acid, pH and alcohol production were monitored to determine the progress of fermentation. Sensory evaluations were carried out to determine the organoleptic acceptability. The result indicated that the combination of *Acetobacter* sp. with *C. fennica* gave the best tea cider. The optimum time of incubation was from 6 to 9 days.

INTRODUCTION

Tea cider is a kind of beverage made from tea extract. A zoogloid mat forms on its surface at the end of production. This kind of beverage is consumed widely in Russia, Japan, Poland, Bulgaria, Germany, Tibet and Indonesia (STEINKRAUS, 1983 and CAMBELL-PLATT, 1987). It has a refreshing, sweet and sour taste with a low alcohol content. In Indonesia, the tea cider was popular before World War II. However, this popularity declined gradually and it is now only consumed by a small section of the populace.

HESSELTINE (1965) reported that the essential microorganisms, in tea cider production, were *Acetobacter aceti* subsp. *xylinum* and two yeasts (NRRL Y-4810 and NRRL Y-4882). Futhermore, the mats or films were formed only when these three organisms were present together. KOZAKI *et al.* (1972) reported that *Acetobacter aceti* subsp. *xylinum* was the principal microorganism. They isolated several types of yeasts: *Saccharomyces* sp., *Torulopsis famata, Pichia membranaefaciens* and *Candida guilliermondii* from Japanese tea cider, and *Klockera apiculata* from Formosan tea cider.

In our laboratory, we isolated from traditionally fermented Indonesian tea cider several types of *Candida,* which included: *C. variabilis, C. famata, C. guilliermondii and C. fennica.* The *Acetobacter* sp. was also found in this beverage.

This study was carried out to characterize tea cider microorganisms. The aim is to scientifically produce a tea cider product in the future with a standard and acceptable quality.

MATERIALS AND METHODS

Preparation of original tea cider

The original tea cider was prepared in Indonesia, as shown in the Fig. 1. The tea preparation was brought to Japan where the microorganisms were isolated.

Isolation of microorganisms

Serial dilutions of 1 ml of the original tea cider was made in sterile 0.85% NaCl solution. Enumeration and isolation was carried out on PCA (malt extract, 2.0%; peptone, 0.1%; glucose, 2.0%; and agar, 2.0%; adjusted to pH 6.0). Acetic acid bacteria was isolated using medium containing 0.5% yeast extract, 1.5% ethanol and 2.5% agar (DE LEY *et al.*, 1984). All incubation was carried out at 30°C for 48 hours. Several yeasts and bacteria were picked up from separate colonies which showed morphological differences under a microscope.

Identification of yeasts and acetic acid bacteria

The methods described by KREGER VAN RIJ (1984) were used in the determination of the morphological and physiological properties of the yeast isolates and their identification. For the acetic acid bacteria, the identification was based on the methods described by J. DE LEY *et al.* (1984). The production of acetic acid from alcohol was tested using ethanol carbonate agar which consisted of 1% yeast extract, 1.5% agar, 2% calcium carbonate and 2% ethanol (SKERMAN, 1967). The same tests were carried out using *Acetobacter aceti* subsp. *xylinum* IFO 3288 obtained from Institute for Fermentation, Osaka, for comparison.

Preparation of tea cider

Tea extract was prepared as shown in Fig. 1, using dry tea leaves obtained from the local market. It was then sterilized and cooled. To select suitable yeast strains, each strain was inoculated in separate samples of sterile tea extract. The samples were incubated at 30°C for 12 days, after which the alcohol contents were determined. The bacterium and selected yeasts were tested for their ability to produce a tea cider product with acceptable quality. All combinations of an *Acetobacter* sp. and one of selected yeast strain were inoculated in samples of the tea extract. The samples were incubated at 30°C for 0, 3, 6, 9, 15 days. The progress of fermentation was monitored by the changes of pH, total acidity, sugar and alcohol production. A sensory evaluation was also carried out on the products.

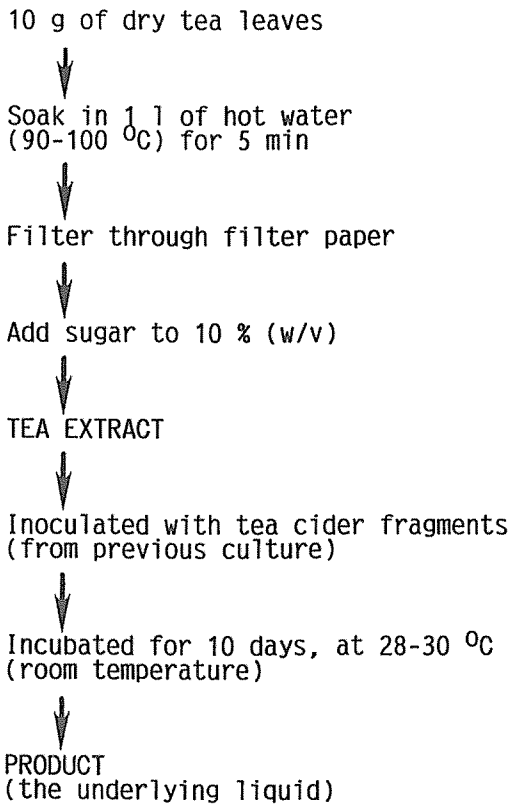

Fig. 1. Preparation of tea cider.

Chemical analysis

The tea cider was centrifuged at 15,000×*g* for 10 min, after which the supernatant was subjected to chemical analysis. Sugars were determined by the ordinary phenolsulphate method. To 1 ml of the diluted sample, 1 ml of 5% (v/v) phenol and 5 ml of conc. H_2SO_4 was added, and kept for 10 min at room temp. After 10 min, it was shaken and kept for another 15 min at 27°C after which its absorbance was read at 490 nm on a spectrometer (Hitachi model 100-20 Spectrometer). The pH was determined with a pH meter. Alcohol and total acidity were measured by AOAC methods (AOAC, 1984), alcohol with pycnometer after distillation, and total acidity was expressed as grams of acetic acid per 100 ml sample after titration with 0.1 N NaOH.

Sensory evaluation

The organoleptic acceptability was determined by sensory evaluation using the Hedonic method (LARMOND, 1977) with 6 panelists and the results were analyzed statistically.

RESULTS

The processes occuring in tea cider fermentation are thought to be the conversion of sugars to alcohols and other metabolites due to the activity of the yeasts, and then further alcohol conversion due to the activity of *Acetobacter*. Based on this presumption 7 yeast strains were selected out of 12 for their ability to produce high amounts of alcohol. As shown in Table 1, strains no. Y1, Y2, Y6 and Y7 produced higher concentrations of alcohol (1.63-1.73%) after 12 days of incubation. The second group of high alcohol production included strains no. Y3, Y4 and Y5 (0.95-1.07%). Strains no. Y8, Y9, Y10, Y11, Y12 produced the lowest amounts of alcohol (0.47%). The yeast strains no. Y1, Y2, Y3, Y4, Y5, Y6 and Y7 were thus selected for further studies.

On the basis of morphological (Table 2) and physiological (Table 3) characteristics, these 7 selected yeast strains were identified as follows: Y1, Y2 and Y7 as *Candida variabilis*; Y3 as *C. famata*; Y4 as *C. guilliermondii*; Y5 and Y6 as *C. fennica* (KREGER VAN RIJ, 1984). As

Table 1. Alcohol production of yeast strain isolates grown on extract tea at various times of incubation at 30°C.

Yeast strain	Alcohol Production(%)					
	Incubation for (days):					
	0	4	8	12	16	20
Y 1	0	0.61	1.35	1.73	2.11	2.75
Y 2	0	0.36	0.86	1.73	2.11	2.63
Y 3	0	0.35	0.47	0.95	0.95	1.43
Y 4	0	0.35	0.95	1.07	2.04	2.29
Y 5	0	0	0.59	1.07	1.07	1.80
Y 6	0	0.73	1.61	1.73	2.37	3.01
Y 7	0	0.71	1.43	1.68	2.54	2.79
Y 8	0	0	0.12	0.61	0.61	0.98
Y 9	0	0	0	0.47	0.47	0.95
Y10	0	0	0.47	0.47	0.83	0.95
Y11	0	0	0.12	0.48	0.48	0.98
Y12	0	0.24	0.48	0.48	0.98	1.11

Table 2. Morphological characteristics of yeast strains isolated from tea cider.

Characteristics	Yeast						
	Y1	Y2	Y3	Y4	Y5	Y6	Y7
Colonies	WW	WW	CS	CS	WW	WW	WW
Mycelium	TM	TM	−	PM	TM	TM	TM
Shape of cells	R−O	O	R	O	R	R	R−O
Size of cells (μm)	(4−6) × (4−7)	(4−5) × (5−6)	3−5	(3−5) × (4−5)	2−5	3−6	3−6
Pellicle formation	+	+	−	−	+	+	+
Ballistospore	−	−	−	−	−	−	−
Vegetative reproduction	MB	MB	MB	MB	MB	MB	MB
Ascospores	−	−	−	−	−	−	−

WW, white-wrinkled; CS, cream-smooth; TM, true mycelium; PM, pseudomycelium; R. round; O, oval; MB, multilateral budding.

Table 3. Physiological characteristics of yeast strains isolated from tea cider.

Characteristics	Yeast						
	Y1	Y2	Y3	Y4	Y5	Y6	Y7
Vitamin free growth	+	+	−	−	+	+	+
Growth at 37°C	+W	+W	+	+	+W	+W	+W
Splitting of arbutin	−	−	+	+	+	+	−
Cycloheximide resistance	−	−	+	+	−	−	−
Urease test	−	−	−	−	−	−	−
Starch test	−	−	−	−	−	−	−
Acid production	−	−	+W	+W	−	−	−
Ester production	−	−	−	−	−	−	−
DBB test	−	−	−	−	−	−	−
Growth on:							
50% Glucose	+S	+S	+S	+S	+S	+S	+S
10% NaCl and 5% glucose	+	+	−	−	+	+	+
Nitrogen utilization:							
Nitrate	−	−	−	−	−	−	−
Nitrite	−	−	−	−	−	−	−
Ethylamine	+	+	+	+	+	+	+
L-Lysine	+	+	+	+	+	+	+
Carbon assimilation:							
Glucose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
D(+)-Cellobiose	+	+	+	+	+	+	+
D(+)-Trehalose	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+
D(+)-Xylose	+	+	+	+	+	+	+
D(+)-Mannose	+	+	+	+	+	+	+
D(+)-Melezitose	+	+	+	+	+	+	+
Lactose	−	−	−	−	−	−	−
L(+)-Rhamnose	−	−	−	−	−	−	−
D(+)-Melibiose	−	−	+	+	−	−	−
Inulin	−	−	+	+	+	+	−
Soluble starch	+	+	−	−	+	+	+
L(+)-Arabinose	+	+	+	+	+	+	+
D(−)-Arabinose	−	−	+	+	−	−	−
L-Sorbose	−	−	+	+	+W	−	+
D-Ribose	+W	+	+W	+W	+W	+W	+
D(−)-Glucosamine	−	−	+	+	−	−	−
Ethanol	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+
Ribitol	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+
D-Glucitol	+	+	+	+	+	+	+
m-Erythritol	+	+	+	−	+	+	+

Galactitol	−	−	+	+	−	−	−
Methanol	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−
Salicin	+	+	+	+	+	+	+
Gluconate	+	+	+	+	+	+	+
D(+)-Glucono-1.5-Lactone	+	+	+	+	+	+	+
Citric acid	−	−	+	+	+	−	+
Succinic acid	+	+	+	+	+	+	+
Valine	−	−	−	−	−	−	−
Arginine	−	−	−	−	−	−	−
Creatine	−	−	−	−	−	−	−
Proline	+	+	+	+	+	+	+
Glycine	−	−	+W	+W	−	−	−
Fermentation:							
Glucose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Galactose	−	−	+	+S	−	−	−
Maltose	+	+	−	−	+	+	+
Lactose	+S	−	−	−	−	−	−
Raffinose	+S	+S	+	+	+	+S	+S
Melibiose	−	−	+	+	−	−	−
Inulin	−	−	+	+S	−	−	−

+, positive; −, negative; +w, weak; +s, slow reaction. DBB test, Diazonium Blue B color test.

shown in Table 2, all yeast isolates could not form spores. These tests were done in the media of acetic acid agar, gorodkowa agar, or vegetable juice agar at 20°C. Incubation was from 3 days to 6 weeks. The lack of the sexual stage led us to classify these yeasts into imperfect states. They were thought to be of the *Candida* sp. as seen from their culture colors of white or cream; producing true mycelium or pseudomycelium; having no ballistospores; and possessing no ability neither to utilize nitrate as a sole source of nitrogen nor to produce strong acetic acid (KREGER VAN RIJ, 1984).

Thirty-five kinds of bacteria were isolated but only one possessed the ability to produce acid and grow on alcohol as a carbon source. This selected bacterium is thought to be of the *Acetobacter* sp. as seen from the morphological and physiological characteristics (Table 4) (DE LEY *et al.*, 1984). However this bacterium differs froms the IFO type by having an optimum growth temperature of 45°C.

Table 4. Morphological and physiological characteristics of bacterium No. B29 isolated from tea cider compared with *Acetobacter aceti* subsp. *xylinum* IFO 3288.

Characteristics	Isolated Bacterium No. B29	*Acetobacter* sp. IFO 3288
Gram strain	−	−
Shape of cells	rod	rod
Size of cells (μm)	0.5×2.5	0.5×3.0
Endospore	−	−
Growth under anaerobic condition	−	−
Pigments production	−	−
Pellicle	+	+
Nitrogen utilization:		
Nitrate	−	−
Ammonium Sulphate	+	+
Oxidizing ethanol	+W	+
Optimum temperature (°C)	45	23

+, positive; −, negative; +W, weak reaction.

Table 5 shows the changes in pH, sugar content, total acidity and alcohol production during fermentations of batches of tea medium with combinations of *Acetobacter* sp. and selected yeasts. The pH generally decreased ranging from 4.38 to 3.33 after 15 days. Total acidity values were also observed to be low even after 15 days, the maximum value being 0.069%.

The decrease of sugar content was followed by the increase in alcohol. The combination of *Acetobacter* sp. with *C. fennica* Y5 and Y6, and also with *C. variabilis* Y7 produced higher concentrations of alcohol. The second in role of high alcohol production was combination of *Acetobacter* sp. with *C. variabilis* Y1 and Y2. *C. famata* Y3 produced lower amounts of alcohol, reaching a value of 0.29% after 15 days. These results indicate that different species of yeasts may play an important role in the use of sugar to produce alcohol in tea cider.

Table 5. Changes in pH, sugar content, total acid and alcohol production occuring in tea cider fermented by *Acetobacter* sp. in combination with various yeasts.

Fermenting organism	Time (days)	pH	Sugar (%)	Total acidity (%)	Alcohol (%)
Unfermented tea extract	0	4.38	10.34	0	0
Acetobacter sp. plus:					
C. variabilis Y1	3	3.87	9.77	0	0.23
	6	3.52	9.30	0.012	0.44
	9	3.52	7.05	0.018	0.88
	15	3.50	7.33	0.042	1.21
C. variabilis Y2	3	3.83	9.12	0	0.15
	6	3.55	9.51	0.006	0.43
	9	3.53	7.49	0.009	0.70
	15	3.47	6.47	0.048	1.16
C. famata Y3	3	3.61	9.00	0	0.31
	6	3.61	9.05	0	0.33
	9	3.74	9.11	0	0.26
	15	3.70	7.93	0.006	0.29
C. guilliermondii Y4	3	3.67	9.73	0	0.13
	6	3.40	9.94	0.018	0.26
	9	3.42	8.88	0.006	0.47
	15	3.33	8.04	0.069	0.89
C. fennica Y5	3	3.65	9.26	0	0.46
	6	3.58	7.79	0.027	0.74
	9	3.53	6.75	0.018	1.10
	15	3.35	4.99	0.033	1.39
C. fennica Y6	3	3.70	9.12	0	0.25
	6	3.60	8.75	0.006	0.58
	9	3.53	6.92	0.018	1.07
	15	3.37	6.81	0.030	1.39
C. variabilis Y7	3	3.63	8.64	0	0.44
	6	3.61	9.52	0.003	0.63
	9	3.53	6.62	0.036	1.23
	15	3.42	5.44	0.027	1.43

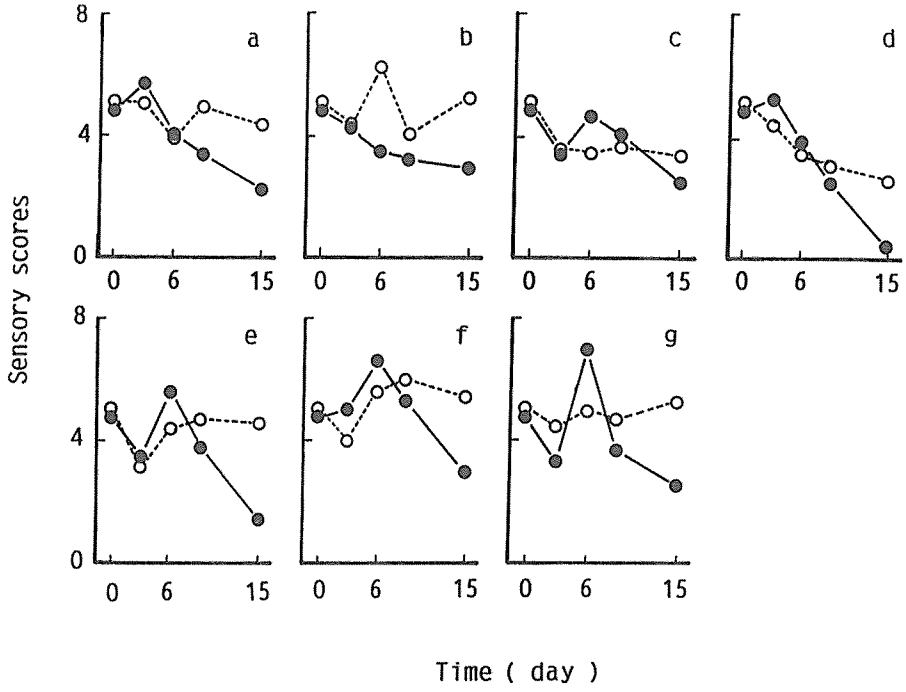

Fig. 2. Taste and smell scores of tea ciders fermented with *Acetobacter* sp. No. B29 in combination with various strains of yeasts (a. *C. variabilis* Y1; b, *C. variabilis* Y2; c. *C. famata* Y3; d. *C. guilliermondii* Y4; e. *C. fennica* Y5; f. *C. fennica* Y6; and g. *C. variabilis* Y7); at various times of incubation at 30°C. Taste scores (—●—) and smell scores (—○—) were average scores from 6 panelists using the Hedonic method.

Fig. 2 shows the results of sensory evaluation of tea cider. The acceptability of the combination between *Acetobacter* sp. with *C. fennica* Y5 and Y6, and also with *C. variabilis* Y7 increased with time of incubation, reaching a maximum at 6 days, after which it gradually declined. The combination with *C. variabilis* Y1 and *C. guilliermondii* Y4 showed a little increase in acceptability after 3 days incubation. Other combinations resulted in a decrease in acceptability with time of incubation. After 15 days incubation with *C. fennica* Y6 and *C. variabilis* Y2 and Y7, the products were slightly unacceptable, whereas *C. guilliermondii* Y4 produced a completely unacceptable product.

From statistical calculations, there was no significant differences between the samples. However, after 15 days incubation, the taste score between the combination *Acetobacter* sp. with *C. fennica* Y6, and with *C. guilliermondii* Y4 were significantly different statistically.

The aroma scores decreased gradually with incubation time, except for *C. variabilis* Y2 and *C. fennica* Y6 (Fig. 2). The aroma scores of the combination with *C. fennica* Y6 increased during incubation, reaching a maximum at 9 days of incubation (moderately liked), and after which it decreased. After 15 days incubation with *C. variabilis* Y2 and Y7, and also with *C. fennica* Y5 and Y6, products were slightly liked, whereas *C. famata* Y3 and *C. guilliermondii* Y4 produced slightly disliked products.

Statistical analysis also show that the combination with *C. guilliermondii* Y4 was significantly much more different from that with *C. fennica* Y6 and *C. variabilis* Y7. The combination with *C. fennica* Y5 after 15 days incubation also produced a product significant-

ly different from that of *C. guilliermondii* Y4.

DISCUSSION

The morphological and physiological classification of yeasts isolated from Indonesian tea cider seemed to include various species of the same genera of *Candida.* These results were different with those reported by KOZAKI *et al.* (1972) that *Klockera* sp. was the type yeast found in the Formosan tea cider. However, *Candida* sp. was one of the type yeast isolated from the Japanese tea cider.

Usually tea cider is traditionally produced using a portion from a previous product as the inoculum. In this study we used pure culture of two kinds of microorganisms isolated from the traditional tea cider product. These two organisms were *Acetobacter* sp. and one of the selected yeast.

The results as seen in Table 5 indicated that after 15 days incubation, 0.29% to 1.43% of alcohol was produced. These values were lower than those in Table 1. In Table 1 only one strain of yeast was used and alcohol contents were 0.95% to 2.54% after 16 days incubation. These differences seemed to be consistent with the presumption that alcohol produced by the activity of yeast was then used as carbon source for the activity of *Acetobacter* sp. in the tea cider fermentation process.

Alcohol and total acidity of the tea cider products were lower than that of traditional tea cider reported by SUGIANTO (1972). In the traditional tea cider, many types of yeast and bacteria (wild types) were used, so that processes occuring in the tea cider fermentation were faster than those processes occuring in this study, resulting in higher alcohol content and total acidity.

Composition of sugar, acid and alcohol seemed to contribute to the taste and odor of tea cider. Fig. 2 shows that the combination of *Acetobacter* sp. and *C. fennica* Y6 was found to be the best in high flavor production in tea cider and the optimal time of incubation was from 6 to 9 days. However, table 5 shows that composition of sugar, acid and alcohol of *C. fennica* Y5 and Y6, and also *C. variabilis* Y7 were almost the same at 6 to 9 days incubation. These results indicated that other metabolites produced during fermentation may play an important role in the product acceptability. Different kinds of strains of yeast may produce different metabolites that results in the differences in product acceptability. However, the metabolites which contribute to the flavor are still unknown. Further research into this metabolites should help in the final selection of suitable microbial strains in tea cider production.

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インドネシアティーサイダーの微生物と化学組成の変化

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インドネシアで自然発酵法で作られているティーサイダーを分離源として，微生物の分離を行い，どのような微生物が関与し，どのような性質を有しているかを明らかにする目的で研究を行った。

その結果，12株の酵母が分離され，その内アルコール発酵能の強い7株を同定し，*Candida variabilis, C. famata, C. guilliermondii* および*C. fennica* に属する菌株であることを明らかにした。また，35株の細菌が分離されたが，その内から，ティーサイダーの酸産生株と考えられている酢酸菌 *Acetobacter* sp. が見い出された。ついで，良い品質のティーサイダーを作る目的で，上記の分離した酵母と細菌とを種々組み合わせて，ティーサイダーを作成した。発酵の過程は糖の消費，アルコールおよび酸の生成，および pH の変化を追跡して検討した。最終的品質は官能テストにより行った。その結果，*Acetobacter* sp. と *C. fennica* Y6 との組合せが最も良かった。また，その場合の至適発酵時間は 30°C，6～9日間であった。