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Changes in major components of tea fungus metabolites during prolonged fermentation

C. Chen and B.Y. Liu

Department of Food Science, National Chung-Hsing University, Taichung, Taiwan, R.O.C.

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C. CHEN AND B.Y. LIU. 2000. Changes in major components and microbes in tea fungus broth (or kombucha; teakwass) prepared from nine different sources during a prolonged fermentation of up to 60 days were investigated. Cell concentrations of both yeasts and acetic acid bacteria in broth were generally higher than those in the cellulosic pellicles. The residual sucrose concentration decreased linearly with time, although the rate fell after the first month. Metabolic fates of glucose and fructose produced as a result of the hydrolysis of sucrose were different. Glucose was not produced in parallel with fructose (0·085 g 100 ml^{-1} d^{-1}) but was produced with a lower initial rate (0·041 g 100 ml^{-1} d^{-1}). Both titratable acidity and gluconic acid increased steadily with time for all samples, although gluconic acid was not generated for 6 days until the fermentation had begun. Acetic acid increased slowly to a maximum value of 1·1 g 100 ml^{-1} after 30 days; thereafter, it decreased gradually. Gluconic acid contributed to the titratable acidity and thus, the taste of tea fungus broth, during the final stage of fermentation. It is concluded that the desired quality or composition of kombucha can be obtained through the proper control of fermentation time.

INTRODUCTION

The tea fungus is a mixed culture of acetic acid bacteria (*Acetobacter xylinum*, *Acet. xylinoides* or *Bacterium gluconicum*) and yeasts (*Schizosaccharomyces pombe*, *Saccharomyces ludwigii*, *Zygosaccharomyces rouxii*, *Candida* sp. or *Pichia membranaefaciens*) (Kozaki *et al*. 1972; Anon. 1983; Blanc 1996; Liu *et al*. 1996; Chen and Liu 1997). The tea fungus broth is composed of two portions: a floating cellulosic pellicle layer and the sour liquid broth. Acetic acid, ethanol and gluconic acid are the major components of the liquid broth (Blanc 1996). The fermentation is traditionally carried out by inoculating a previously grown culture into a freshly prepared tea decoction and incubated statically under aerobic conditions for 7–10 days. Eventually, a pleasantly sour and slightly sparkling beverage called kombucha or teakwass is produced (Reiss 1994). The beverage has been claimed to be a prophylactic agent and to be beneficial to human health; however, this remains to be proved (Fasching 1995; Frank 1995; Blanc 1996). Few studies have been done on changes in the microbial activities and metabolites of tea fungus, particularly for those changes occurring during a prolonged period of fermentation of up to 60 days. In this study, nine samples of tea fungus of various origins in Taiwan were collected, and changes in some major components and microbes in tea fungus broth under static conditions were investigated.

Correspondence to: Dr C. Chen, Department of Food Science, National Chung-Hsing University, 250 Kuo-Kwang Road, Taichung, Taiwan 402, R.O.C. (e-mail: cschen@mail.nchu.edu.tw).

MATERIALS AND METHODS

Samples

Tea fungus samples were collected from nine households scattered throughout Taiwan. Each sample included both the upper pellicle and the lower liquid.

Maintenance of tea fungus samples

The tea fungus samples were periodically maintained or activated by the following procedure. Distilled water (1 litre) was boiled, 100 g of sucrose and two bags of black tea (yellow label tea, Lipton) were added, and the mixture was boiled for 5 min. After removal of the tea bags, the sweetened black tea was immediately dispensed into several 500 ml glass jars (each containing 250 ml tea). Finally, cellulosic pellicle fragments (2·5%, w/v on wet weight basis) and liquid broth (20%, v/v) of each tea fungus sample were

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inoculated together into the above cool tea broth (Reiss 1994). The jars were covered with clean paper towels or cheesecloth and fixed with rubber bands. The fermentation was carried out under ambient temperature (24 ± 3 °C) for 2 weeks, and some samples were used to inoculate new fermentations.

Cultivation method

The preparation of sweetened tea broth was the same as above, except that glass jars (capacity 250 ml) containing 150 ml tea broth were used. These jars were then randomly divided into nine groups with eight jars in each group (i.e., tea fungus sample). About 3·7 g (on wet basis) cellulosic pellicle fragments and 30 ml broth from the above activated tea fungus sample were withdrawn and inoculated into each jar. The subsequent fermentation process (up to 60 days) followed the procedure above. Sampling was performed periodically; each jar was sampled once only in order to avoid potential contamination. All analyses were carried out in duplicate.

Enumeration of acetic acid bacteria and yeasts

About 20 g sample were withdrawn and placed in a sterile plastic bag; 180 ml 0·1% sterile peptone water were added. The samples were then homogenized in a blender (Stomacher Lab Blender 400, Seward Medical Ltd, London, UK) for 9 min. The suspension obtained was used for the enumeration of bacteria and yeasts.

Determination of yeasts and acetic acid bacteria was performed using potato dextrose agar (PDA, Difco) and GYCA media, respectively. The GYCA medium was composed of glucose 30 g, yeast extract 5 g, peptone 3 g, calcium carbonate 10 g, 95% ethanol 30 ml (added after the medium had been autoclaved), agar 20 g, and distilled water to a final volume of 1 litre (Asai *et al.* 1964). Aliquots of 1 ml were taken (the upper pellicle portion was pre-filtered with sterile cheesecloth before sampling to remove the cellulose fibres). A decimal dilution series was prepared with 0·1% sterile peptone water, and 0·1 ml of the appropriately diluted suspension was spread on each of the two media. The colonies were counted after they had been incubated at 30 °C for 3 days (Koburger and Marth 1984). Cell counts were expressed as colony-forming units per millilitre (cfu ml^{-1} or g^{-1}).

Determination of titratable acidity

The titratable acidity was measured according to AOAC (1980). A 10 ml aliquot of tea fungus broth was taken and titrated with 0·1 mol l^{-1} NaOH; the end point was determined by pH at 7·0. The titratable acidity was expressed as the volume consumed in millilitres of 0·1 mol l^{-1} NaOH per 100 millilitre sample.

Analyses of organic acids, sugars and ethanol

A 10 ml aliquot of tea fungus broth was withdrawn and centrifuged (7240 *g*) for 10 min; the supernatant fluid was then filtered through a membrane filter (0·2 μm). The filtrate obtained was subjected to analyses of organic acids, sugars and ethanol. Sucrose, glucose and fructose were determined by HPLC. A 10 μl sample of filtrate was injected into a Hitachi (Tokyo, Japan) L-6000 HPLC system equipped with a refractive index detector (B110, Bischoff, West Germany). The column was packed with Spherisorb NH_2 (250 mm × 4·6 mm, 5 μm) (Phenomenex Inc., Torrance, CA). The mobile phase was a mixture of acetonitrile and water (80:20, v/v). The concentrations of sugars were quantified from standard curves. Acetic acid, succinic acid and gluconic acid were analysed by the same HPLC system, using a Bischoff RP-18 (5 μm, 250 mm × 4 mm) as the column, and detected at 210 nm by a u.v. detector (L-4000, Hitachi Ltd., Tokyo, Japan). The mobile system was mixture of 20 mmol l^{-1} $(NH_4)_2HPO_4$ and methanol (97:3, v/v). Ethanol was determined by gas chromatography. A 2 μl sample of filtrate was injected into a Hitachi G-3000 gas chromatograph equipped with a flame ionization detector. A stainless column (2 m × 2 mm) packed with Carbowax 1500 (GL Sciences, Japan) was used for separation. The column, injector and detector temperatures were 120, 150 and 150 °C, respectively. N_2 was used as the carrier gas at a flow rate of 15 ml min^{-1}.

RESULTS

Changes in population of viable yeasts and acetic acid bacteria

Viable counts of yeasts in nine different samples of tea fungus initially increased with the incubation time, and all samples reached maximum values after 6–14 days of fermentation (Table 1). Although the final cell counts remained high at around 10^5–10^6 cfu ml^{-1} or g^{-1}, the yeasts decreased gradually in the latter period of fermentation. In addition, cell concentrations of yeasts in the broth were generally higher than those in cellulosic pellicles. There were two to three types of colony shape shown on the PDA agar for each sample, which indicated different yeasts growing in the tea fungus (data not shown). Similarly, cell concentrations of acetic acid bacteria in the broth from the various tea fungus samples were generally higher than those in the upper pellicle portion (Table 2). The bacterial growth increased rapidly for the first 6 days,

Table 1 Changes in yeast cell counts in nine tea fungus samples from various sources during prolonged fermentation

Sample		Cell counts ($\times 10^6$ cfu ml^{-1} or g^{-1}) at			
		0*	6	14	30
A	l†	2·03 ± 0·34‡	35·5 ± 6·3	35·0 ± 0·7	34·2 ± 2·7
	s		15·0 ± 1·4	2·10 ± 1·41	2·30 ± 0·14
B	l	1·25 ± 0·05	16·3 ± 1·7	21·6 ± 1·8	20·9 ± 2·8
	s		24·5 ± 6·3	10·0 ± 0·0	3·20 ± 0·83
C	l	2·74 ± 0·10	13·0 ± 0·7	7·05 ± 0·50	13·9 ± 0·5
	s		12·5 ± 3·5	2·50 ± 0·71	2·80 ± 0·71
D	l	0·62 ± 0·02	24·9 ± 1·4	14·3 ± 0·1	9·00 ± 1·70
	s		4·50 ± 0·71	8·00 ± 0·00	10·6 ± 0·8
E	l	0·82 ± 0·06	78·5 ± 0·7	3·00 ± 0·28	10·6 ± 2·4
	s		20·0 ± 8·4	5·00 ± 0·00	10·6 ± 0·7
F	l	0·21 ± 0·05	18·2 ± 2·1	55·0 ± 1·4	6·95 ± 0·21
	s		10·5 ± 0·7	10·5 ± 2·1	8·35 ± 1·49
G	l	0·35 ± 0·14	12·0 ± 0·5	43·0 ± 5·6	21·6 ± 0·2
	s		17·5 ± 0·7	7·50 ± 2·12	5·90 ± 0·85
H	l	1·17 ± 0·07	55·0 ± 7·0	7·10 ± 1·41	0·94 ± 0·12
	s		20·0 ± 1·4	12·5 ± 0·7	10·6 ± 0·3
I	l	0·28 ± 0·02	13·2 ± 0·9	12·6 ± 0·2	5·75 ± 0·35
	s		8·50 ± 2·12	5·50 ± 0·70	10·3 ± 2·4

*Number indicates the day when sampling was performed.
†l: Liquid broth; s: cellulosic pellicle.
‡Mean ± S.D.; $n = 3$.

Table 2 Changes in acetic acid bacteria cell counts in nine tea fungus samples from various sources during prolonged fermentation

Sample		Cell counts ($\times 10^3$ cfu ml^{-1} or g^{-1}) at			
		0*	6	14	30
A	l†	8·45 ± 0·07‡	32·5 ± 4·9	1·95 ± 0·50	–§
	s		34·5 ± 2·1	0·80 ± 0·14	–
B	l	2·40 ± 0·17	17·7 ± 0·2	4·75 ± 0·07	1·60 ± 0·28
	s		0·85 ± 0·21	0·45 ± 0·06	1·01 ± 0·05
C	l	2·40 ± 0·50	91·5 ± 7·7	3·10 ± 0·00	1·10 ± 0·10
	s		9·15 ± 0·78	5·30 ± 0·41	–
D	l	3·15 ± 1·48	68·5 ± 6·3	4·80 ± 0·14	3·80 ± 0·85
	s		19·5 ± 1·4	7·80 ± 0·20	6·11 ± 0·90
E	l	5·05 ± 0·64	55·5 ± 9·1	22·0 ± 0·6	0·88 ± 0·21
	s		89·0 ± 7·0	2·00 ± 0·42	0·55 ± 0·18
F	l	1·50 ± 0·14	76·5 ± 1·9	0·80 ± 0·00	0·62 ± 0·02
	s		16·5 ± 3·0	1·90 ± 0·28	0·73 ± 0·30
G	l	18·8 ± 3·2	88·0 ± 9·9	1·60 ± 0·28	8·55 ± 1·20
	s		35·5 ± 0·7	0·85 ± 0·25	5·95 ± 0·35
H	l	4·30 ± 1·98	9·30 ± 0·00	3·85 ± 0·78	2·25 ± 0·50
	s		6·25 ± 0·21	1·85 ± 0·35	2·60 ± 0·85
I	l	1·71 ± 0·62	12·5 ± 2·1	1·45 ± 0·07	5·35 ± 0·78
	s		25·4 ± 3·1	0·45 ± 0·07	0·65 ± 0·08

*Number indicates the day when sampling was performed.
†l: Liquid broth, s: cellulosic pellicle.
‡Mean ± S.D.; $n = 3$.
§Not detectable.

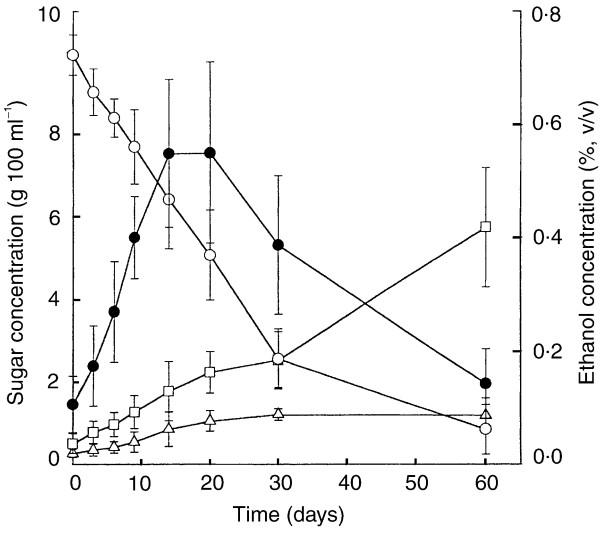

Fig. 1 Changes in residual sucrose, glucose, fructose and ethanol concentrations in tea fungus metabolites during prolonged fermentation. Data presented are the averages of nine samples of tea fungus from different sources, and error bars represent the standard deviations from nine samples. (○), Sucrose; (△), glucose; (□), fructose; (●) ethanol

followed by a gradual decrease until the end of fermentation.

Changes in sugar content

The residual sucrose concentration decreased linearly with time for the first 30 days of the cultivation period, followed by a decrease at a slower rate (Fig. 1). The average glucose concentration increased gradually and reached a plateau (approximately 1·2%) after 30 days. On the other hand, the concentration of fructose increased steadily during the fermentation period, the final concentration reaching about 5·5% (w/v).

Ethanol production

Changes in ethanol concentration in the tea fungus samples during fermentation are shown in Fig. 1. The concentration of ethanol increased initially with time to reach a maximum value of about 0·55% (w/v), followed by a slow decrease.

Changes in organic acids

Figure 2 shows changes in titratable acidity in tea fungus samples during fermentation. Apparently, the acidity increased steadily with time for all samples. The final average acidity was 41 ml of 0·1 mol l^{-1} NaOH 100 ml^{-1}. Among the organic acids produced, the production of acetic acid, varying with time, is shown in Fig. 2. The concentration of acetic acid increased slowly with time until a maximum value of 1·1 g 100 ml^{-1} was reached after 30 days, followed by a slow decrease to the final concentration of 0·8 g 100 ml^{-1}. Gluconic acid was the other major organic acid found in the metabolites of tea fungus, but it was not generated until the fermentation had proceeded for 6 days; the final concentration was about 3·9 g 100 ml^{-1}.

DISCUSSION

Until now there have been few reports about the influence of microbial activity on the component changes of the tea fungus metabolites. Some workers have shown that the composition of different teakwass preparations is greatly affected by the individual tea fungus used (Reiss 1994; Blanc 1996). This probably results from the variability of the normal microflora found in different tea fungus samples. For example, two species of acetic acid bacteria and three species of yeasts were isolated from samples of tea fungus Haipao in three cities of Taiwan (Liu *et al.* 1996). The bacteria isolated were identified as *Acetobacter aceti* and *Acet. pasteurianus*, while the yeasts were *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii* and *Brettanomyces bruxellensis*. The dominant yeast and bacterium during the fer-

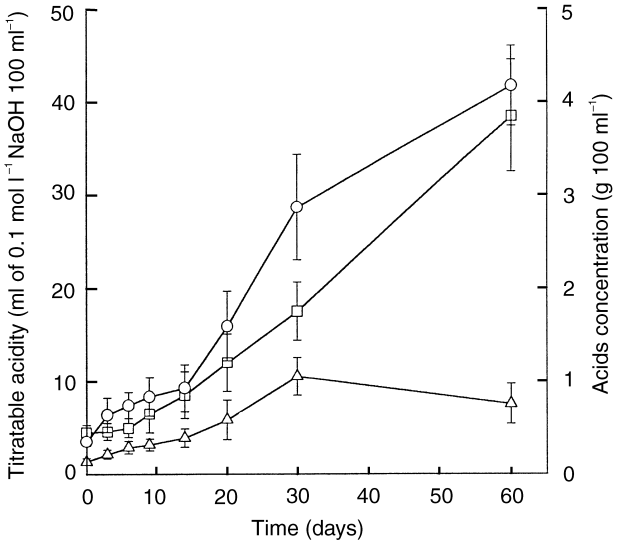

Fig. 2 Changes in titratable acidity, acetic acid and gluconic acid concentrations in tea fungus metabolites during prolonged fermentation. (○), Titratable acidity; (△), acetic acid; (□), gluconic acid. For an explanation of data, see Fig. 1

mentation were *Z. bailii* and *Acet. pasteurianus*, respectively. However, *Pichia membranaefaciens* and *Schizosaccharomyces pombe* were found as major yeasts, while *Acet. xylinum* and *Acet. aceti* were the major bacteria in our nine samples of tea fungus (Chen and Liu 1997). *Acetobacter xylinum* often occurs as a contaminant in vinegar fermentation, and is known to produce cellulose in culture (Brown *et al.* 1976; Fontana *et al.* 1991). Moreover, Reiss (1994) reported that the cell concentration of acetic acid bacteria in the upper pellicle portion was higher than that in the liquid broth due to a greater oxygen supply. On the contrary, the results of the present study indicated that viable counts of acetic acid bacteria in the broth of various tea fungus samples were generally higher (Table 2). In addition to the different types of tea fungus used, this was also probably because most acetic acid bacteria were entrapped within the floating cellulosic matrix, rendering the exact enumeration of bacteria difficult.

The final pH value of the liquid broth after 30 days was about 2·5 (data not shown), which was much lower than the pH for optimum growth (pH 5·4–6·3) of yeasts. Furthermore, the carbon dioxide generated as a result of alcohol fermentation by yeasts accumulated in the interface between the pellicle and broth; this separated the pellicle from the broth and eventually, blocked the transfer of nutrients from the broth to the top and the transfer of oxygen from the surface of the pellicle. These two deleterious effects led to an anaerobic and starved environment. Few genera of yeasts and bacteria could survive such conditions. Therefore, viability of both yeasts and aerobic acetic acid bacteria decreased gradually during fermentation (Tables 1 and 2).

The sucrose was hydrolysed to glucose and fructose by yeast invertase. However, compared with the higher production rate of fructose (0·085 g 100 ml^{-1} d^{-1}), the production of glucose did not increase in parallel with that of fructose, but at a lower initial rate (0·041 g 100 ml^{-1} d^{-1}) (Fig. 1). The final concentrations of both sugars were also different. This indicated that the metabolic fates of both glucose and fructose produced during fermentation were different. Although both sugars can be utilized by yeasts via glycolysis and produce ethanol and glycerol, their further utilization by *Acet. xylinum* was different. Part of the glucose was also directed towards production of organic acids, such as gluconic acid, and to biosynthesis of cellulose (Ross *et al.* 1991). Most *Acetobacter* strains are known to oxidize glucose and produce gluconate (Seto *et al.* 1997). In contrast to glucose, fructose was poorly metabolized by *Acet. xylinum* and thus, it accumulated in the broth. Phosphofructokinase was absent in *Acet. xylinum*, rendering glycolysis by this bacterium either absent or very weak (Kai *et al.* 1994). Furthermore, *Acet. xylinum* was incapable of utilizing sucrose to produce acid (Brown *et al.* 1976). As variations in sucrose concentrations among the nine samples during fermentation were small (Fig. 1), this indicated that the consumption rates of sucrose were almost the same. Moreover, the final sucrose concentration was low (about 1·0 g 100 ml^{-1}), which suggested that the yeasts were probably more active and durable than the acetic acid bacteria.

Acetic acid bacteria utilized ethanol to grow and produced acetic acid. Moreover, the presence of acetic acid could stimulate the yeasts to produce more ethanol (Liu *et al.* 1996). Such a symbiotic interaction was also observed between the yeast *S. cerevisiae* and the bacterium *Gluconobacter oxydans* in a natural fermentation of reconstituted orange juice (Cancalon and Parish 1995). Compared with the steady increase in both titratable acidity and gluconic acid, the concentration of acetic acid only increased to a limited degree; thereafter it decreased gradually (Fig. 2). This was probably due to the further utilization of acetic acid as a carbon source by *Acet. xylinum* when the sugars in the tea broth were running short. The other possibility was that ethanol fermentation by the yeast was reduced by the low pH value or low sugar concentration in the broth as the fermentation proceeded. A similar observation was also made by Reiss (1994), that ethanol production increased to a maximum after 6 days with a subsequent decline. On the other hand, the production of gluconic acid by *Acet. xylinun* was not affected and therefore, it contributed to the titratable acidity determined during the final stage of fermentation.

Sucrose is split by yeasts into glucose and fructose. In addition to production of ethanol by yeasts and cellulose by *Acet. xylinum*, glucose is mainly converted into gluconic acid via the pentose phosphate pathway by acetic acid bacteria; most fructose is metabolized to acetic acid, and little or none to gluconic acid. However, *Acetobacter* is also capable of hydrolysing sucrose, by the action of levansucrase, into glucose and a polysaccharide of fructose, levan (Alvarez and Martinez-Drets 1995). The type of sugar (sucrose, lactose, glucose or fructose) had a distinct influence on the formation of ethanol and lactic acid, but the concentrations of the individual sugars had only a minor effect on the flavour of the tea fungus preparations (Reiss 1994).

The above results indicated that the patterns of change in determinations such as concentration of sucrose, glucose, fructose or gluconic acid, and titratable acidity during fermentation, were similar among the nine samples. These measurements basically reflected the composition changes of the liquid broth during fermentation, and eventually the interaction between yeasts and acetic acid bacteria. The composition of the liquid broth determines the flavour and taste of tea fungus products. This includes concentrations of residual sugars, carbon dioxide and organic acids, espe-

cially the ratio of acetic acid to gluconic acid. Volatile acetic acid produces an astringent and acidic flavour, while the flavour produced by gluconic acid is mild. Reiss (1994) reported that teakwass was a refreshing beverage with a fruit-like taste when produced within 6–10 days of incubation; prolongation of fermentation yielded a distinct vinegar-like flavour. The results of the present study showed that the composition of tea fungus broth varied with fermentation time to give a different taste. It is therefore possible to obtain the desired quality of tea fungus broth by controlling the fermentation conditions.

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