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Factors Affecting Organic Acid Production by Sourdough (San Francisco) Bacteria

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Previous workers from this laboratory observed considerable variation in the proportions of acetic and lactic acids produced in pure broth culture as compared to consistently high proportions of acetic acid produced in the sourdough and flour suspension systems. In the latter the proportion of acetic acid was always in the range of 20 to 35% of the total, whereas in pure broth culture frequently less than 5% acetic acid was produced. In the natural environment, the sourdough bacteria, tentatively identified as lactobacilli, coexist with a yeast, *Saccharomyces exiguus*, and this study was undertaken to determine whether this yeast or flour ingredients including glucose or other factors were involved in this variable production of acetic acid. The proportion of acetic acid produced in broth culture on maltose, the preferred carbohydrate source, was found to depend almost entirely on the degree of aeration. Essentially anaerobic conditions, as obtained by thorough evacuation and flushing with CO_2 or N_2, resulted in very low (5% or less) proportions of acetic acid. Aerobic conditions, achieved by continuous shaking in cotton-plugged flasks, yielded high levels (23 to 39% of the total) of acetic acid. Similar effects of aeration were observed with glucose as the substrate, although growth was considerably slower, or in nonsterile flour suspension systems. It is theorized that, under aerobic conditions, the reduced pyridine nucleotides generated in the dissimilation of carbohydrate are oxidized directly by molecular oxygen, thereby becoming unavailable for the reduction of the acetyl phosphate intermediate to ethyl alcohol, the usual product of anaerobic dissimilation of glucose by heterofermentative lactic acid bacteria. Comparative studies with known strains of homo- and heterofermentative lactobacilli showed similar effects of aeration only on the heterofermentative strains, lending additional support to the tentative grouping by previous workers from this laboratory of the sourdough bacteria with the heterofermentative lactobacilli.

The microorganisms responsible for the leavening and souring activities in the dough used in perpetuating the San Francisco sourdough bread process were successfully isolated recently and described by Kline and Sugihara of this laboratory. The leavening function was numerically correlated with the occurrence of a yeast identified as *Torulopsis holmii*, the asporogenous form of *Saccharomyces exiguus* (8), and the souring activity was correlated with the presence of a bacterium tentatively identified as a lactobacillus, whose requirement for maltose, at least for isolation purposes, and other differences suggest it to be a previously undescribed species (3).

As reported by Kline and Sugihara (3), discrepancies were observed between the proportions of acetic and lactic acids produced in doughs or other flour culture systems and that produced in the pure broth cultures. Thus in flour systems, whether a natural "starter" or pure cultures of yeast and bacteria were used as inocula, the acetic acid produced was between 20 and 30% of the total whereas in pure broth cultures inoculated with the bacterium alone the proportion of acetic acid was frequently less than 10% and highly variable. The sourdough yeast per se does not produce significant amounts of acids in the dough (6), but it was not known whether it, or factors contributed by the flour, exerted any influence on the type of acidity produced by the bacterium. Accordingly, the present studies were undertaken to determine the conditions responsible

for the variable production of acetic acid by the sourdough bacteria. For reference purposes, comparative studies were also made on known strains of heterofermentative and homofermentative lactobacilli.

MATERIALS AND METHODS

Pure culture media. The SDB (sourdough bacteria) agar and broth were prepared as described by Kline and Sugihara (3). Although these workers used only maltose as a fermentable carbohydrate source, I found glucose to be utilized also, although more slowly, and the medium was made up to contain either 2% (w/v) maltose or glucose and designated as SDBM or SDBG, respectively. Initial *p*H of the medium was adjusted to 5.5 with HCl. Fresh yeast extractives (FYE) prepared as described previously (3) were routinely used at a concentration of 0.5% unless stated otherwise.

Flour cultures. Preparation and incubation of sour bread doughs were as described by Kline, Sugihara, and McCready (4). Flour slurry cultures were also made by mixing 100 g of flour, 2.2 g of NaCl, and 250 ml of distilled water and adjusting the *p*H of the slurry to 5.5 with HCl. The slurry was inoculated with a bacterial cell suspension freshly prepared from 20 ml of an overnight culture in SDBM broth by centrifuging at 15,000 rev/min for 10 min in a Sorvall refrigerated centrifuge and suspending the cells in 5 ml of sterile physiological saline.

Methods of cultivation. All cultures were incubated at 30 C. For achieving essentially anaerobic conditions, broth cultures (100 ml) in loosely capped 250-ml Erlenmeyer flasks or slant cultures in loosely capped test tubes were placed in vacuum desiccators which were evacuated to about 50 mm Hg followed by refilling with either pure CO_2 or N_2 to atmospheric pressure; the process was repeated three times. The tubes or flasks were then removed from the desiccators, the screw caps were tightened, and the cultures were incubated without shaking. For aerobic conditions, cultures were incubated in cotton-plugged flasks and shaken in a New Brunswick rotatory shaker-incubator operating at a frequency of about 100 excursions/min. The same methods were applied to flour slurry cultures, except these were shaken in all cases during incubation to prevent settling of the flour.

Organisms. The sourdough bacteria used in these experiments were those isolated and described by Kline and Sugihara (3) and designated by them as strains B, C, L, and T according to the source (San Francisco sourdough bakery from which dough was obtained). These were maintained on SDBM slants. For comparative purposes, known species of lactobacilli obtained as lyophilized cultures from the Northern Regional Research Laboratory culture collection (courtesy of W. C. Haynes) were also studied. These included: *L. delbrueckii* NRRL 3-763 (ATCC 9649) and *L. acidophilus* NRRL B-2178 (ATCC 11506) as homofermenters; *L. brevis* NRRL B-1834, *L. buchneri* NRRL B-1837, and *L. fermenti* NRRL B-585, as heterofermenters; and *L. plantarum* NRRL B-1928 (ATCC 10776) and *L. casei* NRRL B-442 (ATCC 7469) as facultative homofermenters. The lyophilized cultures were taken up in SDBM broth, and, after growth at 30 C, loopfuls were streaked out on SDBM agar plates where well isolated colonies were selected and transferred to SDBM agar slants. A lactobacillus strain isolated by T. F. Sugihara of this laboratory from a commercial frozen concentrated sourdough starter culture, designated as Sardo SF, was also evaluated.

Organic acid analysis. For determining acids in doughs, the doughs were extracted by blending a weighed piece of dough (about 50 g) with about 50 to 60 ml of distilled water in a 250-ml osterizer jar for about 1 min. Sodium hydroxide (1.0 N) was then added to bring the *p*H of the extract to about 8, and the mixture was again blended for 1 min. The extract was then centrifuged at 2,000 rev/min for 30 min, and the supernatant fluid was decanted. The pellet was washed with another 20 to 30 ml of water and again centrifuged. The supernatant fluid and washing were combined and made up to a volume of 100 ml. An acidified sample was used for analysis as described below. Flour slurries were extracted in a similar manner. Alkaline extraction was found to result in more complete extraction of the acids than when distilled water was used, but the proportion of acetic to lactic acid was not significantly affected.

Acids were analyzed by chromatographic separation on a celite column and titration according to the procedure described by Wiseman and Irvin (10). All extracts or samples to be chromatographed were acidified by addition of H_2SO_4 (1.0 N) to an approximate concentration of 0.1 N, and a 2-ml sample was placed on the column. The eluates were titrated to a cresol red end point by using standardized 0.01 N NaOH and agitation with CO_2-free air. Values obtained on culture filtrates were corrected for the blank values in the uninoculated SDB medium which contained average concentrations of 7.3, 4.5, and 0.71 μmoles/ml, respectively, of acetic, lactic, and formic acids.

Diacetyl determination. Diacetyl was determined by the method of Westerfield as described by Neish (5). The reaction time selected was such that equal weights of diacetyl and acetoin would give equal color intensities. Therefore, the "diacetyl" values reported are the sum of these two compounds, and no attempt was made, for the purpose of this report, to distinguish between the two. Diacetyl (2,3-butanedione) obtained from Eastman Chemicals was employed as the standard.

RESULTS

Organic acids in sour bread dough. Table 1 shows the amount and proportions of acetic and lactic acids produced in fully developed sour bread doughs or in the "starter sponges" which are special pieces of dough serving as inocula for preparing these bread doughs (4). No other acids except for a barely measurable trace of formic acid were detected. Acetic acid is seen to represent 25 to 35% of the total

TABLE 1. *Organic acids in various sourdoughs*

Sample	Micromoles/g of dough			Per cent acetic
	Acetic	Lactic	Sum	
Fully developed bread dough[a]	16.1	48.2	64.3	25
Fully developed bread dough[b]	20.3	40.2	60.5	34
Fully developed starter sponge C[c]	27.2	58.0	85.2	32
Fully developed starter sponge L[c]	25.7	57.5	83.2	31

[a] Made in conventional fashion by using a natural starter sponge as the inoculum (starter sponge is a special piece of dough which is constantly rebuilt with fresh flour and water to perpetuate the yeast and bacterial activities). Incubated at 30 C for 7 hr.

[b] Made with pure cultures of yeast (strain C) and bacteria (strain C).

[c] C and L refer to source (bakery).

acidity produced. The higher total acidity figures for the starter sponges, as compared to the bread doughs, are attributed to the fact that these starters are a more concentrated system in terms of flour substrate and initial bacterial counts.

Organic acid production in SDBM under anaerobic conditions. Since Kline and Sugihara reported stimulatory effects of CO_2 on growth (3), their usual procedure was to evacuate and gas with CO_2 before incubation. In the present study this evacuation plus gassing was repeated three times providing essentially anaerobic conditions. As shown in Fig. 1, all four strains (B, C, L, and T), growing on SDBM broth, produced only low levels of acetic acid and for three of the strains (B, C and T) amounting to less than 5% of the total throughout the 60-hr period of fermentation. Accordingly, it was decided to evaluate factors contributed by the flour or dough systems which might account for their higher content of acetic acid. These included, as described below, effects of the glucose contributed by the flour, degree of aeration, and concentration of fresh yeast extractives, the latter relating to a possible contribution from the sourdough yeast moiety.

Effect of glucose and of FYE concentration. Sourdough bacteria, which prefer maltose as a fermentable carbohydrate and appear to require it for isolation (from the dough) purposes, will ferment glucose after a lag of about 24 hr (Ng, *unpublished data*). Since flour does contain a small amount of glucose and more is released after the dough is formed (6), the effect of glucose as a substrate on the proportion of acetic acid formed was studied. As shown in Table 2 with strain B, this proportion was not significantly increased by glucose used separately or in combination with maltose under the essentially anaerobic conditions used. The slow release of acids associated with the lag in growth on glucose is also apparent. Two other strains tested, C and L, reacted similarly to growth on glucose. Table 2 also shows that the concentration of FYE used in the medium does not affect the proportion of acetic acid produced, although the total quantity of acids produced is higher, reflecting the heavier growth observed at the higher FYE level.

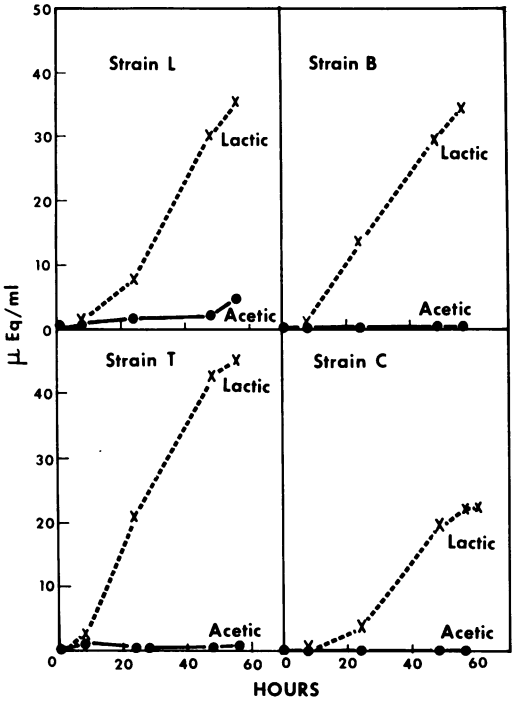

FIG. 1. *Production of organic acids by sourdough bacteria in SDB broth with maltose as carbon source. The cultures were incubated at 30 C under CO_2 atmosphere. Samples were removed at the indicated times and acetic (O) and lactic (×) acids determined as described in Materials and Methods.*

Effects of aeration on organic acid production. In the conventional mixing procedure for preparing a dough, ample opportunity is provided for considerable trapping of air in the structure. Flour also routinely contains small amounts of oxidants such as potassium bromate which could increase the oxidation-reduction potential. Since studies in pure culture under essentially anaerobic conditions consistently resulted in minimal production of

acetic acid, it was decided to evaluate the effects of exposure to variable amounts of air during culturing. As shown in Table 3 for maltose as the substrate, and Table 4 for glucose, aeration had a profound effect on increasing the proportion of acetic acid produced by the sourdough bacteria; thus for strain L (Table 3) the proportion of acetic acid produced was increased to 36% by aeration as compared to only about 3% under CO_2. The percentage of acetic acid was also about 3% when incubated under N_2, so that the low proportion of acetic acid is very likely a result of anaerobic conditions and not unique to CO_2 incubation. That shaking or interchange with the air atmosphere is essential was shown by the results on strains B and T (Table 3) where use of semi-anaerobic conditions (cotton plug but not shaking) yielded acetic acid values of only 6 to 9% as compared to 23 and 39% when shaking was employed. Thus aeration is shown to increase the proportion of acetic acid produced in pure culture on maltose by the sourdough bacteria to approximately that reported for the dough systems. No diacetyl was produced by the sourdough bacteria under either aerobic or anaerobic conditions.

Of the known lactobacilli included for comparative purposes, only the heterofermentative *L. brevis* showed any similarity to the sourdough bacteria in its response to aeration, and the effect was even more striking with the proportion of acetic acid produced on SDBM broth being increased from 1 to 64%. *L. brevis*, also like the sourdough bacteria, produced no diacetyl under either condition. Aeration did

TABLE 2. *Effect of type of carbon source and of concentration of fresh yeast extractives (FYE) on the production of organic acids[a] by strain B of sourdough bacterium in SDB medium under anaerobic conditions (CO_2)*

Time (hr)	2% Maltose + 0.5% FYE		2% Maltose + 1.5% FYE		2% Glucose + 1.5% FYE		1% Maltose + 1% glucose + 1.5% FYE	
	Total acids	Per cent acetic	Total acids	Per cent acetic	Total acids	Per cent acetic	Total acids	Per cent acetic
24	26.7	2.3	48.3	3.1	1.8	—[b]	41.6	4.6
48	42.7	2.1	62.3	5.1	26.4	7.6	61.1	4.9
72	51.3	3.1	65.0	5.5	41.7	6.2	66.0	5.0

[a] Total acids represent the sum of the acetic and lactic acids in micromoles per milliliter of culture supernatant fluid. Percent acetic is calculated by dividing the acetic acid value by the total acids and multiplying by 100.

[b] The amount of acids produced was too low to get an accurate value.

TABLE 3. *Effect of aeration on the production of organic acids and diacetyl by strains of sourdough bacteria and lactobacilli in SDBM (maltose) medium[a]*

Organism	Anaerobic or semianaerobic[b]					Aerobic				
	Acetic	Lactic	Total	Per cent acetic	Diacetyl	Acetic	Lactic	Total	Per cent acetic	Diacetyl
Sourdough bacteria										
Strain B[b]	3.5	36.7	40.2	8.7	0.0	9.6	32.1	41.7	23.0	0.0
Strain T[b]	2.1	36.1	38.2	5.5	0.0	15.4	24.5	39.9	38.6	0.0
Strain L	1.7	48.2	49.9	3.4	0.0	19.6	34.2	53.8	36.4	0.0
Strain L[c]	1.1	46.6	47.7	2.3	0.0					
Other lactobacilli										
L. brevis (heterofermentative)	0.6	53.4	54.4	1.1	0.0	55.5	31.8	87.3	63.6	0.0
L. delbrueckii (homofermentative)	1.0	28.5	29.5	3.4	1.1	2.9	22.0	24.9	11.6	9.0
L. casei (facultative homo.)	1.0	28.2	29.2	3.4	0.3	2.9	15.8	18.7	15.5	11.9
Commercial Sardo SF	0.6	60.7	61.1	1.0	0.0	3.7	65.8	69.5	5.3	0.0

[a] All values expressed as micromoles per milliliter of culture supernatant liquid.

[b] Semianaerobic: flasks were *not* shaken but were stoppered with cotton instead of screw caps. Anaerobic conditions achieved by evacuating and flushing with CO_2.

[c] Anaerobic condition achieved by evacuating and flushing with N_2 instead of with CO_2.

TABLE 4. *Effect of aeration on the production of organic acids and diacetyl by strains of sourdough bacteria and lactobacilli in SDBG (glucose) medium*[a]

Organism	Anaerobic					Aerobic				
	Acetic	Lactic	Total	Per cent acetic	Diacetyl	Acetic	Lactic	Total	Per cent acetic	Diacetyl
Sourdough bacteria										
Strain B	0.0	14.3	14.3	0.0	0.0	12.4	14.7	27.1	42.6	0.0
Strain T	0.3	23.8	24.1	1.2	0.0	15.2	14.6	29.8	51.0	0.0
Other lactobacilli										
L. delbrueckii (homofermentative)	1.3	79.4	80.7	1.6	1.4	2.8	64.1	66.9	4.2	11.2
L. acidophilus (homofermentative)	0.2	29.0	29.2	0.7	0.1	0.7	15.2	15.9	4.4	0.2
L. casei (facultative homo.)	0.2	72.9	73.1	0.3	0.4	2.7	62.4	65.1	4.2	11.4
L. plantarum (facultative homo.)	0.0	60.7	60.7	0.0	0.3	14.7	44.6	59.3	24.8	4.9
L. brevis (heterofermentative)	2.4	42.9	45.3	5.2	0.0	48.2	30.6	78.8	61.2	0.0
L. buchneri (heterofermentative)	1.8	31.8	33.6	5.4	0.0	38.5	35.4	73.9	52.2	0.0
L. fermenti (heterofermentative)	1.2	45.1	46.3	2.6	0.0	17.2	35.2	52.4	32.8	0.0

[a] All values expressed as micromoles per milliliter of culture supernatant liquid.

have a slight effect on the homofermenters, *L. delbrueckii* and *L. casei*, increasing the acetic acid produced on SDBM from 3.4 to 11.6% and from 3.4 to 16.5%, respectively. In addition, aeration markedly increased the production of diacetyl by these homofermenters.

The isolate from the commercial "sourdough" starter culture appeared dissimilar to the San Francisco sourdough bacterial isolates in producing a minimal proportion of acetic acid (ca. 5%) even under aerobic conditions. It would appear to be a homofermenter except that it did not produce diacetyl as did the particular known species of homofermenters studied.

The results in Table 4, with glucose used as substrate, show that the aeration effect was not unique to maltose as a substrate. Additional known strains of lactobacilli were studied. Thus the two additional heterofermenters, *L. buchneri* and *L. fermenti*, responded to aeration in similar fashion to the sourdough bacteria and *L. brevis* in markedly increasing their production of acetic acid under aeration and in not producing diacetyl under either condition. Results with *L. acidophilus*, a homofermenter, were similar to those obtained with *L. delbruekii*, although *L. acidophilus* did not grow well on the SDBG broth. *L. plantarum*, a facultative homofermenter, showed proportionately more acetic and less diacetyl increases on aeration than did *L. casei*.

The similar effect of aeration in a flour system was demonstrated in the study shown in Table 5. Here the B strain of sourdough bacteria was inoculated into a flour-water slurry with added salt. After 24 hr, the flour was removed by centrifugation and the acids were determined in the supernatant liquid. The proportion of acetic acid produced by aeration was increased from 9.5 (anaerobic) to 19.9% (aerobic). The magnitude of the effect was not as great as that observed in the broth systems, possibly due to the failure to attain complete anaerobiosis resulting from a difficulty of removing occluded air or the fact that the requirement for shaking to keep the flour suspended could result in incorporating air if the screw caps leaked. However, qualitatively, the effect of aeration was significant and similar.

DISCUSSION

The foregoing experiments demonstrate that the proportion of acetic acid produced by the sourdough bacteria, and by known strains of heterofermentative lactobacilli, are dependent upon the degree of aeration to which the cultures are exposed. These observations offer a possible explanation for the higher proportions of acetic acid developed in the dough systems as compared to that observed in essentially anaerobic pure culture fermentations. Varia-

TABLE 5. *Production of organic acids by sourdough bacterium strain B in flour suspension, under aerobic and anaerobic conditions*

Condition	Acids (μmoles/ml)			
	Acetic	Lactic	Total	Per cent acetic
Anaerobic (CO_2)	5.5	52.7	58.2	9.5
Aerobic (air)	12.5	50.5	63.0	19.9

bility in the earlier results with pure cultures reported by Kline and Sugihara are also likely attributable to variability in the degree of "anaerobiosis" achieved in their culturing techniques. This explanation is further supported by the finding that the degree of aeration also affected the proportion of acetic acid produced in a flour slurry system.

The mechanism by which acetic acid is produced by these recently isolated sourdough bacteria has not been studied. However, based on mechanisms which have been postulated for the oxidative metabolism of glucose by heterofermentative lactobacilli, it is presumed that, under aerobic conditions, the reduced pyridine nucleotide generated from the oxidation of glucose to 6-phosphogluconic acid via the hexose monophosphate shunt transfers its electrons directly to molecular oxygen so that the acetyl phosphate intermediate yields acetic acid rather than being reduced to ethanol, the usual product under anaerobic conditions. In this connection, Stamer and Stodola (7) have shown that *L. brevis*, a heterofermenter, produces increased amounts of acetate under aerobic conditions and at the expense of decreased production of ethanol. White and Sherman (9) also have reported that anaerobically *Streptococcus lactis* converts 99.4% of the glucose to lactic acid whereas aerobically only 43.2% of glucose is recovered as lactic acid. No effort was made by these workers to account for the missing carbon, but it is very likely that it has been diverted to acetic acid.

A similar explanation may also be invoked to account for the increased production of diacetyl by the homofermentative lactobacilli under aerobic conditions. In this case, pyruvate which becomes reduced to lactic acid anaerobically is now diverted to diacetyl aerobically for lack of reduced pyridine nucleotides. This is in general agreement with the data in Tables 3 and 4. This postulated mechanism is also supported by the report of Bruhn and Collins (1) who showed that under aerobic conditions, there is an increase in diacetyl and acetoin production by *Streptococcus diacetilactis* with a concomitant increase in production of the enzyme nicotinamide adenine dinucleotide oxidase. This enzyme presumably facilitates the transfer of electrons to molecular oxygen instead of reducing pyruvate to lactate.

The present findings lend additional support to the preliminary tentative positioning of the sourdough bacteria by Kline and Sugihara as heterofermentative lactobacilli (3). The strong preference of the sourdough bacteria for maltose and other differences suggested to these workers a lack of identity with known strains. Work is under way at another laboratory to compare the genetic composition and hybridization relationships of known lactobacilli and the sourdough bacteria. Thus far the preliminary report by Nelson et al. (Bacteriol. Proc., p. 4, 1971) suggests that the guanine plus cytosine content of the sourdough bacteria is about 37% or substantially lower than that of known strains of heterofermentative lactobacilli, lending further support to the view that the sourdough bacteria are previously undescribed strains. Further definitive studies on hybridization, complete analyses of fermentation products, and determination of their enzymatic makeup (2) will help to establish the taxonomic position of these bacteria.

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