

CHAPTER 48

Citric Acid Fermentation in Whey Permeate

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Acid-whey permeate was found to be suitable for the production of citric acid by *Aspergillus niger*. The fermentation proceeded in two phases: growth phase when citric acid was not accumulated, followed by acidogenic phase when citric acid accumulated and mold growth was greatly reduced. Optimal production of citric acid occurred after 8-12 days at 30 C. Maximum citric-acid yields by stock cultures were influenced by initial lactose concentration and reached 10 g/liter when lactose concentration in acid-whey permeate was adjusted to 15% (w/v). Methanol in concentrations of 2-4% (v/v) markedly increased the production of citric acid. After reaching a maximum level, citric acid was frequently depleted from the medium by the culture. Fermentation of acid-whey permeate by a mutant strain, *A. niger* 599-3, was more reproducible and yields of citric acid were substantially improved. The amount of citric acid produced by *A. niger* 599-3 was 18-23 g/liter after 12-14 days, depending on the lactose content of the whey permeate. Throughout the fermentation galactose was apparently co-metabolized with glucose.

INTRODUCTION

Disposal of acid whey presents a serious problem to the cottage-cheese industry which generates over 4 billion pounds of this by-product annually (Clark 1979). Deproteinization by ultrafiltration which removes approximately 96% of nutritionally high-quality lactalbumin and lactoglobulin has offered the industry a new means for disposing of acid whey. However, the deproteinized whey permeate still contains about 97% of the original lactose (40-50 g/liter), organic acids, some low molecular-weight protein, vitamins (thiamine, niacin, panthotenic acid, riboflavin, folic acid, and vitamin B₁₂), minerals, and other minor

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components (Hargrove et al. 1976). Thus, the biological oxygen demand of acid-whey permeate remains high and its disposal is a challenge to the processing industries.

Several approaches have been suggested for direct utilization of whey and whey permeate. These include the use of whey as a substrate for the production of yeast (Wasserman et al. 1961; Webb and Whittier 1970), beverages (Holsinger et al. 1974), alcohol (Rogosa et al. 1947; Gawel and Kosikowski 1978; Moulin et al. 1980), ammoniated organic acids (Gerhardt and Reddy 1977), and microbial polysaccharides (Charles and Radjai 1977).

The production of citric acid by fermentation of cheese whey has not been reported. Although the commercial synthesis of citric acid is carried out in carefully defined media under rigidly controlled conditions (Perlman and Sih 1960; Wold and Suzuki 1976), the feasibility of citric-acid production in complex media such as brewery waste has been described (Hang et al. 1977). In addition, mutant cultures with a high degree of insensitivity to high levels of trace metals in complex media have been developed (Perlman and Sih 1960; Trumpy and Millis 1963). Recent studies suggested that nitrogen-limiting conditions favor citric-acid synthesis (Kristiansen and Sinclair 1978). Since the ultrafiltration process removes most of the protein nitrogen and some nonprotein nitrogen from acid whey, it seemed appropriate to investigate the suitability of acid-whey permeate as a fermentation medium for the production of citric acid. The purpose of the studies reported here was to evaluate the production of citric acid by *Aspergillus niger* in acid-whey permeate.

MATERIALS AND METHODS

Microbiological. The following strains of *Aspergillus niger* were used as test organisms: NRRL-3, NRRL-326, NRRL-372, NRRL-599, NRRL-2001, and NRRL-2270 (from the Northern Regional Research Center, U.S. Department of Agriculture, Peoria, IL). Cultures were maintained on potato-dextrose agar (Difco) slants at 4 C. Inocula for fermentation studies were prepared by growing the test organisms on potato-dextrose agar at 30 C for 5 d. Spore suspensions were prepared by adding 5 ml of sterile 0.05% Tween 80 solution to the slants and shaking gently for 1 min.

Fermentation studies. Acid-whey permeate was prepared according to the method described by Hargrove et al. (1976). The permeate contained (w/v) 4.4-5.0% lactose, 0.025% protein, 0.03% nonprotein nitrogen (NPN), 0.05% citric acid, and 0.4% lactic acid, and had a pH of 4.1 which was not adjusted. Aliquots of 200 ml of acid-whey permeate were dispensed into 1000-ml Erlenmeyer flasks and autoclaved at 121 C for 15 min prior to use. Each flask was inoculated with 1.0-ml inoculum (2×10^7 spores), and incubated at 30 C for 10 to 14 d on a Psychrotherm shaker (New Brunswick Scientific Company, Edison, NJ), at 180 rpm. The pH of the medium was not controlled during fermentation. Aliquots (5 ml) were withdrawn daily and mycelium-free filtrates were analyzed for citric acid and lactose content.

Selection of mutants. Mutants of *A. niger* 599 were developed with N-methyl-N-nitroso-n'-nitroguanidine (NTG), according to the recommenda-

tions of Fantini (1975). Spore suspensions of 5-d-old cultures were prepared in 0.1 M phosphate (K_2HPO_4 - KH_2PO_4) buffer (pH 7.0) containing NTG at 0.5 mg/ml. After gentle stirring at 30 C for 5 h, spores were sedimented by centrifugation in a clinical centrifuge. The supernatant was removed with a Pasteur pipette, the pellet resuspended in buffer, serially diluted, and plated on whey agar (2% agar, w/v), containing 0.001% (w/v) bromphenol blue indicator. Plates were incubated at 30 C for 7 d.

Analyses. Protein content of acid-whey permeates was estimated by the method of Lowry et al. (1951), using bovine serum albumin (Sigma) as a standard. The NPN was determined by a standard Kjeldahl procedure (AOAC 1970). Lactose, citric acid, and galactose concentrations were measured at regular intervals. Lactose was estimated by the dinitrosalicylic-acid technique of Bernfeld (1955). Galactose was measured using a galactose dehydrogenase assay according to the method described in the Boehringer-Manheim Company Manual (1973). Citric acid was measured by the method of Marier and Boulet (1958). Yields were calculated as anhydrous citric acid on the basis of total sugar consumed. Mycelial dry weight was determined by filtering, washing with distilled water, and drying at 90 C for 72 h.

RESULTS AND DISCUSSION

Preliminary experiments showed that after 8 d of incubation the amount of citric acid produced in acid-whey permeate varied, depending on the *A. niger* strain used (Table 1). The *A. niger* 599 produced the highest

TABLE 1. Citric acid production by *Aspergillus niger* in acid whey permeate

Strains	Mycelial Dry Wt (g/liter)	Reducing Sugar Consumed ^a		Yield of Citric Acid	
		(g/liter)	(%)	(g/liter)	(%) ^b
3	14	24.0	54	3.4	14
326	14.5	24.5	56	3.8	15.5
372	13.2	18.5	42	3.4	18.3
599	12.8	23.0	52	5.2	22
2001	10	13.0	29	1.2	9

^aInitial lactose conc.: 4.4%.

^bBased on carbohydrate metabolized.

citric-acid titer (5.2 g/liter) and was selected for further study.

The history of a typical fermentation in acid-whey permeate is given in Fig. 1. During the active growth phase, which lasted 4-6 d, lactose was metabolized slowly. It was very characteristic to find a peak in the pH profile of the fermentation after 2-4 d of incubation. This was followed by a rapid rise in acidity because of the onset of the acid-production phase. The concentration of citric acid usually reached a maximum after 8-12 d. Following the acidogenic phase, citric acid was depleted rather rapidly from the medium.

Mycelium-free fermentation samples also were analyzed for free galactose to establish whether hexose utilization by *A. niger* 599, following the hydrolytic cleavage of lactose by β -galactosidase, had a diauxic pattern. The results showed that highest galactose concentra-

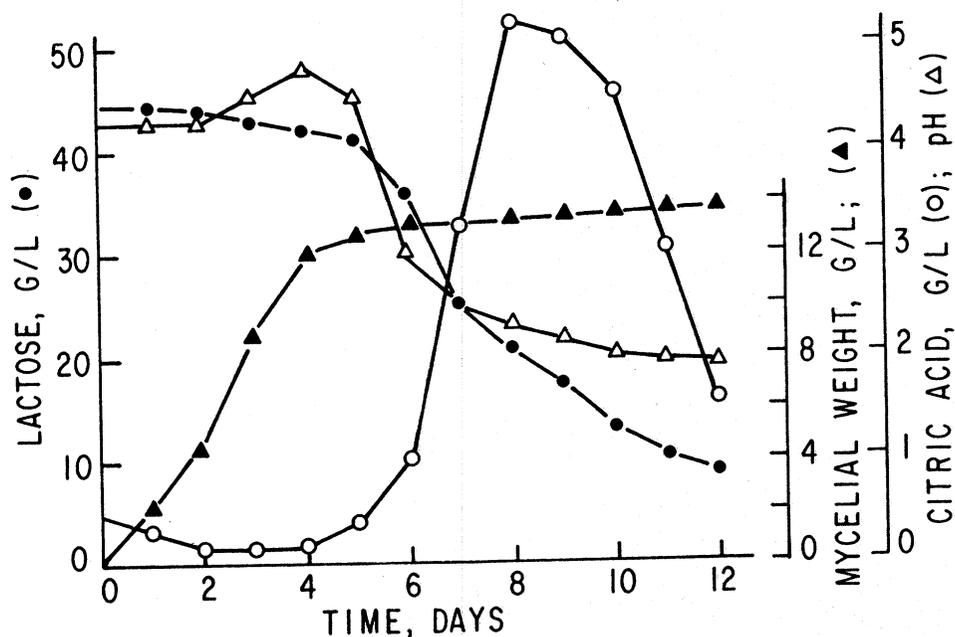


FIG. 1. Biological changes during fermentation of acid-whey permeate by *Aspergillus niger* 599.

tion in the acid-whey permeate medium was reached after about 6-7 d. However, the amount of galactose detected (4 g/liter) was only 18% of the theoretically possible concentration (22 g/liter). This was taken as evidence that the galactose moiety of lactose was co-metabolized with glucose throughout the fermentation.

The amount of citric acid produced was influenced by the initial lactose concentration of acid-whey permeate. Supplementation of the acid-whey permeate with crystalline lactose to a sugar concentration of 10% or 15% (w/v) resulted in 38% and 102% increase in citric-acid titers, respectively. With lactose adjusted to 10%, citric-acid yield was 7.2 g/liter after 10-12 d, whereas a 15% initial lactose content resulted in the accumulation of 10.5 g/liter of citric acid after 12-14 d of incubation. As before, after peak levels of citric acid were reached, the culture rapidly depleted citrate from the medium.

Citric-acid synthesis in acid-whey permeate was stimulated by the addition of methanol which is not assimilated by *A. niger*. The addition of 2% and 4% (v/v) methanol to the fermentation medium after 48-72 h of incubation resulted in a 70% (8.8 g/liter) and 136% (12.3 g/liter) increase in citric-acid yield, respectively. Although methanol stimulated citric-acid accumulation, it had no effect on the depletion of citrate from the medium and the overall history of fermentation remained nearly identical to that shown in Fig. 1, except that there was a 12-15% decrease in the mycelial dry wt attained per liter. The enhancement of citric-acid synthesis by methanol was similar to that reported by Moyer (1953) to occur in the fermentation of crude carbohydrate sources such as blackstrap molasses, wheat starch, and corn starch. Hang et al. (1977) also have described a similar enhancement of citric-acid production from brewery waste in the presence of methanol.

The function of methanol in stimulating citric-acid accumulation is not understood.

Attempts also were made to improve and stabilize citric-acid yields by using mutant strains of *A. niger* 599. Following treatment of spore suspensions with NTG, mutant colonies were selected on the basis of altered colony morphology and growth characteristics, such as loss of pigmentation, scantiness of aerial mycelium, slow growth, and slow sporulation, and on the basis of high acid production which was indicated by an intense yellow zone around colonies in whey agar-bromphenol blue indicator plates. By these criteria, 40 colonies were selected and checked for citrate production in acid-whey permeate. The mutant culture finally selected was *A. niger* 599-3. This relatively slow-sporulating strain produced an increased amount of citric acid and accumulated 18 g/liter citrate after 12 d at 30 C in acid-whey permeate with an initial lactose concentration of 48 g/liter (Fig. 2). On the

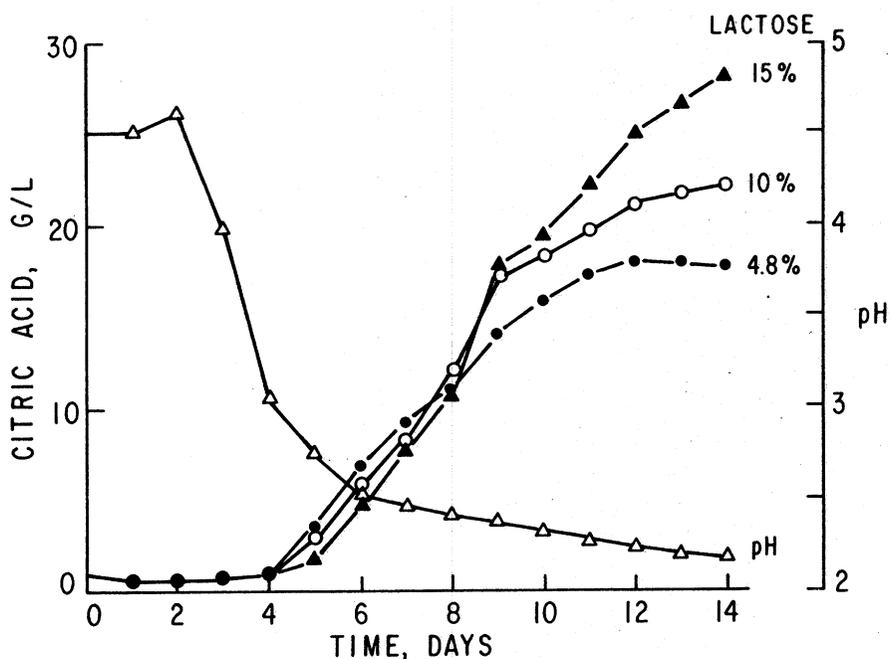


FIG. 2. Effect of initial lactose concentration on production of citric acid in acid-whey permeate by *Aspergillus niger* 599-3.

basis of lactose metabolized (44 g/liter), this represented a 41% yield of citric acid. During fermentation, the pH of the medium gradually decreased from pH 4.3 to pH 2.1. As before, a characteristic transient increase in medium pH occurred during the first phase of fermentation. The addition of methanol at concentrations of 2% and 4% (v/v) resulted in a 27% (22.8 g/liter) and 38% (24.8 g/liter) increase in the amount of citric acid produced, respectively.

The adjustment of initial lactose concentration in the acid-whey permeate to 10% and 15% (w/v) resulted in the accumulation of 23 g/liter and 28 g/liter citric acid, respectively, as shown in Fig. 2. On the basis of lactose consumed (52 g/liter and 49 g/liter), the cor-

responding citric-acid yields were 44% and 57%, respectively. Thus, similarly to citric-acid production in chemically defined media (Shu and Johnson 1948), a high concentration of carbohydrate was required in acid-whey permeate to produce high yields of citric acid. Of course, an increase in initial lactose content may also be attained by concentrating the whey permeate. For example, an approximately 15% initial lactose concentration would require a threefold concentration of acid-whey permeate. This also would increase the concentration of every other component of whey permeate, including nitrogen sources and salts, which may deleteriously influence the accumulation of citric acid. The effect of such modifications will have to be studied separately.

The above findings indicate that on the basis of displaying two distinct phases, the active growth phase and acidogenic phase, and being influenced by the initial concentration of assimilable carbohydrate, the fermentation pattern of citric-acid production in acid-whey permeate was similar to the sucrose-based process described by Shu and Johnson (1948). However, yields in acid-whey permeate fluctuated and citric acid apparently was further metabolized by *A. niger*, as evidenced by its depletion from the medium. The *A. niger* 599-3, a mutant culture selected after NTG treatment of spores, was more suitable since it accumulated 18 to 28 g/liter citric acid, depending on the initial concentration of lactose, and did not deplete the product from the medium.

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