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The effect of the sugar source on citric acid production by *Aspergillus niger*

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Summary. Under otherwise identical fermentation conditions, the sugar source has been shown to have a marked effect on citric acid production by *Aspergillus niger*. Sucrose was the most favourable source, followed by glucose and fructose and then lactose. No citric acid was produced from galactose. Strong relationships were observed between citric acid production and the activities of certain enzymes in mycelial cell-free extracts prepared from fermentation samples. When sucrose, glucose, or fructose was the sugar source pyruvate carboxylase activity was high, but 2-oxoglutarate dehydrogenase activity was not detected. When galactose was the sugar source pyruvate carboxylase activity was low, but 2-oxoglutarate dehydrogenase activity was high. It is suggested that whereas glucose and fructose repress 2-oxoglutarate dehydrogenase, thereby causing accumulation of citric acid, galactose does not. The activity of aconitase showed a direct relationship to the citric acid production rate. Thus, the activity was highest when sucrose was the sugar source, and lowest when galactose was the source. It is suggested that when large amounts of citric acid are lost from the cell the activity of aconitase increases as a response to the diminished intracellular supply of its substrate.

Introduction

Substrates commonly used for commercial production of citric acid by *Aspergillus niger* are molasses (sucrose) or starch hydrolysates (glucose) (Miall 1978). In this laboratory we have been investigating the use of whey permeate (lactose) as a substrate for this process, but the yields of citric acid observed have been low (up to 23% using standard fermentation conditions) (Hossain et al. 1983). These results may be due to the strain of organism used, the nature of the sugar source, or the other nutrients present, e.g., metal ions. As a first step towards the solution of this problem, we decided to investigate the effect of the sugar source on citric acid production, using a synthetic growth medium and a selected strain of *A. niger*. During this study it was considered appropriate to monitor the levels of selected enzymes in mycelial cell-free extracts prepared from fermentation samples, since their activities may bear a relationship to citric acid accumulation (Röhr and Kubicek 1981). There have been few previous studies on the effect of sugar source on citric acid production. Bernhauer (1928) reported that more citric acid was produced from sucrose than from glucose or fructose, and only a trace amount was produced from galactose. Noguchi (1962) observed similar effects. In the present study, the sugars investigated were sucrose, glucose, fructose, lactose, and galactose.

Materials and methods

Organism. A. niger MH 15-15, a mutant strain of *A. niger* IMI 41874, was isolated as described previously and maintained at −20° C as a spore suspension in nutrient broth containing 30% (v/v) glycerol (Hossain et al. 1983).

Chemicals. The sugars used for media preparation were all of analytical grade. Sucrose was obtained from Ajax Chemicals (Sydney, Australia), fructose and lactose from B. D. H. Chemicals Ltd., (Palmerston North, New Zealand), and galactose and glucose from Sigma Chemical Co. (St. Louis, Missouri, USA). For experiments using decationized sugars as medium constituents, decationization was performed by passing the sugar solution (140 g/l) through a column (43 cm height and 3.2 cm diameter) of Amberlite IR-120 (H) (B. D. H. Chemicals Ltd.). The remainder of the medium components were of analytical grade, obtained from B. D. H. Chemicals Ltd.

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Reagents for enzyme assays using cell-free extracts were obtained from Sigma Chemical Co.

Cultivation. The synthetic medium used was based on that described by Kristiansen and Charley (1981) and contained (g/l): sugar, 140; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; Fe^{3+} [as $(NH_4)_2SO_4 \cdot Fe_2(SO_4)_2 \cdot 24H_2O$], 0.1×10^{-3}; Zn^{2+} (as $ZnSO_4 \cdot 7H_2O$), 0.1×10^{-3}; Cu^{2+} (as $CuSO_4 \cdot 5H_2O$), 0.06×10^{-3}; made to volume with glass-distilled water and adjusted to pH 6.5 using 1 M NaOH.

Shake-flask culture experiments were performed in 250-ml Erlenmeyer flasks containing 100 ml of medium. All media and equipment were sterilized at 121° C for 20 min. A spore suspension in distilled water (approx. 1×10^8 spores), prepared by scraping spores off slopes of sucrose-beef extract agar (Sanchez-Marroquin et al. 1970), was used as inoculum and the flasks were incubated at 30° C on an Environ-Shaker Model 3597 (Lab-Line Instruments Inc., Illinois, USA) at an operating speed of 180 rpm.

Fermenter experiments were performed in a Microferm fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey, USA), using a 6-l glass vessel with a working volume of 5 l, as previously described (Hossain et al. 1983). The aeration rate was 3 l/min during the initial 48 h of fermentation, and was then increased to 5 l/min. The agitation speed was 200 rpm during the initial 48 h, and was then increased to 300 rpm. The temperature was controlled at 30° C, and the dissolved oxygen tension was continuously recorded. The pH was continuously recorded but not controlled, since previous results had shown this to be unnecessary (Hossain et al. 1983). The inoculum (10% v/v) was a 40-h shake-flask culture containing mycelial pellets of 0.2–0.3 mm diameter.

Analyses. Fermenter samples (30 ml), or whole shake-flask contents (to eliminate effects of evaporation), were withdrawn as required and filtered through Whatman No. 45 filter paper to remove mycelium. The fungal mass was washed three times with distilled water (250 ml) and dried to constant weight at 105° C. Citric acid and sugar in the filtrate were determined using high performance liquid chromatography as previously described (Hossain et al. 1983). The procedure used for citric acid also allowed the detection of isocitric, 2-oxoglutaric and succinic acids, while the procedure used for the sugars allowed the detection of gluconic acid.

Enzyme assays. These were performed using cell-free extracts prepared from fermenter cultures. Samples (50 ml) were filtered through Whatman No. 54 filter paper and the mycelium was washed with cold water followed by 0.1 M potassium phosphate buffer, pH 7.4. The washed mycelium was then suspended in the same buffer containing 1 mM EDTA (10 ml solution/g wet wt. mycelium) and the suspension was transferred to the cell of a rotary cell homogenizer (B. Braun, Melsungen, Germany) containing glass beads of 0.5 mm diameter (5 g/g wet wt. mycelium). The equipment was operated for 1 min at 4,000 rpm, at a temperature of approx. 4° C, and the homogenate was then separated from the beads by filtering through a glass sinter. Cell debris were removed by centrifugation and the supernatant liquid was assayed for enzyme activity.

All assays were performed at 23° C using a Cecil CE-272 Spectrophotometer (Cecil Instruments, Cambridge, England) and were conducted in triplicate. The activity was expressed as μmoles/min/mg protein.

Aconitase (E.C. 4.2.1.3), NAD-linked isocitrate dehydrogenase (E.C. 1.1.1.41) and NADP-linked isocitrate dehydrogenase (E.C. 1.1.1.42) were assayed as described by La Nauze (1966). Pyruvate carboxylase (E.C. 6.4.1.1) was assayed according to Feir and Suzuki (1969), and 2-oxoglutarate dehydrogenase (E.C. 1.2.4.2) as described by Reed and Mukherjee (1969). The protein content of the cell-free extracts was estimated using the method of Schacterle and Pollack (1973).

Results

Initial experiments were performed in shake-flask culture using the five different sugars (non-decationized) as carbon sources. The fermentation parameters recorded after 14 days of incubation are summarized in Table 1. Results obtained from similar experiments using decationized sugars as substrates were in good agreement to those in Table 1, indicating that the differences observed among the sugar sources were not due to contaminating metal ions. Thus, sucrose was the most favourable substrate, followed by glucose and fructose and then lactose. No citric acid was produced from galactose despite the fact that this is a readily utilizable carbon source. During fermentations involving sucrose, it was observed that complete hydrolysis to glucose and fructose occurred within the initial 30 h of the fermentation. The monosaccharides were then utilized simultaneously. No such hydrolysis was observed when lactose was the sugar source. In all

Table 1. Effect of different sugar sources on citric acid production in shake-flask culture

Sugar	pH[a]	Mycelial dry wt. (g/l)	Sugar utilized[b] (g/l)	Citric acid (g/l)	Citric acid yield[c] (%)	Maximum observed citric acid production rate (mg/g dry wt. · h)	Biomass yield[c] (%)
Sucrose	1.8	18.8	110	53	48	24	17
Glucose	1.9	19.5	90	31	35	19	21
Fructose	2.0	17.8	92	23	25	10	19
Lactose	2.0	9.8	68	5	7.5	4	14
Galactose	2.4	12.5	73	0	0	0	17

Results are expressed after 14 days of incubation
[a] Initial pH 6.5
[b] Initial sugar concentration 140 g/l
[c] Based on sugar utilized

fermentations, the only acid detected in the medium by the analytical methods employed was citric acid.

Similar experiments using non-decationized sugars were performed in fermenter culture and the results after 10 days of fermentation are summarized in Table 2. It is apparent that the citric acid concentrations obtained from sucrose, glucose, and fructose were less than those obtained in shake-flask culture. It is possible that this was due to insufficient aeration during the later stages of the fermenter cultures, since the maximum observed production rates of citric acid were reasonably similar in both culture modes (Table 1 and Fig. 1). However, in fermenter culture the rates decreased markedly after 4 days of fermentation. For all sugar sources in fermenter culture, the dissolved oxygen tension decreased to approx. 15% of saturation after 4 days and then remained constant at this level. Kubicek et al. (1980) have demonstrated the importance of dissolved oxygen tension to citric acid production.

From Table 2 and Fig. 1, it is clear that the sugar source exerts a strong effect on citric acid production. As in shake-flask culture, sucrose was the most favourable source, followed by glucose, fructose, and lactose, while no citric acid was produced from galactose. For all sugar sources, however, the biomass yields were similar. After 3.5 days of fermentation, when citric acid production rates were maximal, the rates of sugar utilization were 150, 80, 80, 80, and 120 mg sugar/g dry wt. h for sucrose, glucose, fructose, lactose, and galactose, respectively. In the case of sucrose, complete hydrolysis to glucose and fructose was observed after 1.5 days of fermentation and the monosaccharides were then utilized simultaneously at rates comparable to when each was present on its own. No such hydrolysis was observed with lactose. In all cases the only acid detected was citric acid.

The results of the enzyme assays performed on mycelial cell-free extracts at various stages of the fermentations are shown in Table 3. The relative activities of aconitase observed after 2 days of fermentation showed a strong relationship with the citric acid production rates. Thus, the activity was highest when sucrose was the sugar source and lowest with galactose. As the fermentation proceeded, however, the activities decreased, except in the presence of galactose, and these decreases coincided with decreased citric acid production rates. Similar effects were seen with both NAD- and NADP-linked

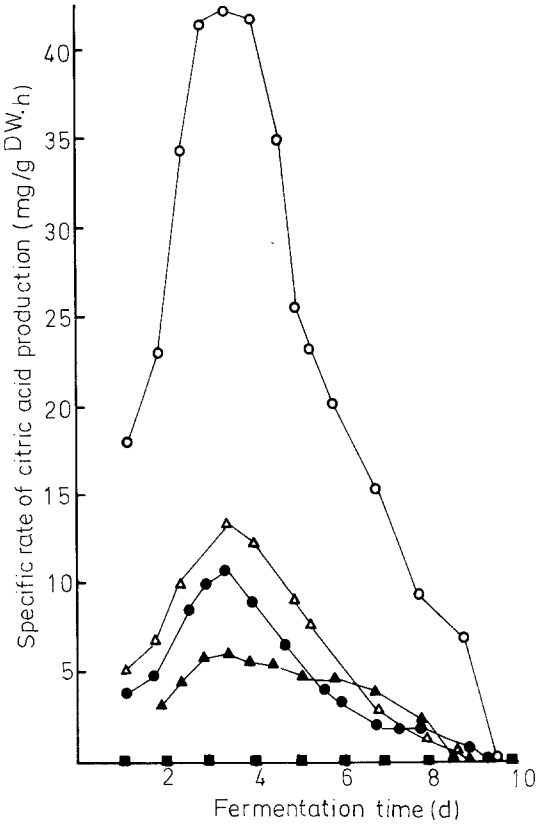

Fig. 1. The specific rate of citric acid production from different sugars in fermenter culture. ○ sucrose; △ glucose; ● fructose; ▲ lactose; ■ galactose

Table 2. Effect of different sugar sources on citric acid production in fermenter culture

Sugar	pH[a]	Mycelial dry wt. (g/l)	Sugar utilized[b] (g/l)	Citric acid (g/l)	Citric acid yield (%)[c]	Biomass yield[c] (%)
Sucrose	1.7	13.5	112	38	34	12
Glucose	1.8	14.5	100	13	13	15
Fructose	1.9	13.0	105	9.5	9	12
Lactose	2.0	10.5	70	5	7	15
Galactose	2.2	9.0	80	0	0	11

Results are expressed after 10 days fermentation
[a] Initial pH 6.5
[b] Initial sugar concentration 140 g/l
[c] Based on sugar utilized

Table 3. Activities of certain enzymes in mycelial cell-free extracts at various stages of fermenter culture

Sugar source	Day of culture	Activity (μmoles/min/mg protein)				
		Aconitase	Isocitrate dehydrogenase (NAD-linked)	Isocitrate dehydrogenase (NADP-linked)	2-Oxoglutarate dehydrogenase	Pyruvate carboxylase
Sucrose	2	2,200	28	160	0	50
	4	900	13	50	0	750
	6	400	5	25	0	1,050
	8	200	3	25	0	750
Glucose	2	1,300	23	215	0	150
	4	500	17	125	0	300
	6	300	13	45	0	600
	8	300	12	40	0	500
Fructose	2	1,500	30	90	0	100
	4	700	26	70	0	250
	6	200	10	40	0	500
	8	200	9	20	0	300
Lactose	2	200	17	70	12	50
	4	50	11	40	10	50
	6	50	9	20	8	50
	8	50	6	10	4	20
Galactose	2	100	11	25	29	20
	4	150	14	30	37	5
	6	150	15	35	45	0
	8	150	13	30	41	0

isocitrate dehydrogenase. Thus, in the early stages of the fermentation there appears to be some relationship between the levels of activity of these three enzymes and the citric acid production rate.

In the case of 2-oxoglutarate dehydrogenase, the sugar source exerted a strong effect on the activity. This enzyme was never detected when sucrose, glucose or fructose was the sugar source. With lactose, activity was at a maximum after 2 days of fermentation, after which time it decreased. With galactose, however, relatively high activity was detected, and this increased as the fermentation proceeded.

There was also a strong relationship between the activity of pyruvate carboxylase and citric acid production from the various sugars. Initial activities were low, but they increased markedly with time when sucrose, glucose, or fructose was the sugar source. With lactose, the activity remained low throughout the fermentation, and with galactose it was barely detected after the second day.

Discussion

The results demonstrate that, under otherwise identical fermentation conditions, the nature of the sugar source exerts a strong effect on levels of enzyme activity and citric acid production. Of particular note is the lack of citric acid production from galactose, a sugar which is utilized readily by the organism under study. Since the analytical methods employed detected no other TCA cycle acids or gluconic acid in the fermentation medium, the fate of the carbon is unknown. It is probable that the galactose moiety of lactose contributed to the relatively poor citric acid production from the disaccharide. A possible explanation for the superiority of sucrose over glucose and fructose is the higher rate of sugar utilization caused by the simultaneous uptake of the monosaccharides after hydrolysis.

With regard to a biochemical mechanism to explain the observed results, Röhr and Kubicek (1981) have suggested that citric acid accumulation is due, at least in part, to repression of 2-oxoglutarate dehydrogenase, thus causing a block in the TCA cycle. The present findings support this view and it might be further suggested that whereas glucose and fructose can cause repression, galactose cannot. The role of pyruvate carboxylase in citric acid accumulation is well established, as this enzyme serves to produce oxaloacetate when the TCA cycle is blocked (Röhr and Kubicek 1981). The present data agree with this concept.

The roles of aconitase and isocitrate dehydrogenase in citric acid accumulation are more controversial. Early work suggested that these enzymes disappeared during citric acid production but this has now been

discounted (Röhr et al. 1983). Thus, these enzymes continue to operate throughout the fermentation. The present findings demonstrate that when the specific citric acid production rate is high, the activities of these enzymes are also high. The decrease in enzyme activity that occurred as the fermentation proceeded coincided with decreased specific citric acid production rates. Similar decreases in enzyme activity as the fermentation proceeds have been observed by Szczodrak (1981). However, this author does not provide sufficient data to allow accurate calculation of citric acid production rates and he suggests that since the bulk of the citric acid is formed when the enzyme activities have decreased, then these decreased activities contribute to citric acid accumulation. Although the present results also show that the bulk (approx. 66%) of the citric acid is formed when these enzyme activities are decreasing (after 3.5 days of fermentation), the maximum production rates occurred while the activities were relatively high. Ahmed et al. (1972) reported increases in the activities of these enzymes during citric acid accumulation, but, again, insufficient data were provided to allow accurate calculation of citric acid production rates.

Since the data show a strong relationship between citric acid production rate and the activities of aconitase and isocitrate dehydrogenase, an explanation must be sought. One possibility is that the high levels of enzyme activity observed, as when sucrose was the sugar source, are a response to citric acid loss from the cell. When the specific growth rate is high, the requirement for biosynthetic intermediates is also high. Thus, more enzyme is synthesized to scavenge the remaining citrate to produce isocitrate and 2-oxoglutarate for biosynthesis. Then, as mycelial growth and biosynthesis decrease, so the activities of aconitase and isocitrate dehydrogenase also decrease. Thus, the level of aconitase activity has no effect on citric acid accumulation; rather, the reverse may be true. Interestingly, during citric acid-producing fermentations, the activity of pyruvate carboxylase reached a maximum after 6 days of fermentation, at which time the citric acid production rate and activities of aconitase and isocitrate dehydrogenase were decreasing. The reason for this delayed maximum is not clear. One possibility is that when growth and biosynthetic reactions are occurring oxaloacetate is produced by some other route. Then when growth slows, pyruvate carboxylase activity increases to provide the necessary oxaloacetate to maintain citrate production.

In conclusion, the results demonstrate that the nature of the sugar source has a strong effect on citric acid production and these findings can be related to the activities of certain enzymes. The key enzyme in citric acid accumulation appears to be 2-oxoglutarate dehydrogenase and it is suggested that this is repressed during growth on glucose and fructose, but not on galactose. In comparison with previous work using whey permeate as a substrate (Hossain et al. 1983), citric acid production from lactose in the present study was poor. This indicates that whey permeate is, in fact, a more suitable substrate than the synthetic medium.

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