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Influence of impeller speed on citric acid production and selected enzyme activities of the TCA cycle

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SUMMARY

The effect of impeller speed on citric acid production and selected enzyme activities of the TCA cycle was studied. The highest yield of citric acid (28 g/l) was obtained in culture agitated at lower speed (300 rpm). The activity of citrate synthase decreased with the increase of speed of agitation, while the activity of aconitase and isocitrate dehydrogenase increased with the increase in agitation speed.

INTRODUCTION

Citric acid, a tricarboxylic acid, is used in the pharmaceutical, food and beverage industries as an acidifying and flavour-enhancing agent. It is mainly produced from beet and cane molasses by surface and submerged fermentation using *Aspergillus niger* [4,6–8,15]. Beet molasses is a suitable raw material for citric acid production because it is readily available and relatively low priced.

The effect of agitation on citric acid production is extremely important for the successful progress of the fermentation. It not only assists mass transfer between the different phases present in the culture, but also maintains homogeneous chemical and physical conditions in the culture by continuous mixing [11]. Ng et al. [10] studied the effect of dilution rate on the specific activities of selected enzymes of the tricarboxylic acid (TCA) cycle and demonstrated that the activity of enzymes involved in the TCA cycle has an important influence on the progress of the citric acid fermentation.

This study examined the influence of agitation speed on citric acid production and the activity of enzymes of the TCA cycle during citric acid production from beet molasses by *A. niger* in submerged culture.

MATERIALS AND METHODS

Organism and culture conditions

Aspergillus niger ATCC 9142 was used throughout the investigation and cultivated as described previously [13]. Beet molasses, from a Greek sugar factory (Platy, Thessaloniki), was diluted with distilled water in a 1 : 4 ratio to 14% (w/v) total sugar concentration. The medium was adjusted to pH 6.0 with concentrated HCl, sterilized at 121 °C for 2 h and treated while hot with potassium ferrocyanide to encourage the precipitation of heavy metals [5]. The pH of the cooled medium was adjusted to 6.5.

The fermentation was carried out in a 12-l stirred tank fermenter (S.K. Fermenters Ltd., Manchester, U.K.) with a working volume of 9 l and a temperature of 30 °C. The fermenter consisted of a glass vessel with stainless steel end plates and had four equally spaced vertical baffles. The aeration rate was 1 vvm and agitation was provided by a six-flat-blades turbine operating at 300–600 rpm. The impeller was located 5 cm above the bottom of the vessel. During the initial 24 h of fermentation polypropylene glycol was used to control heavy foaming. A spore inoculum [14] was used to the fermenter to give a final concentration of approximately 2.7×10^5 spores/ml.

Analytical techniques

Citric acid, biomass dry weight, residual sugars as sucrose, pH, protein and the specific activity of citrate synthase (CS), aconitase ACH_1 (reaction: citrate → *cis*-aconitate) and ACH_2 (reaction: *cis*-aconitate → isocitrate), NAD-linked isocitrate dehydrogenase (NAD-ICDH) and NADP-linked isocitrate dehydrogenase (NADP-ICDH) were determined as previously described [12]. Dissolved oxygen concentration was measured using an autoclavable oxygen electrode (Uniprobe Ltd.). The values of the readings were expressed as percentage of the initial level of saturation. Shear-dependent (apparent)

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viscosity was measured by means of a portable viscometer (Ferranti) at a shear rate of 68 s^{-1}. The reported data are the average values of two separate experiments.

RESULTS AND DISCUSSION

Effect of agitation speed on citric aid production

As shown in Fig. 1, the concentration of citric acid decreased as agitation speed increased from 300 to 600 rpm. This was due to the changes noted in the enzyme activities which are connected to the citric acid accumulation (Figs. 5–7). The highest concentration of citric acid (28 g/l) was obtained in the culture agitated at 300 rpm, while in cultures agitated at 400, 500 and 600 rpm the maximum concentration of citric acid was lower by 7, 21 and 28%, respectively. Chopra et al. [3] found a maximum concentration of citric acid (42 g/l) at low speed of agitation (100 rpm) during citric acid production from sucrose by *A. niger* RRL 12-6/1-2. In contrast, Clark and Lentz [4] reported a maximum concentration of citric acid (86 g/l) at high speed of agitation (600 rpm) when *A. niger* NRC A-1-233 was grown on beet molasses medium. There are several possible reasons for these differences including the chemical composition of substrate, the strain of organism used and physical environmental factors such as dissolved oxygen tension.

Effect of agitation speed on culture morphology and dissolved oxygen concentration

At agitator speed 300 rpm or higher, pellets (2–3 mm diameter) were formed within 24–48 h of inoculation which crushed later into pieces of mycelia (100–250 μm length) in which the hyphae formed a homogeneous suspension dispersed through the fermentation broth.

The concentration of dissolved oxygen increased with the increase of speed of agitation (Fig. 2). In all cultures the concentration of dissolved oxygen fell rapidly during the first 2 days of fermentation after which it increased slower. This was due to the rapid increase of biomass concentration observed at the same time. In the cultures agitated at 300, 400, 500 and 600 rpm the concentration of dissolved oxygen from the 2nd–12th day remained at about 22, 30, 40 and 50% of the initial saturation level, respectively.

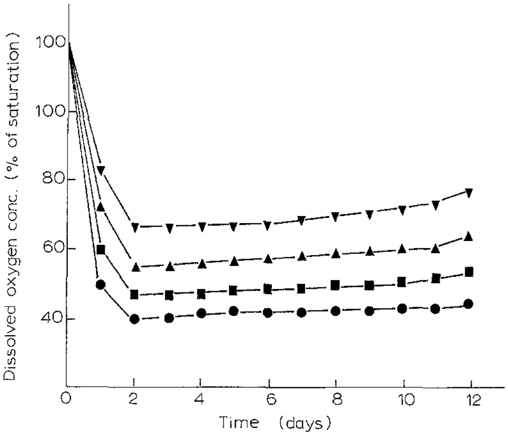

Fig. 2. Effect of stirred speed on dissolved oxygen concentration during citric acid production by *A. niger* in batch culture (●) 300 rpm; (■) 400 rpm; (▲) 500 rpm; (▼) 600 rpm.

Effect of agitation speed on biomass and residual sugars concentration

The results of biomass and residual sugars concentration as function of speed of agitation are shown in Fig. 3. The biomass concentration increased with the increase in the speed of agitation from 300 to 600 rpm. This

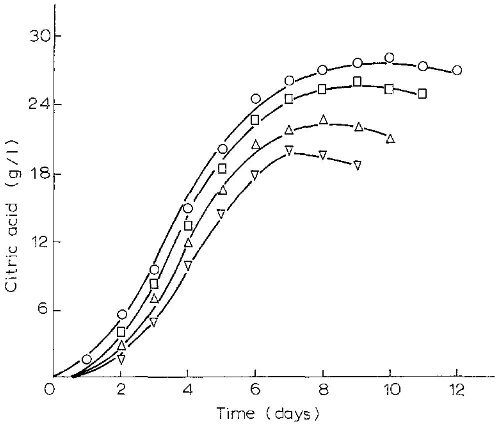

Fig. 1. Effect of stirred speed on citric acid production from beet molasses by *A. niger* in batch culture (○) 300 rpm; (□) 400 rpm; (△) 500 rpm; (▽) 600 rpm.

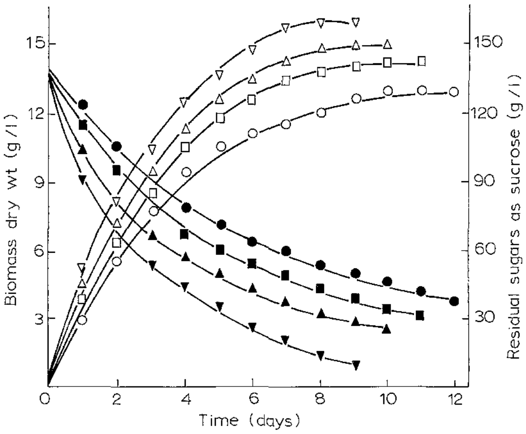

Fig. 3. Effect of stirred speed on biomass and residual sugars concentration during citric acid production by *A. niger* in batch culture. Symbols as in Fig. 1 (biomass dry weight), symbols as in Fig. 2 (residual sugars).

may be explained by the fact that the crushing of the pellets into small pieces of mycelium, resulted in the surface of mycelium having much better contact with nutrients and oxygen. In culture agitated at 300 rpm, maximum dry weight was obtained on the 10th day, while in cultures agitated at 400, 500 and 600 rpm, maximum dry weight was found to be 1, 2 and 3 days earlier, respectively. This means that, the better the condition of aeration was, the earlier the maximal mycelial weight occurred. The maximum biomass concentrations of the cultures agitated at 300, 400, 500 and 600 rpm, were 13.0, 14.3, 15.0 and 16.0 g/l, respectively.

As expected, the concentration of residual sugars decreased during the fermentation coinciding with an increase in biomass and citric acid production. As shown in Fig. 3 the assimilation of residual sugars increased as agitation speed increased. This was expected also since there was an increase in biomass concentration when the agitation speed was increased. The concentration of residual sugars fell rapidly during the first 4 days of fermentation, after which it decreased slower. When the maximum production of citric acid was achieved in cultures agitated at 300, 400, 500 and 600 rpm, 29.5, 26.0, 21.5 and 17.0% of sugars consumed were converted to citric acid, respectively.

Effect of agitation speed on shear-dependent viscosity and pH

The shear-dependent viscosity decreased with the increase of speed of agitation (Fig. 4). This was unexpected, because the biomass concentration increased with the increase of speed of agitation. This may be explained by the fact that the filamentous pellets were crushed into smaller pieces by the increased speed of agitation as a result of the shear stress between the fermentation broth and the impeller. In the culture agitated at 300 rpm the maximum shear-dependent viscosity was 220 cp, while in cultures agitated at 400, 500 and 600 rpm the maximum shear-dependent viscosity was lower by 10, 18 and 27% respectively. In general, the reduction in viscosity with increasing stirred speed indicated that the culture morphology is the major determinant of the rheological properties of the fermentation broth.

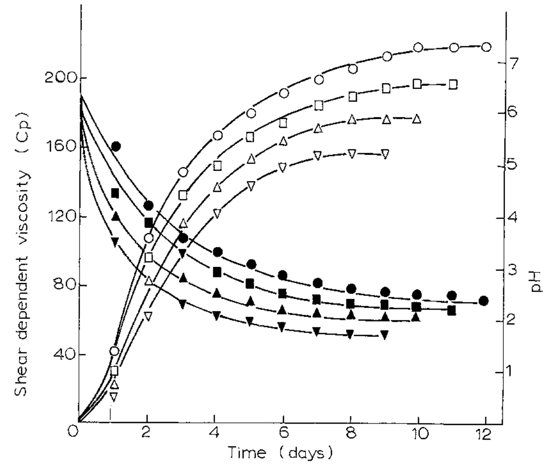

Fig. 4. Effect of stirred speed on shear-dependent viscosity and pH during citric acid production by *A. niger* in batch culture. Symbols as in Fig. 1 (shear-dependent viscosity), symbols as in Fig. 2 (pH).

The pH decreased during the fermentation. In cultures agitated at 300, 400, 500 and 600 rpm, the pH decreased from an initial value of 6.5 to 2.4, 2.2, 2.0 and 1.8, respectively (Fig. 4).

Effect of agitation speed on the activity of CS, ACH and ICDH

CS activity decreased with increasing agitation speed between 300 and 600 rpm (Fig. 5). It followed a pattern similar to citric acid concentration with maximum activity observed at the same time as the maximum concentration of citric acid was observed.

Both ACH and ICDH activities increased with the increase of speed of agitation from 300 to 600 rpm, although the specific activities of ACH_1 and NAD-ICDH were lower than that of ACH_2 and NADP-ICDH, respectively (Figs. 6 and 7). Szczodrak [15] found that the specific activity of NADP-ICDH was higher than the activity of the NAD-ICDH during citric acid fermentation by *A. niger* in surface and shake flask cultures. The lowest ACH and ICDH activities were obtained at the same time as the maximum concentrations of citric acid were observed. The above results show that the increase in agitation speed resulted in the changes in the activity of enzymes, which are connected to the citric acid accumulation. This was due to the change of dissolved oxygen concentration, biomass concentration, viscosity and pH with the increase of speed of agitation (Figs. 2–4).

In general, our results show that the accumulation of citric acid is accompanied by an increase in the activity of CS and a decrease in the activity of ACH and ICDH responsible for the degradation of citric acid in the TCA cycle. These results agree with those of Szczodrak [15] who studied the activity of selected enzymes of the TCA cycle during citric acid production from beet molasses by *A. niger* I_{13} in surface and shake flasks culture, but are not in agreement with Ahmed et al. [2] who showed an increase in the activity of ACH and ICDH when *A. niger* IMI 41873 was grown on a chemically defined medium in submerged culture. As shown in Figs. 5, 6 and 7 the activities of CS, ACH and ICDH were never inactivated during the fermentation. This is in agreement with the results of La Nauze [9] who studied the activity of selected enzymes of the TCA cycle during

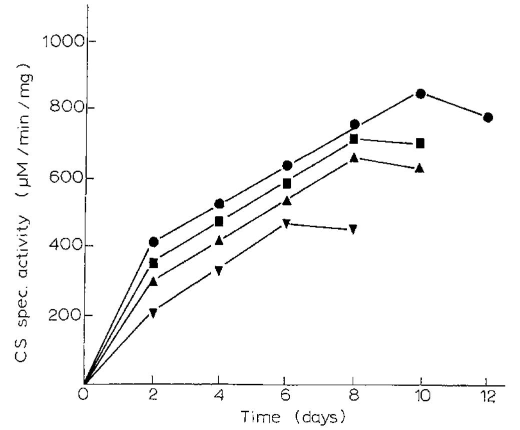

Fig. 5. Effect of stirred speed on the activity of CS during citric acid production by *A. niger* in batch culture. Symbols as in Fig. 2.

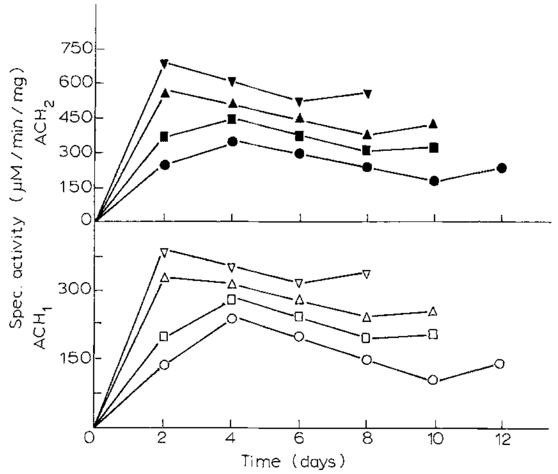

Fig. 6. Effect of stirred speed on the activity of ACH during citric acid production by *A. niger* in batch culture. Symbols as in Fig. 1 (ACH_1). Symbols as in Fig. 2 (ACH_2).

citric acid accumulation by *A. niger* strains (72–4 and 72–44) in submerged culture, but are in contrast to those of Agrawal et al. [1] who found that CS, ACH and ICDH activity totally disappeared during citric acid accumulation by *A. niger* strain A in surface fermentation. There are some hypotheses for these differences including the chemical composition of substrate, the fermentation system, the design of fermenter and the fermentation conditions (temperature, pH, oxygen supply, speed of agitation, etc.). For instance, at low speed of agitation, the oxygen transfer rate may be a problem. Thus, a decrease in the activity of enzymes may be in connection with the insufficient oxygen supply of the culture. At high agitator speeds, either mycelial damage or too high concentration of dissolved oxygen may be problems.

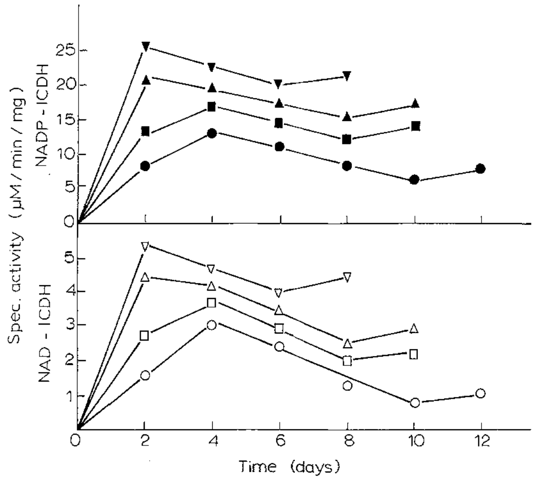

Fig. 7. Effect of stirred speed on the activity of ICDH during citric acid production by *A. niger* in batch culture. Symbols as in Fig. 1 (NAD-ICDH), symbols as in Fig. 2 (NADP-ICDH).

In conclusion, our results have brought to light three important aspects of citric acid fermentation from beet molasses by *A. niger* in submerged culture. Firstly, increase in speed of agitation caused the decrease in citric acid concentration. Secondly, increase in speed of agitation resulted in the decrease in CS activity and increase in ACH and ICDH activities, respectively. Third, citric acid accumulation is accompanied with the increase in CS activity and decrease in ACH and ICDH activities.

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