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EFFECT OF LOW pH ON CONTINUOUS CITRIC ACID FERMENTATION BY *ASPERGILLUS NIGER*

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Abstract

The present study is concerned with the effect of low pH on continuous citric acid fermentation by *Aspergillus niger* strain SK-17. Microbial cultivations were carried out in a stainless steel stirred bioreactor of 15 L total capacity. The bioconversion of glucose to citric acid at low pH was investigated on cellulose fabric. Sucrose solution (100 g/l) was made to flow through capillaries of a vertical fabric support and oxidized to citric acid at the interface. Conditions of temperature, humidity, airflow and glucose feed rate were optimised. The emerging broth contained a product concentration of 120-140 g/l of citric acid, which is higher than the expected [maximum of 109 g citric acid per 100 g sucrose] as a result of evaporative concentration during the downward flow.

Introduction

An appropriate initial pH is important for the progression and successful termination of fermentation. A higher initial pH leads to the accumulation of oxalic acid. A lower pH of cane molasses, however, has been found to be inhibitory for the growth of *A. niger* (Rohr *et al*., 1996; Hess *et al*., 2000). According to Guebel & Torres (2001), a close energetic coupling relation exists between the citric acid production and pH regulation. So, for each set of fermentation conditions, the optimisation of initial pH should be made. During the fermentative formation of citric acid, the pH of the broth falls resulting in lowered bioconversion. To overcome this problem, neutralizing agents such as Sodium hydroxide, calcium carbonate or lime are developed to maintain the pH between 5-6 facilitating the action of mycelial glucose oxidase. Lee *et al*., (1987) used a semi-continuous mode of fermentation by recycling mycelial biomass in successive cycles and obtained citrate bio production in a rotary fermentor.

In the present study, we report the bioconversion of sucrose to citric acid using *Aspergillus niger* on cellulosic fabric as a support matrix. The substrate was dripped on to the upper end of the fabric and during its downward movement through the capillaries has abundant opportunity to be bio converted at the interface by the mycelium entangled with the capillaries. The product formed emerges as free acid from the lower end.

Materials and Methods

Organism and culture maintenance: The strain *A. niger* SK-17 procured from our culture collection was used throughout the study. The strain was maintained on potato-dextrose-agar slopes (PDA).

Growth and fermentation media: Thirty gram glucose anhydrous; 0.25 g $MgSO_4.7H_2O$, 0.3 g KH_2PO_4 and 0.8 g $(NH_4)_2HPO_4$ in 1 L distilled water, 2.5 g Calcium

carbonate was sterilized separately and added to the medium to maintain the pH between 4 and 6. The fermentation medium consists of 100 g glucose anhydrous purified, 0.035 g $MgSO_4.7H_2O$, 0.05 g KH_2PO_4 and 0.1 g $(NH_4)_2HPO_4$ in 1 L tap water. The pH of the medium was adjusted to 6.0 using 1 N H_2SO_4.

Starter medium: This was a low pH (2.8-3.0) medium, which was fed to the *A. niger* in the first 24 h of fermentation period to enhance productivity. The synthetic medium was prepared by acidifying the fermentation medium using 1 N H_2SO_4 to pH 2.8-3.0. The partially fermented medium (PFM): submerged stage growth medium (resulting after mycelial growth), which had been depleted of nitrogen and attained a pH of 2.8-3.0 was filtered, autoclaved and used directly.

Mycelium at surface and submerged modes: One hundred grams of glucose anhydrous purified, 0.156 g $MgSO_4.7H_2O$, 0.188 g KH_2PO_4 and 0.388 g $(NH_4)_2HPO_4$ in 1 L tap water was used. The pH of the medium was adjusted to 6.0 with 1 N H_2SO_4. The medium was sterilized at 15 psi for 15 min and other minerals were not added during growth and fermentation. A cut piece of cellulosic fabric (68 x 8 x 0.5 cm) was used.

Fermentation technique: The fermentation unit consisted of an enclosed chamber (15 L stainless steel bioreactor) equipped with humidifier, temperature controller and inlet and outlet for air. For the purpose of comparison, citric acid production using free (filamentous-or pellet form) mycelia was carried out in 500 ml Erlenmeyer flasks containing 75 ml of the above mentioned fermentation medium. Conidia of 7 days old *A. niger* SK-17 grown on a PDA slope were inoculated in to the flask and incubated either on a shaker (210 rpm) or stationery at 30ºC. In the case of citric acid production with mycelia, the cellulosic support on which *A. niger* mycelia were grown was fixed into the fermentation unit.

Analyses: Residual sugar was analysed by di-nitro-salicylic acid method (Miller, 1959). Most routine analyses were performed by simple titration. Mycelial morphology was studied. Samples were fixed with ultra-violet light and mounted on brass stubs. Specimens were coated with a thin layer of gold (100 Å) in a gold coating unit; model E 5000, Porland Equipment. Mycelial cell mass was determined by washing the support with distilled water until all traces of citric acid were removed, and thereafter drying it at 80ºC for 48 h. During the steady state fermentation (50-400 h) the fabric support was progressively cut at 10 cm intervals and emerging fluid was analysed for pH conversion of glucose.

Results and Discussion

In a submerged culture at pH 6.0, citric acid productivity of *A. niger* increased when the mycelial growth and fermentation was carried out at higher dissolved oxygen (DO) concentration (Fig. 1). In the present study, *A. niger* grown in air or in oxygen phase was on a fabric support and its ability to bring about continuous bioconversion at low pH was studied (Fig. 2). Air grown mycelia attained the peak productivity later than in the case of mycelia grown on oxygen. There was a lag period of about 8 h in respect of mycelia grown on oxygen. In both cases, there was a marginal decrease in productivity after

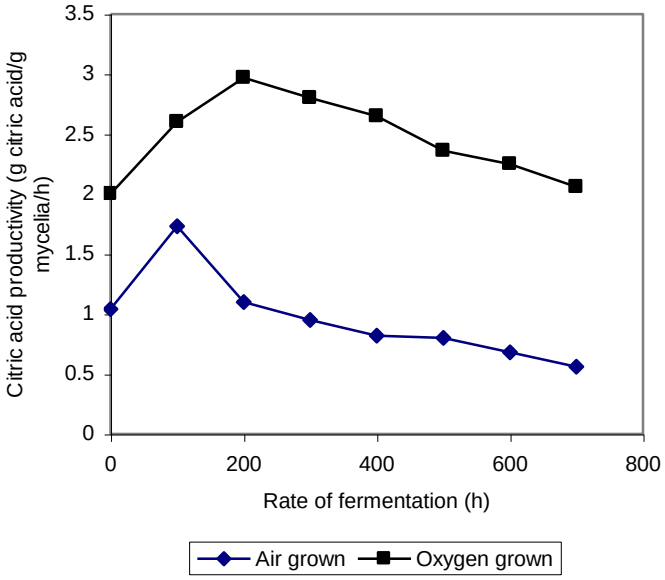

Fig. 1. Citric acid production rate by *A. niger* grown in the atmosphere of oxygen and in air. Oxygen grown mycelia are durable up to 48 days, whereas air grown mycelia loose the activity at about 14 days.

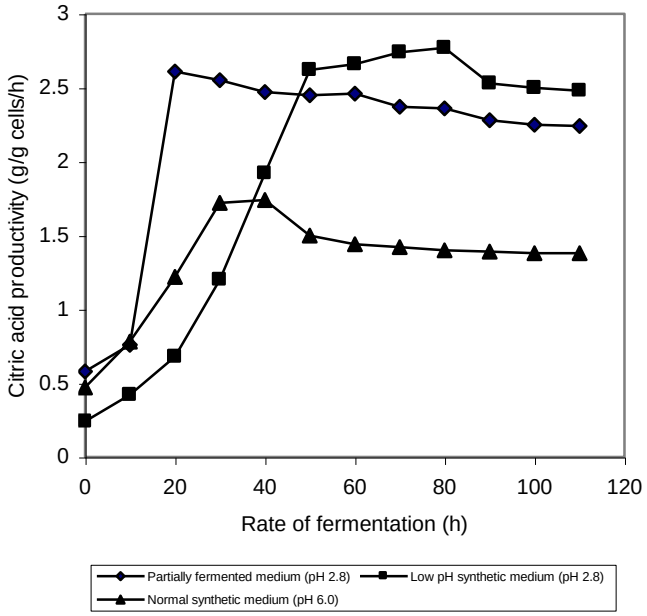

Fig. 2. Use of starter media in the initial stage (24 h) of fermentation and its effect on citric acid productivity by the oxygen grown *A. niger* for partially fermented medium, low pH synthetic medium and normal fermentation medium.

reaching the peak value. In addition, the oxygen grown mycelia showed a sustained productivity for a period of up to 48 days as against 16 days for mycelia which were air grown. When air grown mycelium were deployed there was restricted access of the medium (flowing downwards) to the biomass on the surface due to the nature of the mycelial growth which was thick and uncontrolled. Simultaneously, thicker growth resulted in a restricted access of atmospheric oxygen to the medium/mycelium interface (Mullar, 1986).

The morphological development of the mycelia was studied to determine the type of growth, which favours efficient bioconversion (Vassilev *et al*., 1993). In the first case, mycelial growth resulted in coil formation and its winding around fabric fibers, which permits better interaction at the interface. In the second case the mycelial mat was thick restricted access of the medium to another portion of biomass as well as air. The effect of acclimatizing mycelia on productivity was examined by feeding a starter medium (low pH synthetic medium or partially fermented medium of pH 2.8-3.0) in the initial stage (24 h) of fermentation (Fig. 2,3). Experiments in which low pH synthetic medium was used for acclimatization led to a comparable productivity values after a considerable lag period. In the absence of such acclimatization, the productivity remained at a much lower level.

In the present study, progressive formation of citric acid was observed throughout the fabric support in spite of progressive lowering of pH (Fig. 3). The pH profile on the fabric during fermentation was measured and a pH gradient was observed on the fabric support. The feed pH was 6.0 whereas the exit pH was 2.1. The pH profile on the support indicates incremental formation of citric acid in every successive zone of fabric support. Average formation of citric acid was computed to be 2.2 g citric acid mycelia h^{-1}. Repeated use of the biomass eliminated substrate diversion, reduced the lag phase and improved the overall economics of fermentation (Cai & Yin, 1989). The present study differs in being continuous in nature and was carried out in a surface mode and at low pH over 48 days (Fig. 4). During this period, the productivity dropped marginally as time progressed. This drop lowered the bioconversion efficiency by about 10 %, which was brought back to the original level by reducing the flow rate.

During the initial stage of fermentation (10-50 h) the titrable acidity of the emerging broth was higher than the theoretical value expected on the assumption that all glucose was converted to citric acid. The discrepancy was traced to contaminating citric acid formed during this phase (Schuster *et al*., 2002). In this period of the first 50 h of fermentation exit, broth contained 135 g/l citric acid and 4 g/l. At low pH the appearance of gluconic acid as a contaminant during citric acid fermentation was reported. Citric acid fermentation is highly aerobic, since oxidation of 1.0 mole of glucose requires a half mole of oxygen, which is to be provided in the form of pure oxygen or air (Drysdale & McKay, 1995; Watanabe *et al*., 1998; Haq *et al*., 2001). In the present study on a laboratory scale (68 x 8 x 0.5 cm support) no separate aeration was needed as the temperature in the unit was slightly above the ambient temperature causing a gentle convective airflow through the unit. A separate experiment to estimate the actual aeration need was conducted in a vertical glass reactor having a height of 70 cm and internal diameter of 10 cm. In the surface fermentation described here, during downward passage of the medium a considerable amount of water loss up to 25% was observed. This evaporative loss of the water vapour concentrates the emerging citric acid up to 40 g/l.

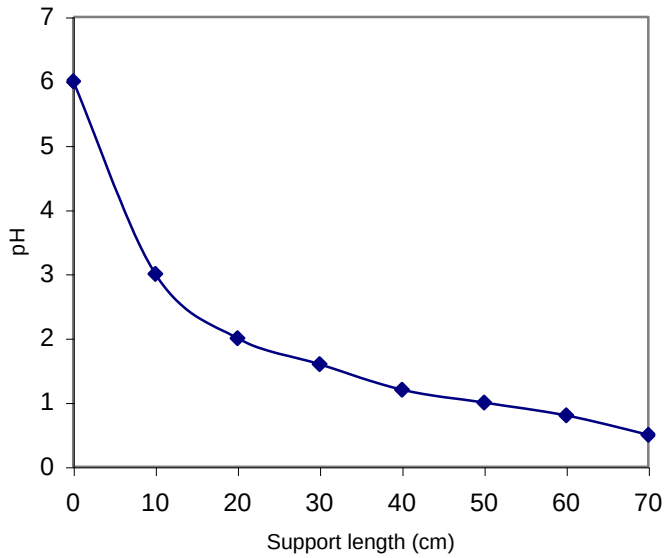

Fig. 3. A pH profile on the fabric support during fermentation of glucose to citric acid by *A. niger*. Mycelia were oxygen grown and the pH was measured in the steady state of fermentation. Feed pH of the medium (at the top of the support) was 6.0 whereas exit broth was 2.1.

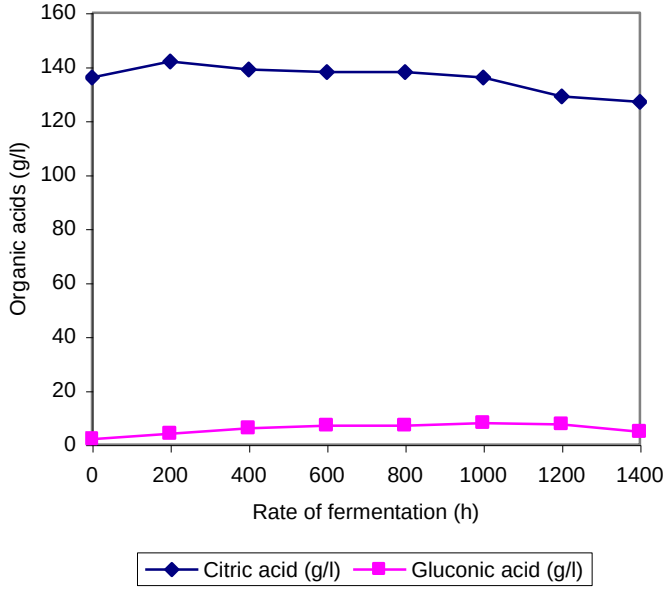

Fig. 4. Formation of gluconic acid during citric acid fermentation at low pH by oxygen grown *A. niger*. In earlier period of 50 h citric acid production was higher but decreased with further incubation.

An experiment was set up in which the flow rate (glucose feed rate) was increased to increase the availability of glucose to values above 2.25 g citric acid g mycelia h^{-1}. The productivity of the biomass was unaltered leading to emergence of some unreacted glucose. Free citric acid obtained as a solution in the fermented broth had a brownish appearance, which was decolourised by 2 g/l of commercial grade powdered activated charcoal. This broth could be concentrated to 50% by evaporation. Similar, kind of findings has also been reported by Sakurai *et al*., (1997). In submerged fermentation the average citric acid productivity at 2-12 days was 0.18 g citric acid g mycelia per hour and utilization of sucrose was 45% at the end of 12 days. When fermentation was carried out in the stationary culture, a thick mat was formed which on an average at 2-12 days gave 0.22 g citric acid per g mycelia per hour at the end of 12 days and 60% sucrose was converted into citric acid. In this mode, sporulation began approximately 24 h after the inoculation. In both modes of fermentation, the biomass formed was reused with fresh medium. The conversion efficiency was found to drop progressively after the second recycle of biomass.

From the results, we hypothesize that it is quite possible to bring about continuous stationary bioconversion of sucrose to citric acid at low pH (2.1) using *A. niger* conidia. The fermentation system had a conversion efficiency of more than 95 % theory based on sucrose utilized. The product obtained was in the form of a solution containing up to 14% free citric acid.

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