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Oxalic Acid Production by *Aspergillus niger:* Influence of Hydrogen Ion Concentration and Nitrogen Source*

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Abstract: Fermentative production of oxalic acid by *Aspergillus niger* NCIM 548 strain depended on the initial medium pH and better yield was obtained at pH 4-7. Yield of oxalic acid augmented (2.5 to 3-fold) if pH of the broth was maintained at 6-7 by occasional addition of alkali. The strain produced more oxalic acid from glucose with ammonium salts, especially diammonium hydrogen phosphate, as the N-source in comparison to other ammonium salts, $NaNO_3$ and urea. With lactose as the C-source, yield of oxalic acid was maximum with ammonium dihydrogen phosphate closely followed by diammonium hydrogen phosphate; the yield was much better with urea than with ammonium nitrate or ammonium sulphate.

Key words: *Aspergillus niger*, oxalic acid, N-source, biomass, medium pH

INTRODUCTION

Oxalic acid has wide applications and is prepared mainly by chemical methods (Pernet, 1991). In nature, it is secreted by many saprophytic and phytopathogenic fungi (Dutton and Evans, 1996; Sayer *et al*., 1999). Emerging applications of this acid in hydrometallurgy for extracting iron and some heavy metals from various resources have renewed the interest of its fermentative production, preferably using a cheap carbon source (Strasser *et al*., 1994; Bohlmann *et al*., 1998; Cameselle *et al*., 1998; Santoro *et al*., 1999). We screened a range of microbial strains in order to upgrade the quality of china clay by lowering its iron content (Mandal *et al*., 2002). The best activity was displayed by the culture filtrate of *Aspergillus niger* NCIM 548 strain (Mandal *et al*., 2002) and the secreted oxalic acid was found to be the causative agent (van de Merbel *et al*., 1994). The immobilized fungal strain could produce sufficient oxalic acid from glucose (Mandal and Banerjee, 2005), which is usually converted to gluconic acid under submerged condition by *A. niger*, in general (Milsom and Meers, 1985; Kubicek and Röhr, 1986). The production of oxalic acid by this fungus is highly regulated depending much on the factors like C- and N-source and the initial medium pH (pH_m) as well as the culture/broth pH during fermentation (pH_c) (Strasser *et al*., 1994; Bohlmann *et al*., 1998; Cameselle *et al*., 1998; Santoro *et al*., 1999). In view of the fact that immobilized *A. niger* NCIM 548 strain could produce high yield of oxalic acid from glucose and lactose, the two widely available cheap C-sources (Mandal and Banerjee, 2005), we undertook this work on reviewing the effect of factors like (I) the hydrogen ion concentration of the medium (pH_m) and culture (pH_c) and (ii) the N-source, both of which might have influence toward oxalic acid production by the *A. niger* strain. We had sequentially studied these aspects and report the results in this article. We surmise that this study will immensely help in standardizing the fermentation conditions of oxalic acid production from glucose, lactose or other cheap sources like whey by *A. niger*.

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Materials and Methods

This study was conducted in the Molecular Genetics Laboratory of Indian Institute of Chemical Biology, Kolkata, India during 2004.

Microbial Strain and Medium Composition

Aspergillus niger NCIM 548 strain used in this study was kindly provided by Prof. A. K. Guha, Indian Association for the Cultivation of Science, Kolkata, India.

The medium usually contained the following components in (g L^{-1}): glucose (105.5); $NaNO_3$ (1.5); KH_2PO_4 (0.5); $MgSO_4.7H_2O$ (0.025); KCl (0.025); yeast extract (1.6) (Strasser *et al*., 1994). In view to studying the effect of N-sources, $NaNO_3$ was replaced with other nitrogenous compounds, viz., diammonium hydrogen phosphate (1.18 g L^{-1}), ammonium dihydrogen phosphate (2.08 g L^{-1}), ammonium sulfate (1.18 g L^{-1}), urea (0.54 g L^{-1}), each containing the same amount of nitrogen (0.025%) as $NaNO_3$. The amount of lactose in the medium was 102.5 g L^{-1} when it was used as the C-source. Medium pH (pH_m) was adjusted to 6 with 4 M NaOH before sterilization. Universal pH indicator solution (20 mL per liter; Merck, India) was added to the medium (before sterilization) to observe its pH which was maintained within 6 to 7 by adding alkali during fermentation whenever necessary.

Spore Suspension

Spores from 7-day stationary culture at 30°C in potato-dextrose broth were suspended in Triton-X100 solution (0.001%, v/v) and were counted under microscope.

Fermentation

Fifty milliliter culture medium taken in a 250 mL Erlenmeyer flask was inoculated with spores of *A. niger* (5×10^6 spores per mL medium) and was incubated at 30°C on an orbital shaker at 215 rpm for 7 days or as desired. Culture filtrate from each triplicate set was analyzed for oxalic acid content. During fermentation, pH of the culture (pH_c) was maintained at 6.5±0.5 with 4 M NaOH whenever necessary. Growth and oxalic acid production were monitored during the course of fermentation and the data presented were average of two or more experiments.

Analytical Methods

Broths after fermentation were filtered through 0.2 μm membrane. Oxalate in the filtrate was estimated by titration with $KMnO_4$ after precipitating it with $CaCl_2$ (Bhattacharyya, 1992; Vogel, 1943). In limited cases, it was estimated by HPLC using deproteinized culture filtrates in Shodex KC-811 (bed volume, ca. 15 mL) column (Waters, USA). This column is packed with a sulfonated rigid styrene-divinyl benzene copolymer providing high efficiency separation of low molecular weight water-soluble organic compounds including organic acids. The column provides ion exclusion and reversed phase mode of chromatography. Mobile phase used for the best resolution was 0.1% phosphoric acid (14.8 mM) in water at a flow rate of 1 mL min^{-1} at 60°C. Depending upon the amount of organic acid present in the culture filtrate, sample volume of 12.5, 25 and 50 μL was used for analysis of different organic acids. Each organic acid was eluted from the column after a definite Retention Time (RT) and measured as absorbency at 214 nm (Model 2487 Dual λ Absorbance Detector, Waters, USA). The observed RTs for oxalic, citric and fumaric acid corroborated closely with the literature supplied by manufacturer of the column; the RT for gluconic acid was determined as 6.56 min (average of 4). The amount of each organic acid was measured from the peak area in

comparison with the area of known concentrations of standard organic acids by the integrated Millennium[32] software (Waters USA) according to the manufacturer's bulletin.

Sugars were estimated colorimetrically using orcinol (Brown and Anderson, 1971) as described previously (Mandal and Banerjee, 2005). Biomass of *A. niger* grown under different conditions was measured gravimetrically after separating the mycelia from fermented broth by filtration, washing with water and drying at 80°C to constant weight.

RESULTS AND DISCUSSION

Production of oxalic acid has been reported to be influenced by medium pH and fermentable sugars (Strasser *et al*., 1994; Bohlmann *et al*., 1998). Therefore, the effect of pH (range 2-8) on oxalic acid production from glucose ($NaNO_3$ as the N-source) by this specific strain was investigated first. Table 1 shows that the production increased steadily with increase in pH up to 6 during 7 days. However, the amount of oxalic produced was almost same at pH 3, 4 and 7 after 9 days of fermentation and the maximum production was noted at pH 6. There was slight or no change in the medium pH during initial 36 h of fermentation, which may be due to slow growth of the strain in this period. Thereafter, it changed to varying extent depending on the pH_m; the final pH was within 1.7 to 2.1 on the 9th day.

Since the medium pH dropped significantly during fermentation that might have strong effect on oxalic acid production, we investigated this issue by monitoring the oxalic acid production on different days of fermentation at the optimum initial medium pH of 6 by adjusting the broth pH (pH_c) at this level. When pH_c was not adjusted, production of oxalic acid increased linearly with time at a constant rate, while at controlled pH the production rate was very high during 3rd to 5th day and decreased thereafter (Fig. 1). It is evident from this Fig. 1 that 2.5-fold increase in average yield of the acid (74.8 vs. 29.8 mM) occurred on the 7th day when pH_c was maintained at ca. 6.5 compared to that without pH control where it decreased. In the later case, average pH_c was 3.54, 2.73 and 1.88 on 3rd, 5th and 7th day, respectively. The amount of sugar utilized was higher under pH-control experiment in comparison to the uncontrolled set (ca. 38%) indicating better conversion of sugar to oxalic acid.

Our results corroborate nicely with the previous observations reporting strong influence of the medium and broth pH on oxalic acid production by *A. niger* (Cleland and Johnson, 1956; Lenz *et al*., 1976; Kubicek *et al*., 1988; Strasser *et al*., 1994). The reasons behind are: (I) induction of the enzyme oxaloacetate hydrolase at pH ~4 and (ii) maintenance of pH_c above 6 favouring accumulation of oxalic acid by *A. niger*. On the basis of these results, we conducted all succeeding experiments on oxalic acid production under controlled pH.

In order to know whether the present strain produced other organic acids also we analyzed by HPLC some randomly selected fermented broth samples. Figure 2 represents comparative analysis of two such samples as Retention Time (RT) in minutes of different organic acids, such as oxalic, citric and fumaric eluted from the KC-811 column. The peak of oxalic acid (RTs 5.141 and 5.136) was preceded and succeeded by a peak of unknown identity. The peak preceding that of oxalic

Table 1: Oxalic acid production by the *A. niger* strain at different initial medium pH

	Oxalic acid (mM) at the medium pH of						
Day	2	3	4	5	6	7	8
7th	12.45±0.55	23.6±1.6	30.30±2.8	33.2±1.9	37.4±1.8	33.00±0.75	32.00±1.3
9th	16.1±1.1	36.9±1.9	41.9±2.5	41.7±1.7	47.9±2.0	42.4±0.95	36.9±0.1

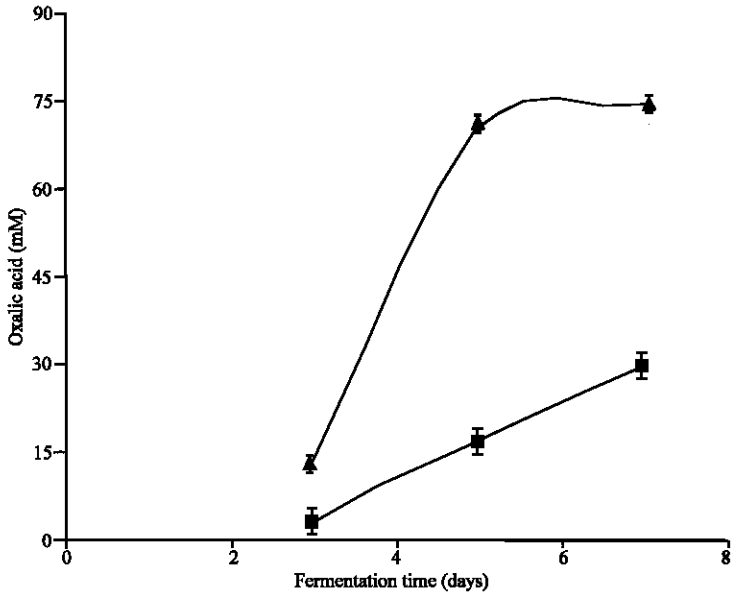

Fig. 1: Oxalic acid production from glucose vs. fermentation period at controlled pH 6 (▲) and uncontrolled pH (■)

Table 2: Production of different organic acids from glucose by *A. niger* NCIM 548

	Amount of organic acids (mM) produced after 7 days		
Condition (Broth pH)	Oxalic acid	Citric acid	Fumaric acid
Maintained within 6-7	67.55[a](74.75[b])	20.04[a]	0.20[a]
Not maintained	21.80[a](29.75[b])	20.66[a]	0.06[a]

[a]Estimated by HPLC; [b]measured by titration

acid was also seen in the standard sample. It is evident from the Fig. 2A and B that more than 3-fold oxalic acid was produced by the fungus after 7-day fermentation when pH_c was maintained at ca. 6.5 in comparison to the culture where pH was not adjusted (Table 2). The amount of citric acid (RTs- 6.12, 6.09) produced was almost the same under both the conditions, while more fumaric acid (Rts- 8.837, 8.779) was produced under controlled pH (Table 2). Interestingly, no gluconic acid (RT- 6.544) was produced where pH was not maintained (Fig. 2A), but a compound with very close retention time (6.721) to gluconic acid was produced. Under pH-controlled condition (Fig. 2B), this compound was also produced along with gluconic acid. Thus, although gluconic acid was produced under pH-adjusted condition, its amount could not be ascertained because a large area (including the 6.721 peak) was taken into account for this acid. It is to be noted that a difference in the amount of oxalic acid was measured by the two methods (Table 2) in repeated experiments. Although at this point, it is difficult to understand the reason behind this anomaly, it may be mentioned that the area for the same amount of standard oxalic acid peak in HPLC differed greatly when the acid was used alone and in combination with other acids (data not shown). Comparative analysis of oxalic acid by $KMnO_4$ titration and HPLC in different samples with a view to resolve this apparent anomaly is needed.

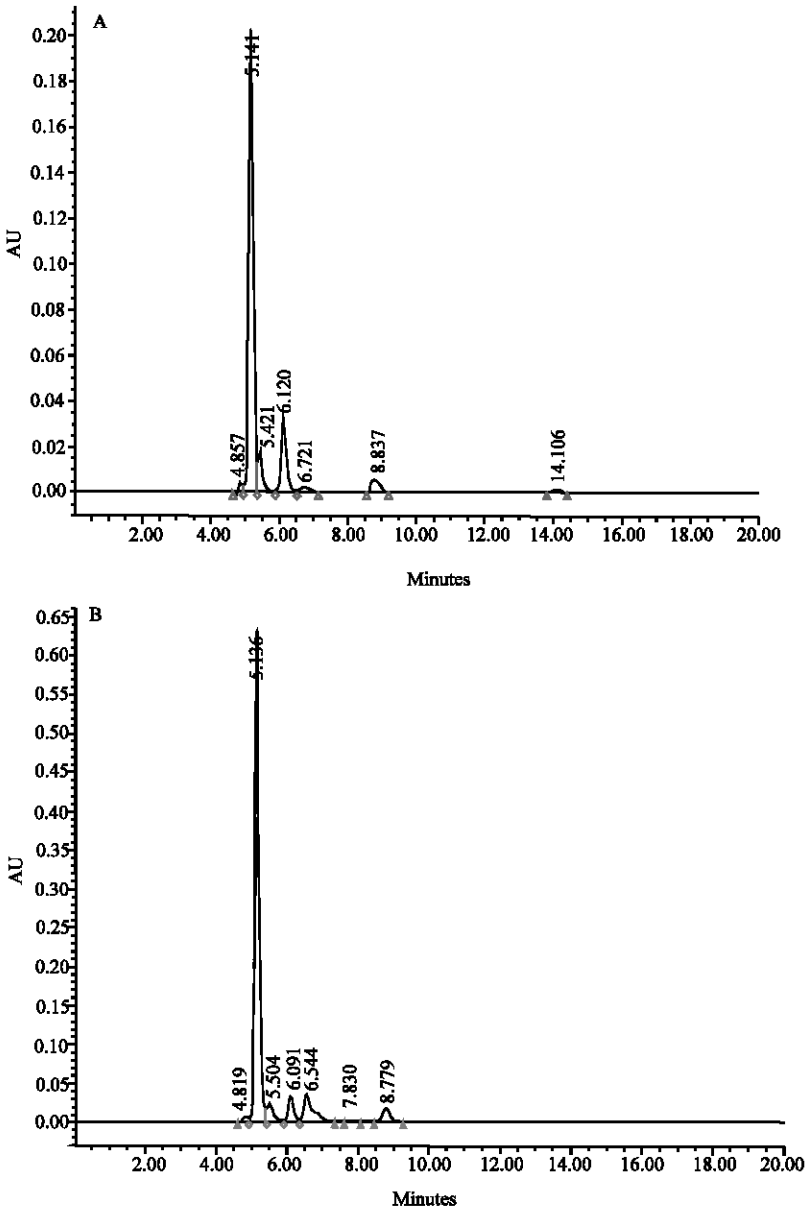

Fig. 2: HPLC analysis of culture filtrates after 7 days fermentation: (A)-pH of the broth was not adjusted; (B)-pH of the broth was adjusted and kept at 6.5±0.5 during fermentation

In previous studies (Strasser *et al*., 1994; Santoro *et al*., 1999) nitrate was used as the N-source for oxalic acid production from sucrose and the influence of other N-sources on this fermentation was not reported. Therefore, we examined the efficacy of other simple N-sources on this fermentation, which were added at a concentration equivalent to 0.25 g of N per liter medium (Strasser *et al*., 1994).

Table 3: Oxalic acid and biomass production from glucose with various N-source by *A. niger*

N- source	Oxalic acid (mM)	Biomass (g L^{-1})	Average yield of oxalic acid/g biomass
$NaNO_3$	45±4.5	5.7±0.2	7.89
Urea	62±7	5.4±0.1	11.48
$(NH_4)_2SO_4$	78±5	5.2±0.1	15.00
$(NH_4)_2HPO_4$	92±8.5	6.0±0.2	15.22

Table 4: Oxalic acid and biomass production from lactose in presence of various N-source by *A. niger*

N- source	Oxalic acid (mM)	Biomass (g L^{-1})	Average yield of oxalic acid/g biomass
NH_4NO_3	62.6±4	6.66±0.1	9.34
Urea	102.6±5.5	5.86±0.2	17.38
$(NH_4)_2SO_4$	47.1±3	4.98±0.15	9.42
$(NH_4)H_2PO_4$	131.6±6	4.88±0.35	26.85
$(NH_4)_2HPO_4$	126.6±7	5.32±0.35	23.88

Table 3 shows the profile of oxalic acid production from glucose using different N-sources after 7-day fermentation at pH 6.5±0.5. The yield was much better when ammonium salts (ca.78±5 and 92±8.5 mM), especially diammonium hydrogen phosphate (ca. 92±mM), were used as the N-source in comparison to $NaNO_3$ (ca. 45±4.5 mM) and urea (ca. 62±7 mM). Glucose consumption by the fungus was also better with ammonium salts than with nitrate; it was in the range of ca. 53, 69 and 65%, respectively with nitrate, urea and ammonium salts. It was noted that urea supported better mycelium growth than ammonium sulphate (Table 3); this explains why the strain consumed more glucose with urea as the N-source. Since the average yield of oxalic acid per g biomass was much better in case of ammonium salts (Table 3), we omitted nitrate in the succeeding experiments with lactose as the C-source.

Our observations comply with the fact that in a fermentation medium ammonium salts are better as the N-source than nitrate in general, because these are assimilated directly into cellular organic components, whereas nitrate is first reduced to ammonia and then assimilated. Urea is also a rich source of fixed nitrogen which is enzymatically cleaved directly to ammonia and CO_2.

Previously we reported that lactose, the sugar component of whey-a polluting waste of diary industry, is a better C-source for oxalic acid production by the *A. niger* strain. Using various N-sources, production of oxalic acid was the best with ammonium dihydrogen phosphate followed closely by diammonium hydrogen phosphate (131.6±6 vs. 126.6±7 mM); it was much better with urea (102.6±5.5 mM) compared to ammonium nitrate and ammonium sulfate (Table 4). The two ammonium phosphates produced less biomass from this sugar and the yield per g biomass was much higher in case of these salts than other compounds (Table 4). This implicates that more sugar was available for the production of oxalic acid. It may be mentioned that four-fold increase of ammonium salts concentration in the medium enhanced oxalate production only to a limited extent (10-30%). On the other hand, significant decrease in the production was noted when ammonium dihydrogen phosphate concentration was reduced to 0.01% N (data not shown). These experiments also indicated that medium phosphate concentration in the range of 1.2-2.0 g L^{-1} had no significant influence on the oxalic acid production because the medium with ammonium dihydrogen phosphate containing nearly 2-fold more phosphate than in the same with diammonium hydrogen phosphate produced almost same amount of oxalic acid (Table 4). The results also show that ammonium hydrogen phosphates are more suitable than urea because the amount of oxalic acid produced per g dry weight of mycelium was higher in case of ammonium salts with both glucose and lactose. It may be mentioned that previous studies were mostly conducted with sucrose as the C-source which is costly than either glucose or lactose.

In summary, *A. niger* NCIM 548 strain produces (I) maximum amount of oxalic acid at initial medium pH of 6, (ii) at controlled broth pH of 6.5±0.5 that may be adjusted by addition of alkali and (iii) either of the ammonium hydrogen phosphates is the best N-source in stead of $NaNO_3$. Various wastes containing free glucose or lactose are polluting agents and their conversion to useful products, such as organic acids through fermentation, may be considered as an effective measure toward abatement of environmental pollution (Mukhopadhyay *et al*., 2005). The *A. niger* NCIM 548 strain is better than other reported strains of this species because of its ability to converting polluting sugars to oxalic acid efficiently.

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