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Nitrogen-Dependent Regulation of Gluconic and/or Citric Acid Production by *Aspergillus niger*

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Abstract Surface culture fermentation using *Aspergillus niger* was studied for gluconic and citric acid production at different C/N ratios. A culture of *A. niger* was found to produce either gluconic acid alone, a mixture of gluconic and citric acid, or citric acid alone depending on the level of nitrogen in the medium (4 to 18 mM). Glucose oxidase from the mycelial mat was also analyzed at different levels of nitrogen in the media. By choosing the level of nitrogen in the medium at the start of fermentation, it is possible to produce either of the two acids as the dominant product or the two together as a mixture.

Key words: *Aspergillus niger*, surface fermentation, metabolism, nitrogen/ammonium effect, gluconic acid, citric acid, glucose oxidase.

Aspergillus niger produces commercially important organic acids such as gluconic and citric acid. Today, about 80% of the production of citric acid is carried out by submerged culture [22]. However, in parts of the world where labor costs are inexpensive, surface culture in shallow pans are practiced. This method requires two-third less energy and has simpler operations as compared to the submerged process [1].

The basic metabolism by which *A. niger* accumulates these organic acids is well known. *A. niger* is also able to accumulate other organic acids. Oxalic acid is a toxic byproduct of citric acid fermentation, although its biosynthetic regulation is controversial [10, 19].

Selective formation of gluconic and citric acid depends to a great extent on the strain used, and its response to the consumption of the medium can show a great deal of variability [8]. Several articles appearing on this topic show the importance of levels of N and P for optimum acid production. Citric acid accumulation is shown to be stimulated by exogenous addition of nitrogen/NH_4^+ to the medium [2, 31]. Furthermore, in our earlier study on sugarcane juice fermentation using *A. niger* NCIM 545, citric acid was the only acid observed at higher nitrogen levels [25].

The objective of this work was to study the formation of gluconic and citric acid by *A. niger* at different nitrogen levels and to optimize its level. The results of these investigations may be relevant while using higher nitrogen containing raw materials like molasses and cane juice for surface as well as submerged fermentation.

Materials and Methods

Microorganism and Medium

A. niger NCIM 545 procured from the National Collection of Industrial Microorganism (NCL, Pune, India) was cultivated on PDA (Potato-Dextrose-Agar) slants at 28°C for 7 days, and was used throughout the study of gluconic and citric acid fermentations.

Fermentation medium: 100 to 120 g sucrose, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4, and 0.8 g $(NH_4)_2HPO_4$ in 1 l of water was used. The nitrogen content was progressively raised by increasing $(NH_4)_2HPO_4$ in this fermentation medium upto 2.4 g/l (~18 mM). Before sterilization, the pH of the medium was adjusted to 6.0 using 1.0 M H_2SO_4.

Di-ammonium hydrogen ortho-phosphate (DAHP) was used as the sole source of nitrogen in the medium (4 to 18 mM) keeping all the other medium constituents unchanged. While DAHP levels were altered in different experimental setups, for the sake of simplicity, these changes have been referred to as changes in nitrogen levels.

Effect of phosphate was studied at DAHP levels of 4, 8, and 12 mM by raising the phosphate levels of medium using KH_2PO_4 at the levels of 3, 6.1, 12.25, and 24.5 mM.

Fermentation Procedure

Surface culture fermentation was carried out in triplicate sets of 1-l Erlenmeyer flasks containing 150 ml of the

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sterile medium. Spores of a 7 days old culture of *A. niger* grown on PDA slope were harvested and were suspended in distilled water to obtain a spore density of $3-5\times10^7$/ml. A 5-ml spore suspension was transferred to each flask and incubated at 28°C. A mycelial mat was allowed to grow for the first 2 days and medium samples at 24-h intervals were collected thereafter for analyses until 12 days.

Chemicals

All chemicals used in this study were of analytical grade and procured from Hi-Media Laboratory and Loba Chemie, India.

Determination of Proteins and Glucose Oxidase (GOD) Activity from Mycelia

Activity of GOD and total protein content in the mycelial mat were studied at varying levels of nitrogen content of the medium. On the 8th day of fermentation, the mycelial mat was harvested from the two media sets. Fermented broth was decanted, and the mycelial mat was gently rinsed with 30 ml of water to remove spores and conidia. After two such rinsings most of the spores were removed. Then, the mycelial mat was suspended in 50 ml of water and washed several times until all traces of glucose were removed. It was macerated using 30 ml of 50 mM acetate buffer, pH 5.6, for 10 min using a conventional laboratory glass homogenizer at ~1,000 rpm. The disrupted mat was kept for one day at 10°C for further release of proteins into solution. The homogenate was centrifuged at 10,000 ×g for 15 min to remove cell debris, yielding clear solution with a yellow tinge, which was used for enzyme and protein analyses.

The GOD activity was determined by the method of Müller [20]. Protein was determined by method of Lowry [14].

Sugar and Acids Analyses

Feed and unconverted sucrose was hydrolyzed by 2 N HCl at 90°C for 15 min into glucose and fructose and analyzed by the dinitrosalicyclic acid method [17]. The appearance of gluconic acid and citric acid during fermentation was routinely monitored by TLC (mobile phase: chloroform, methanol, and ammonia in the ratio of 3: 5: 3). Citric acid was analyzed by the pyridine-acetic anhydride method [15].

Unreacted sugar was monitored usually by TLC using ethyl acetate:1-propanol:water at the ratio of 8:7:2 as a mobile phase. Spray reagent used for visualization contained 4% α-naphthol in ethanol: 2 N sulfuric acid (20:80). This method helped visualization of sucrose and fructose down to 0.05% in the medium. Gluconic acid and citric acid present in the medium were analyzed by HPLC using an AMINEX HPX 87H column. However, most routine acid analyses were done by simple titration. Nitrogen present at the different stages of fermentation was analyzed by the Micro-Kjeldhal method [13]. Phosphate was determined by the molybdate method [5].

Results and Discussion

Effect of Nitrogen Concentration on Batch Fermentation

In earlier work, it has been reported that citric acid can be induced by NH_4^+ ions [2, 31] provided that these additions were made after sufficient growth of the mycelia. In chemostat culture, under a phosphate limited growth condition, less citric acid was produced as compared to a nitrogen limited steady state condition. The level of excess nitrogen was a more important factor than the lack of phosphate, because the greater the nitrogen excess, the less citric acid was produced [9]. Under the limited phosphate growth condition of *A. niger*, citric acid production was subject to nitrogen catabolite repression [3]. In our earlier study on sugarcane juice fermentation by a strain of *A. niger* capable of producing either gluconic or citric acid, it was observed that the strain preferentially produced citric acid when the medium had higher levels of nitrogen [25]. In order to obtain better insight into the role of the N and P metabolic behavior of *A. niger* capable of producing either of these two acids, batch experiments were performed to investigate the role of nitrogen during the mycelial development, growth, and subsequent fermentation. As shown in Fig. 1, gluconic acid was produced satisfactorily at low nitrogen content (4 to 8 mM). Formation of citric acid was first noticed when the nitrogen level was higher than 8 mM, and rose proportionately with increasing nitrogen levels up to 15 mM.

Considering these observations for gluconic acid production, the nitrogen level has to be kept in the range of 4 to 8 mM. Beyond these limits, either less yields of gluconic acid or a mixture of both the acids were obtained. This nitrogen

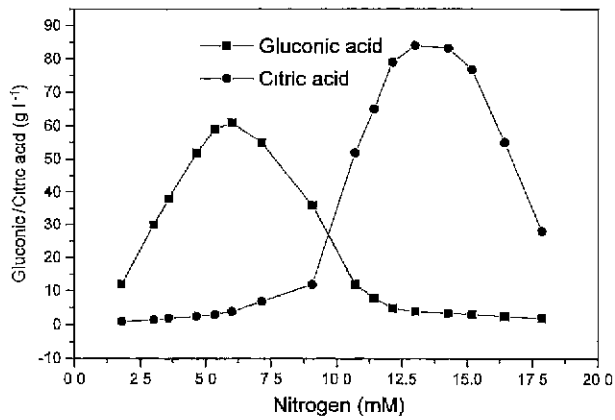

Fig 1. Production of gluconic acid and citric acid by surface culture of *A. niger* at low pH and different nitrogen (nitrogen of DAHP) levels

At 4 to 8 mM, the major product was gluconic acid and at 11 to 17 mM nitrogen concentration, citric acid is the major product.

dependent shifting of metabolism can thus be used as a key factor in deciding the course of acid production in this strain for the selective production of gluconic or citric acid.

Production of Gluconic and Citric Acid at Optimal Nitrogen Concentrations

Gluconic acid is produced by surface fermentation, up to a yield of 57% of theoretical based on utilization of sugar by free mycelia [16] and more than 98% by immobilized mycelia [26]. The deployed nitrogen was exhausted by the 4th day, the biomass growth was then suspended, and the mixture analyzed showed a considerable amount of gluconic acid in the broth (Fig. 2B). This observation agrees with the earlier reports stating that at diminished levels of nitrogen, gluconic acid can be found to accumulate in the broth, or gluconic acid formation is stimulated at low nitrogen levels [20]. Between 4 and 8 mM nitrogen levels, gluconic acid was the major product. The pH of the fermented broth drops to 2.6 (Fig. 2A) and almost 82% sucrose was utilized. After 10 days of fermentation, the gluconic acid concentration reached up to 60.0 g/l with 56.4 g/l fructose. Associated citric acid levels were around 4 g/l.

It was observed that, as the level of nitrogen in the medium was raised, the mat thickened and led to faster consumption of sucrose. As shown in Fig. 2C, during the progress of fermentation, biomass continues to increase up to the 5th day. At this stage, nitrogen in the medium is almost exhausted and the citric acid accumulation starts. This is in agreement with the earlier report [24] that for citric acid production, nitrogen depletion of the fermentation medium is important. On the 9th day, there is a peak accumulation of citric acid with the nitrogen level at 13 mM, and most of the sucrose is found to be consumed. As shown in Fig. 1, at the nitrogen levels of 11 to 17 mM, citric acid was the predominant product and gluconic acid was present only in trace amounts.

On the 10th day, 92% of the sucrose was utilized, fermented broth contained 97 g/l citric acid, and the pH of the broth fell below 1.98. This increase in the citric acid concentration was associated with an increase in the maximum biomass formation and to higher levels of nitrogen. Nitrogen levels in excess of 18 mM led to continued growth of the mycelia and delayed citric acid formation.

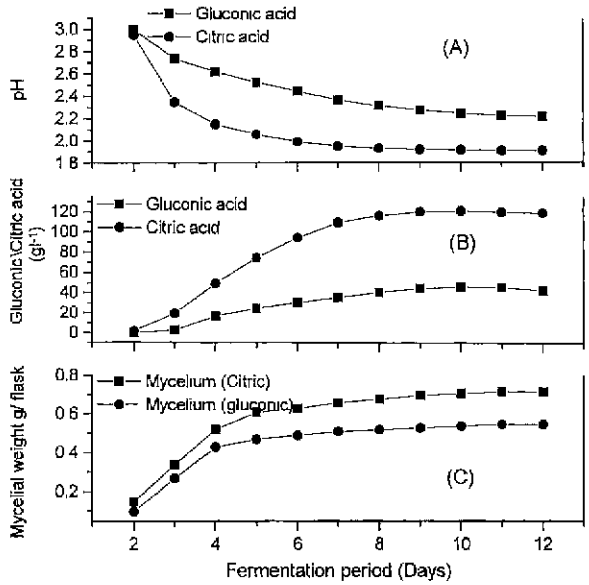

Fig. 2. Accumulation of gluconic acid and citric acid by *A. niger* at surface culture fermentation, broth analyses at different time intervals, and at 6 and 13 mM nitrogen levels for gluconic and citric acid, respectively.

(A) pH of the broth during citric acid and gluconic acid fermentation. (B) Gluconic\citric acid concentration accumulated at optimum nitrogen levels. (C) Mycelial weight (dry) present at the respective day of fermentation.

Effect of Nitrogen Concentration on Formation of Mycelial GOD and Proteins

Formation of gluconic acid and citric acid by *A. niger* was further studied at the cellular level. GOD is the enzyme involved during gluconic acid fermentation and its content in the mat was estimated in flasks grown for 8 days on media containing increasing levels of nitrogen. Along with GOD activity, the protein content of the mat was also estimated. As shown in Fig. 3, the specific activity of GOD reached a peak of 3.6 µmol/min/mg protein at the nitrogen level of 6 mM. This was also the level of nitrogen at which gluconic acid production was at its peak. It progressively dropped to 0.25 µmol/min/mg protein as the nitrogen level in the medium was raised to 10 mM. The total GOD activity per flask was computed to be 190 U and 35 U at 6 mM and 13 mM of nitrogen levels, respectively. Mycelial protein synthesis increased with the higher nitrogen level and reached to 35 mg/ml. At 13 mM nitrogen, citric acid was the major product with maximum cellular protein levels.

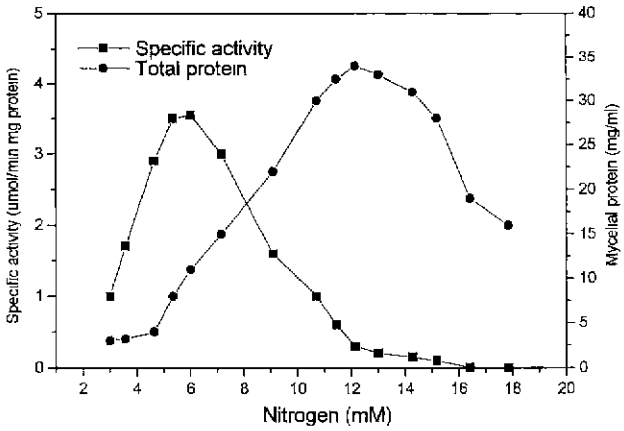

Fig. 3. *In vitro* activity of glucose oxidase at different nitrogen levels.

Mycelial extracts were analyzed for total protein (soluble) and specific activity of glucose oxidase at the 8th day of fermentation

It is reported that at high glucose levels and aeration, GOD is induced in *A. niger* mycelia [4, 18, 23]. Under typical conditions of citric acid fermentation, extracellular GOD is formed in the initial phase and a significant amount of glucose is diverted to gluconic acid. The released GOD is directly influenced by external pH and it is deactivated at pH<3.5 [18, 24]. Nevertheless, the *A. niger* NCIM 545 is able to produce free gluconic acid in high yields with negligible amounts of citric acid at low pH [26]. In previous studies [20], it has been shown that *A. niger* grown on nitrogen rich medium shows pronounced ability to consume gluconate which was initiated by gluconokinase, and that the PP and EM pathways and the TCA cycle were involved in further metabolism. This may be the reason why low gluconate during citric acid production was observed. However, the conversion of gluconate to citric acid is still unclear in the reported literature.

Thus, having the knowledge of the medium constituents, the higher nitrogen containing cheap raw materials like molasses and cane juice can be used to produce gluconic or citric acid by monitoring nitrogen content and, thus, the contamination of either acid in the final product can be avoided.

The Metabolic Shift

A. niger is known to follow the hexose-bisphosphate pathway and the pentose phosphate shunt. In addition, *A. niger* forms glucose oxidase to produce gluconic acid. Metabolic steps involved in TCA-related organic acids require at least three steps; glycolytic breakdown to pyruvate and acetyl-CoA, formation of oxaloacetate from pyruvate, and possible steps within the TCA cycle [12].

The regulation of the hexose-bisphosphate pathway in *Aspergillus* involves three key regulatory enzymes; hexokinase (EC 2.7.1.1), 6-phosphofructokinase (EC 2.7.1.11; PFK1), and pyruvate kinase (EC 2.7.1.40) [11, 28]. PFK1 is activated by Fru-2,6-P_2, AMP, and NH_4^+ ions. It is well known that citric acid accumulation can occur under conditions where PFK1 is unable to control glycolytic flux efficiently.

PFK1 regulation by Fru-2,6-P_2 may not be the only parameter to regulate citric acid [32]. Citrate inhibition of PFK1 seems to be antagonized by ammonium ions [6]. Furthermore, this antagonism is linked to trace metal ions during citric acid accumulation [27, 29, 30]. As discussed earlier, citric acid accumulation was stimulated by exogenous addition of nitrogen/NH_4^+ to the medium, which is consistent with this effect of NH_4^+ on PFK1. Some strains of *A. niger* show different yields at the similar concentrations of nitrogen and phosphorus [21].

In order to verify whether the phosphate content of the medium was contributing to the metabolic shift, experiments were conducted for varying levels of DAHP (4, 8, and 12 mM). At each concentration of DAHP, phosphate levels were elevated to 6.1, 12.25, and 24.5 mM to simulate the phosphate levels by addition of KH_2PO_4 to the medium. As shown in Fig. 4, the elevated phosphate levels show the identical amounts of acids at three different nitrogen levels and had no effect on bringing about a metabolic shift; thus higher phosphate levels are not necessary during formation of gluconic and citric acid.

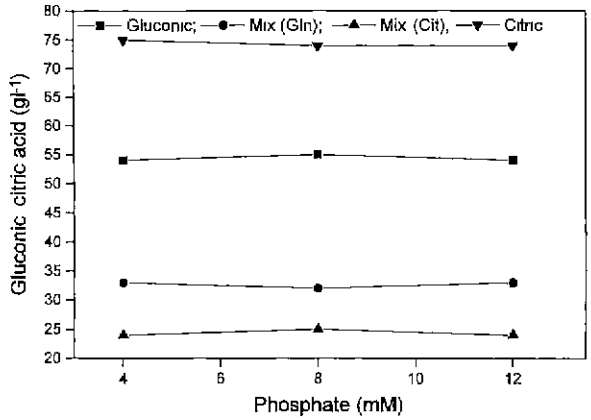

Fig. 4. Effect of phosphate on gluconic and citric acid yields. At 4, 8, and 12 mM of nitrogen levels, additional phosphate levels (KH_2PO_4) added were 6.1, 12.25, 24.5 mM. Solid circles and uptriangles show mixture of gluconic and citric acid, respectively, at intermediate nitrogen levels (8 mM).

During citric acid fermentation, with the pH of the medium between 1.9–2.5, gluconic acid was not produced [24]. However, in our earlier study at pHs 2.2 to 3.6, gluconic acid was obtained using a strain of *A. niger* [26]. Since the gluconic acid did appear under our experimental conditions of low pH, it would seem that change in pH might not be the reason to activate operation of the TCA cycle. On the other hand, nitrogen levels of the fermentation appear to result in the metabolic shift leading to the formation of either of the two acids. A related observation was reported earlier [7], although more detailed insight into the metabolic events is still being awaited.

Conclusions

A. niger NCIM 545 has the ability to produce both citric and gluconic acid and can be used to produce either acid by manipulating the level of nitrogen. The organism responds predictably to a shift in the amount of nitrogen available in the medium, yielding citric acid at high levels and gluconic acid at low levels.

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