Bioconversion of Colocasia antiquorum and Aponogetonnatans to Citric Acid by Aspergillus niger — Effect of Metal Ions and Kinetics

A R Angumeenal, P Kamalakannan, H J Prabhu† & D Venkappaya*,
Department of Chemistry, †Department of Chemical Engineering, Regional Engineering College, Tiruchirappalli 620 015

Received: 11 July 2002, rev. rcd: 17 February 2003, accepted: 03 March 2003

Tuber crops belonging to the family Araceae namely Colocasia antiquorum and Aponogetonnatans are cultivated in large quantities for their edible portion. In this work, tubers are suitably treated and used as efficient substrates for citric acid production by fermentation using Aspergillus niger. The quantities of citric acid produced using these materials as substrates for bioconversion using Aspergillus niger are compared with those produced in synthetic medium comprising glucose as substrate. Transition metal ions such as, chromium, molybdenum, cadmium and lead are added at optimum concentration as nutritional supplements and their effect on the biosynthetic route of the citric acid cycle are discussed. Experimentally observed growth stages are used for mathematical modeling to evaluate the kinetic parameters. The values obtained by calculation agree well with the observed ones.

Introduction

Several sugar sources such as molasses, whey permeate, date syrup, banana extract, and root crops were being used as substrates for citric acid production. In recent years, a great upsurge has occurred in search of alternative materials as substrates for citric acid production. This made us to select Colocasia antiquorum and Aponogeton natans (vernacular names — Seppankizhangu/seppan and Koraikkizhangu/korai) as substrates for citric acid production. Earlier work described the effect of metal ions on citric acid production by Aspergillus niger with synthetic substrates. In this work, an attempt is made to study the effect of chromium, molybdenum, cadmium and lead ions on the degradation of these natural substrates during the metabolism of Aspergillus niger.

Experimental Procedure

Aspergillus niger used in the present study was procured from NCL, Pune (NRRL 322 — Species for Citric Acid Production CM1 31276; NCTC 594; WB 322).

The fermentation medium comprising (in per cent) glucose (14), KNO₃ (0.8), K₂HPO₄ (0.125), MgSO₄·7H₂O (0.02) and ZnSO₄·7H₂O (0.02) was used as the control. The selected tubers were characterised for their crude fibre, cellulose, hemicellulose, starch and mineral content (determined using AAS). Steaming the pulped tuber with 0.4 N HCl for 1.5 h was found to be the optimum condition for hydrolysing the maximum soluble sugars. The pH of the hydrolysed substrate was adjusted to be between 2 and 3. Batch fermentation by surface culture was initiated using the spore suspension of Aspergillus niger. From the third day of inoculation onwards up to the 11th day, the culture broth was filtered and analysed for total titrable acidity (volume of 0.1 N NaOH consumed by 1 mL of the filtrate), citric, succinic and malic acids (using HPLC), extracellular soluble proteins and residual metal ion contents (using AAS). The biomass was analysed for amylase, lipid and protein contents.

Results and Discussion

The morphological views of the tuberous species used as substrates for the study are shown in Figure 1 and Figure 2. Seppan was found to contain 55.5 per cent starch, 2.8 per cent crude fibre and 2.4...
per cent cellulose, whereas korai was found to contain 60 per cent starch, 3.8 per cent fibre and 2.8 per cent cellulose. Hemicellulose fraction was present in same amounts (0.5 per cent) in both the species.

Results with Colocasia

With Colocasia as substrate A.niger was found to exhibit the best amylase activity (68 U / mL / min) and the substrate sugar concentration was not depleted till the end of fermentation. The results of protein and lipid metabolism are given in Table 1. The metabolic state of A. niger was found to be influenced by adding cadmium (in the form of cadmium chloride, 40 ppm) lead (in the form of lead nitrate, 40 ppm), molybdenum (in the form of ammonium molybdate, 10 ppm) and chromium (in the form of potassium dichromate, 20 ppm) to the
media, as observed earlier with the synthetic substrates. The products of the microbial metabolism are shown in Table 2. With the control medium 25 g/L of citric acid was produced, whereas it was 18.4 g/L with seppan as substrate. The quantity of citric acid produced was considerably increased with the metal ion supplemented media. The biomass production was also high with metal ions in the medium with increase in extracellular protein content. This could be due to the interaction of these metal ions with the cell wall contents like peptidoglycans and lipoproteins resulting in the leakage of peptides, nucleotides and other small molecules across the membrane. The highest citric acid production was in the presence of molybdenum (52 g/L) as nutrient.

Results with Aponogeton natans

The underground tuber portion of this species was used as the substrate for the production of citric acid. The products of the microbial metabolism after fermentation are shown in Table 3. The amount of citric acid produced was higher with this substrate than with the synthetic medium (37.6 g/L). The metal ion supplemented media were found to produce more quantities of citric acid in a shorter time of 3-5 d. The quantities of acids, proteins and biomass produced by A. niger with this substrate are given in Table 4. The

<table>
<thead>
<tr>
<th>Incubation period, d</th>
<th>Total titrable acidity, mL/mL</th>
<th>Citric acid, g/L</th>
<th>Protein</th>
<th>Lipids, g/100 mL</th>
<th>Biomass, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.5</td>
<td>12.5</td>
<td>-</td>
<td>4.0</td>
<td>0.6660</td>
</tr>
<tr>
<td>5</td>
<td>4.4</td>
<td>15.0</td>
<td>3.4</td>
<td>4.5</td>
<td>0.7080</td>
</tr>
<tr>
<td>7</td>
<td>4.8</td>
<td>15.0</td>
<td>6.6</td>
<td>4.7</td>
<td>0.9440</td>
</tr>
<tr>
<td>9</td>
<td>5.0</td>
<td>18.4</td>
<td>3.0</td>
<td>23.0</td>
<td>1.200</td>
</tr>
<tr>
<td>11</td>
<td>3.4</td>
<td>8.2</td>
<td>2.2</td>
<td>10.0</td>
<td>0.860</td>
</tr>
</tbody>
</table>

ICP= Intracellular proteins, ECP= Extracellular proteins

<table>
<thead>
<tr>
<th>Control</th>
<th>Cd (40 ppm)</th>
<th>Pb (40 ppm)</th>
<th>Mo (10 ppm)</th>
<th>Cr (20 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTA, mL/mL</td>
<td>5.0</td>
<td>8.0</td>
<td>5.6</td>
<td>5.2</td>
</tr>
<tr>
<td>CA, g/L</td>
<td>18.4</td>
<td>30.6</td>
<td>31.2</td>
<td>52.0</td>
</tr>
<tr>
<td>ICP, per cent</td>
<td>6.6</td>
<td>5.6</td>
<td>4.4</td>
<td>2.8</td>
</tr>
<tr>
<td>ECP, g/100 mL</td>
<td>4.7</td>
<td>18.0</td>
<td>17.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Biomass, g/100 mL</td>
<td>1.200</td>
<td>2.148</td>
<td>2.130</td>
<td>2.214</td>
</tr>
</tbody>
</table>

TTA = Total titrable acidity, CA = Citric acid, ICP = Intracellular proteins, ECP = Extracellular proteins

<table>
<thead>
<tr>
<th>Incubation period, d</th>
<th>Total titrable acidity, mL/mL</th>
<th>Citric acid, g/L</th>
<th>Protein</th>
<th>Lipids, g/100 mL</th>
<th>Biomass, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7.2</td>
<td>34.9</td>
<td>35.3</td>
<td>26.1</td>
<td>2.460</td>
</tr>
<tr>
<td>5</td>
<td>10.3</td>
<td>36.3</td>
<td>28.3</td>
<td>26.1</td>
<td>5.023</td>
</tr>
<tr>
<td>7</td>
<td>9.9</td>
<td>37.6</td>
<td>20.0</td>
<td>18.0</td>
<td>5.944</td>
</tr>
<tr>
<td>9</td>
<td>7.8</td>
<td>20.8</td>
<td>15.5</td>
<td>16.8</td>
<td>7.809</td>
</tr>
<tr>
<td>11</td>
<td>7.2</td>
<td>20.3</td>
<td>11.5</td>
<td>8.0</td>
<td>7.482</td>
</tr>
</tbody>
</table>

ICP = Intracellular proteins, ECP = Extracellular proteins
results reveal that more quantities of citric acid are produced by A.niger in the presence molybdenum and lead. However the total titratable acidity produced was found to be hindered in the metal ion supplemented media; probably due to the electron transport chain being obstructed. Due to this H⁺ ion release is less, and so less total titratable acidity. With lead and molybdenum as nutrients the biomass weight was less and the citric acid produced was high, but it was not so in the presence of chromium and cadmium. This could possibly be due to the absence of active cells taking part in citrate production. The cell wall of A.niger is made up of peptidoglycans and lipoproteins. The initial stages of glycolysis occur in the cytoplasm after which the acetyl-CoA formed enters the mitochondria for further metabolism. Hence, it was found that the nature of the cell wall as well as the mitochondrial permeability alters the secretion of citric acid. The permeability of these are governed by the environment to which they are exposed to. Thus, as A.niger is exposed to the metal ions while using these substrates the permeability is influenced. Metal ions interact with the cell wall components such as glucons and mannans, thereby disrupting their arrangement leading to the formation of pores. Consequently the permeability through the pores is improved.

Kinetics and Computation

To gain further insight into the correlation between the experimental and experiment based theoretical values mathematical modeling was done. On the basis of the kinetic theory of microbial cell growth and the product concentration in fermentation, equations of growth rate and cell concentration were derived from the general equations of growth rate²³, along with equations for product concentration in each phase of microbial cell growth.

The rate equations²³ used for the modeling studies are given below:

**Microbial Cell Concentration**

**Exponential growth phase**

\[
C_x = C_{xe} \exp(kx(\theta - \theta_c))
\]

**Declining growth phase**

\[
C_x = C_{XM} - (C_{XM} - C_{xe}) \exp\left[-kx(C_{XM} - C_{xe})/(C_{XM} - C_{xe})\right] - k_{p2} C_{XM} (\theta - \theta_c) + C_{pc}
\]

where,

\[
C_x \quad \text{- Microbial concentration at exponential and declining growth phases}
\]

\[
C_{xe} \quad \text{- Biomass concentration at critical point}
\]

\[
C_{XM} \quad \text{- Maximum biomass concentration}
\]

\[
kx \quad \text{- Microbial growth rate}
\]

\[
\theta \quad \text{- Time in hours}
\]

\[
\theta_c \quad \text{- Critical point - point with maximum microbial growth}
\]

\[
C_p \quad \text{- Product concentration at exponential and declining growth phases}
\]

\[
C_{pc} \quad \text{- Product concentration at the critical point}
\]

\[
k_{p1} \quad \text{- First production rate constant, and}
\]

\[
k_{p2} \quad \text{- Second production rate constant.}
\]

**Determination of variables**

A plot of \( \theta \) (incubation time) versus the biomass weight (weight of A.niger) was obtained from which
the exponential growth phase and declining phases were identified. The area under the exponential growth phase and declining phase are blown up and analysed with more accuracy. During the above two phases the amount of citric acid produced by *A. niger* was also determined, which gave experimental *Cx* and *Cp* values. The point (on the graph) where the growth of *Aspergillus niger* is steady is labeled as ec (critical point). At this point the corresponding *Cxc* and *Cpe* values were noted. Plots of dCx/dt versus *e* and dCp/dt versus *e* gave the rate constants *kx*, *kP1*, *kP2*. These values along with *ec*, *Cxc* and *Cpe* were applied in the above equations to get the calculated *Cx* and *Cp* values.

**Results of the Modeling Studies**

Using the control/synthetic medium the product formation was found to be associated with growth, which was inferred from the rate constant values obtained (ie., *kP1* is positive and *kP2* is zero). Experimentally determined *Cx* and *Cp* values were in good agreement with the calculated ones.

With *Aponogeton natans* as substrate two production rate constants were obtained viz., *kP1* and *kP2*, one being positive and the other negative which indicates the fermentation process to be associated with growth as well as with non-growth. The calculated and observed cell and product concentrations are given in Table 5. The growth of *Aspergillus niger* was observed to increase under experimental conditions in the initial stages of the exponential phase but the production of citric acid was found to decrease in the later stages. This observation indicates that the active cell concentration in the total cell mass of *Aspergillus niger* is low and hence the total number of microorganism taking part in citric acid formation is low. Two production rate constants were identified with *Colocasia* as substrate whose signs are similar to the previous case (ie, *kP1* is positive and *kP2* is negative). The quantities of cell and product concentration obtained by the modeling studies reveal that citric acid production can be increased if the growth rate can be slightly improved (*kx* > 0.0066). With these tuberous sources too the experimentally determined *Cx* and *Cp* values were in good agreement with the calculated ones. Statistical analysis was done (using computer excel software) to get the correlation between the calculated and observed values. A higher value for *r*² (Table 5) shows better fitness to the kinetic model used.

**Conclusions**

The substrates studied in the current investigation were found to be equal in quality to other substrates reported earlier. The metals, by producing intermediate substances, take part effectively in improving membrane permeability. The amount of citric acid produced with *colocasia* as substrate was 18.4 g/L and with *aponogeton natans* 37.6 g/L. With *colocasia* the acid production was
improved considerably in the presence of the metal ions, the highest production being in the presence of molybdenum (52 g/L). With *A. oryzae* as substrate, lead at a concentration of 40 ppm was found to produce 58.1 g/L of citric acid and molybdenum at a concentration of 10 ppm, 70.8 g/L. Thus the nutritional quality of both the substrates were found to be improved in the presence of metal ions. To arrive at the exact mechanistic path radioactive labeling studies are to be done to identify the activated species (either the enzyme or the organ) in *A. niger* that induces citric acid production.

**Reference**