Malic Acid Production by Aspergillus oryzae: The effect of Alkaline-Earth Carbonate Buffer Identity

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Malic acid is a specialty chemical that is currently mainly used in the food and beverage industry (market value of $182 million) but has a potential market value of $3.5 billion if used to produce maleic anhydride. The results from the study indicated that the production of malic acid by A. oryzae requires the presence of the alkaline earth metals calcium or magnesium in significant quantities. It was observed that replacing an amount of CaCO₃ (240 g.l⁻¹ CaCO₃), significantly over that required for pH buffering (21 g.l⁻¹ CaCO₃), with an equivalent amount of MgCO₃ (192 g.l⁻¹ MgCO₃ based on CO₃²⁻) results in similar malic acid yields and final malic acid titers. In contrast, a marked reduction in glucose consumption and malic acid production rates were observed. These observations are likely due to an evolutionary response to calcareous soils. These soils tend to immobilize minerals in solid precipitates resulting in nutrient depletion, while the production of malic acid solubilizes these minerals making them bioavailable. The higher rates observed for the calcium vs magnesium runs were likely a result of the stimulatory effect of Ca²⁺ on the ATP generating pathways as well as several regulatory responses within the fungal physiology. In addition, it was found that A. oryzae was capable of assimilating malic acid from the environment, therefore, minimizing the loss of valuable carbon due to malic acid excretion. This study provides invaluable information required for economically viable malic acid production by A. oryzae which could markedly reduce reliance on the petrochemical industry.

1. Introduction

Developing a sustainable society requires a significant industrial shift away from petroleum-based products and towards renewable and bio-based technologies. This shift has been supported by renewed interest by consumers in natural, biodegradable, and environmentally friendly products (Mondala, 2015). As part of its drive towards bio-based products, the US Department of Energy has identified 12 priority platform chemicals required for bio-based chemical production; malic acid – along with the other two four carbon diprotic organic acids succinic- and fumaric acids- being considered among these (Werpy and Petersen, 2004). The current worldwide demand for malate is reported to be 200 kt/a (Chi et al., 2016) while the current international supply of L-malic acid is estimated at 40 kt/a (Liu et al., 2018). Currently, malic acid is commercially produced by the catalytic hydration of maleic or fumaric acid, both derived from maleic anhydride. Maleic anhydride is, in turn, produced from vapor phase oxidation of hydrocarbons, most prominently butane (Hermann and Patel, 2007). Unfortunately, this synthetic pathway produces a racemic mixture of L– and D–malic acid which is unsuitable for the food and beverage industry where malic acid is utilized as an acidulant (Knuf et al., 2014).

The biological production of malic acid provides stereo selectivity since L–malic acid is a key intermediate in the tricarboxylic acid cycle (TCA) present in most microorganisms (Liu et al., 2017). Filamentous fungi of the genus Aspergillus are superior producers of various bio-based chemicals including lipases, (Melo et al., 2011) xylanase, (Park et al., 2002), and various organic acids; Aspergillus flavus and A. oryzae widely considered the best biological producers of malic acid (Ochsenreither et al., 2014). However, A. flavus is known to produce hazardous amounts of carcinogenic aflatoxin, making the malic acid produced unsuitable for the food industry. In contrast, A. oryzae is a GRAS (Generally Regarded as Safe) organism that does not produce mycotoxins and has therefore been used in the production of sake, shochu, soy sauce, and miso for centuries (Payne et al., 2006).
Currently, laboratory-scale methods for malic acid production are limited due to the use of pellet morphology and CaCO₃ as the go-to buffering agent which complicates downstream processing. Geyer et al. (2018) observed a maximum malic acid production rate of 0.09 g·l⁻¹·h⁻¹ for 20 g·l⁻¹ CaCO₃ was initially dosed as compared to a maximum malic acid production rate of 0.23 g·l⁻¹·h⁻¹ for 100 g·l⁻¹ CaCO₃. This compares well with the results obtained by Kövilein et al. (2021) who measured a maximum malic acid production rate of 0.052 g·l⁻¹·h⁻¹ for 10 g·l⁻¹ CaCO₃ and 0.121 g·l⁻¹·h⁻¹ for 90 g·l⁻¹ CaCO₃. These results indicate a likely synergistic effect of excess CaCO₃ on the excretion of malic acid in addition to its role as a pH buffer. To assess the effect of exchanging Ca²⁺ for Mg²⁺ on A. oryzae malic acid fermentation, while maintaining the buffering capacity and bicarbonate source in the medium, two different carbonate buffers were compared by adding the same amount of CO₃²⁻ and changing only the cation (Ca²⁺ vs Mg²⁺). The resulting buffer concentrations used were 240 g/l CaCO₃ and 192 g/l MgCO₃ (impurities of each taken into consideration).

### 2. Materials and Methods

#### 2.1 Micro-organisms and fermentation in shake flasks

A. oryzae NRRL 3488 was obtained from the Agricultural Research Service Culture Collection in Illinois, USA. The stock cultures were stored at -40 °C in a 50 % w/w glycerol solution. Potato dextrose agar (PDA) (Merck KgaA, Darmstadt, Germany) plates were inoculated with the stock solution and incubated at 30 °C for 7 days. Approximately 60 mg of spores (circa 6.7 × 10⁸ spores (Smith et al., 1988)) were harvested from two agar plates with sterilized distilled water. The inoculum was prepared by adding the spore solution to a 10 % w/w glycerol solution which was subsequently stored at -40 °C.

Production was done using a one-step method and fermentation media adapted from Shigeo et al (1962) consisting of (in g/l): 60 glucose, 1.2 (NH₄)₂SO₄, 0.75 KH₂PO₄, 0.75 K₂HPO₄, 0.1 MgSO₄·7H₂O, 0.1 CaCl₂·2H₂O, 0.005 FeSO₄·7H₂O and 0.005 NaCl. A 250 ml un baffled Erlenmeyer flask was loaded with the medium, sterilized mixed, inoculated with the spore glycerol solution, and stirred (final spore concentration of 240 mg/l or 2.7 × 10⁶ ml⁻¹) and cultivated at 35 °C and 150 rpm for ~24 hours in an incubator shaker.

There were 2 batches of shake flasks: one set used 240 g/l CaCO₃, and one set used 192 g/l MgCO₃ for pH buffering, each added at the start of the fermentation to the relevant flask. Both buffers were tested in triplicate. The choice of buffer amounts was 1) to ensure a complete excess of buffer at all times in the system, and 2) to ensure that equivalent amounts of CO₃²⁻ (~140 g/l) were loaded in both.

#### 2.2 Sample preparation

It was hypothesized that there was a synergistic effect between pH control by CaCO₃/MgCO₃ dissolution and the precipitation of calcium salts (calcium–malate, –citrate, –fumarate). Due to this, the concentration of acids in the fermentation broth would be lower which in turn would allow A. oryzae to continue producing acid without possible adverse effects (pH, osmotic pressure, etc.). To determine how much acid, if any, had precipitated out of solution in the form of calcium/magnesium salts, 2 sets of HPLC and ICP-OES analyses were run on each sample: before acidification and after acid treatment. For this, a fraction of the supernatant was extracted and used for metabolite and cation analyses before acidification. Each sample remnant was then treated with 1 M HCl and incubated in the oven at 90 °C for 30 min (vortexed every 5 min) and then centrifuged. The subsequent supernatant was used for metabolite and ICP-OES analyses.

#### 2.3 Analytical methods

Samples from the flasks were collected in pre-weighed CELLSTAR® tubes (Greiner Bio-One, Sigma Aldrich, St. Louis, MO, USA). The concentrations of glucose, glycerol, ethanol, and organic acids in the samples were determined with an Agilent 1260 Infinity HPLC (Agilent Technologies, Santa Clara, CA, USA) fitted with a refractive index detector. Samples (± 1 ml) were centrifuged at 16 600 × g for 90 s and filtered with 0.45 µm Nylon (Ministart®, Sigma Aldrich, St. Louis, MO, USA) syringe filters into HPLC vials and loaded into the autosampler tray of the HPLC. Samples (5 µl) were injected into a Micro-Guard® Cartridge (30 m × 4.6 m) that was attached to a 300 mm × 7.8 mm Aminex HPX-87H ion-exchange column (Bio-Rad Laboratories, Hercules, CA, USA) maintained at 60 °C. Organic acids and glycerol were measured with mobile phase A (0.02 M H₂SO₄) and glucose, lactic acid, and ethanol with mobile phase C (0.002 M H₂SO₄) both at a flow rate of 0.6 ml/min. Each sample was analyzed twice: the control supernatant and the pre-treated supernatant. The concentration of calcium and magnesium in the solution was measured using inductively coupled plasma mass spectrometry (ICP-OES, Perkin-Elmar, Waltham, MA, USA) using an Argon plasma to ionize the samples and measure the relevant concentrations. This was repeated for both control and pre-treated samples.
2.4 Curve fitting

Time-dependent concentration profiles were approximated in the GraphPad Prism 7.0 (GraphPad Prism Software, San Diego, CA, USA) environment using a four-parameter logistic function

\[ C_i(t) = C_{i,max} + \frac{(C_{i,min} - C_{i,max})}{1 + \left( \frac{t}{T_{50}} \right)^k} \]  

Consumption/production rates were determined by differentiating the fitted functions.

3. Results & Discussion

The results for the malic acid concentration curves can be seen in Figure 1(a) for the CaCO\textsubscript{3} experiment and in Figure 1(b) for the MgCO\textsubscript{3} experiment. According to the proposed hypothesis, it was anticipated that the measured malic acid concentration would change significantly between the untreated and acid-treated samples. However, a Wilcoxon matched-pairs signed-rank test (α = 0.05) showed that there was not a statistically significant difference for the CaCO\textsubscript{3} (Z = 0.257; p = 0.7969) or MgCO\textsubscript{3} (Z = 0.873; p = 0.383) sets. This suggested that calcium-malate and magnesium-malate were not present in as significant concentrations as previously predicted. This contradicted the hypothesis that the precipitation of insoluble acid salts decreased the acid concentration in the fermentation broth which would decrease the inhibition of further acid production and therefore the effect of the CaCO\textsubscript{3} or MgCO\textsubscript{3} was likely related to either the supply of CO\textsubscript{2} for anaplerotic reactions required for malate synthesis (Kövilein et al., 2021) or the effect of the alkaline earth cation identity itself.

![Figure 1](image-url)
the produced organic acids required one carbonate ion for every proton neutralized. In addition, all produced acids would be fully dissociated for the pH range. From the results, it was observed that 0.88±0.22 M and 1.08±0.30 M of CO$_3^-$ were liberated in the CaCO$_3$ and MgCO$_3$ runs, respectively. In comparison, total proton production of 0.79±0.15 M and 0.80±0.05 M were measured for the CaCO$_3$ and MgCO$_3$ runs, respectively. While CO$_2$ demands of 0.40±0.08 M and 0.40±0.03 M for the synthesis of malate and succinate were determined. From the known equilibrium reactions for CaCO$_3$ and MgCO$_3$ in water (Aina et al., 2020), the neutralization of proton results in an equivalent molar formation of HCO$_3^-$ from CO$_3^{2-}$ resulting in the dissolution of CaCO$_3$/MgCO$_3$. In addition, the assimilation of CO$_2$ (as HCO$_3^-$) by the fungi results in an equivalent decrease in HCO$_3^-$ in solution which results in the conversion of CO$_3^{2-}$ to HCO$_3^-$ with the concomitant dissolution of CaCO$_3$/MgCO$_3$ to satisfy the prevailing equilibrium conditions.

Therefore, it can be seen that nearly all the CO$_2$ for anaplerotic reactions was obtained from MgCO$_3$, while a very small fraction of CO$_2$ for these reactions came from CaCO$_3$. This implies that the main source of CO$_2$ for malate synthesis in the CaCO$_3$ was respiration, while in the MgCO$_3$ system this was satisfied by the buffer.

The results further demonstrate that a nearly constant concentration of calcium (as Ca$^{2+}$ and CaCO$_3$) and magnesium (as Mg$^{2+}$ and MgCO$_3$) were continuously present in the system, corresponding to an average of 96.2±20.1 g/l and 55.4±13.2 g/l for Ca$^{2+}$ and Mg$^{2+}$, respectively. These values correspond well with the originally loaded amounts of these ions.

The fitted malic acid concentration curves with their corresponding malic acid production rates can be seen in Figures (a) and Figure 2(b). The glucose concentrations for control samples can be seen in Figure 2(c) with the consumption rate curves in Figure 2(d). The fitted parameters were summarised in Table 1. The CaCO$_3$ set had significantly higher rates of glucose consumption and malic acid production compared to the MgCO$_3$ set. Despite this, the measured malic acid concentrations were similar between the MgCO$_3$ and CaCO$_3$ experiments and the distributions in the group did not differ significantly (Mann-Whitney U = 32.5; n$_1$ = 9; n$_2$ = 8; p = 0.765 two-tailed). This indicated that the identity of the corresponding metal cation (Ca$^{2+}$ or Mg$^{2+}$) influenced the production/consumption rates. The glucose was completely consumed for the CaCO$_3$ set after 250 hours with a decrease in malic acid seen after the same time.

Figure 2: Malic acid (c) concentration curves and (d) production rate curves, glucose (e) concentration curves, and (f) consumption rate curves for the MgCO$_3$ and CaCO$_3$ control shake flask experiments. Curves were fitted using the logistic model (Equation 1) with the standard deviation of triplicates shown by the error bars.
Table 1: Curve fitting parameters for the concentration profiles in Figures (a) and (c)

<table>
<thead>
<tr>
<th>Dataset</th>
<th>240 g/l CaCO₃</th>
<th>192 g/l MgCO₃</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Malic acid</td>
<td>Glucose</td>
</tr>
<tr>
<td>C&lt;i&gt;&lt;sub&gt;i&lt;/sub&gt;&lt;sub&gt;min&lt;/sub&gt; (g/l)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C&lt;i&gt;&lt;sub&gt;i&lt;/sub&gt;&lt;sub&gt;max&lt;/sub&gt; (g/l)</td>
<td>95.3</td>
<td>43.3</td>
</tr>
<tr>
<td>k</td>
<td>-3.12</td>
<td>3.44</td>
</tr>
<tr>
<td>r&lt;i&gt;&lt;sub&gt;t&lt;/sub&gt;&lt;sub&gt;50&lt;/sub&gt; (h)</td>
<td>142</td>
<td>108</td>
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<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.956</td>
<td>0.876</td>
</tr>
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</table>

The results presented in Figures 1 and 2 indicate that the effects of alkaline-earth buffer identity are extremely complicated. The results demonstrated that the hypothesis of immobilization of malate as Ca-malate or Mg-malate was not a significant factor in the system as negligible amounts of these complexes were detected. However, from the ionic dissolution measurements, it appeared that the type of buffer affected the source of the CO₂ required for malate biosynthesis. This links strongly to the significantly increased rate of glucose consumption, with concomitant increased respiration rate, when comparing the CaCO₃ and MgCO₃ runs. The Ca²⁺ ion has been labeled the second messenger omnipresent in all fungi (Navazio and Mariani, 2008). This increased Ca²⁺ concentration within the external medium results in a coordinated range of intracellular signals to regulate the Ca²⁺ concentration within the mitochondria to regulate ATP synthesis (Tarasov et al., 2012). This Ca²⁺ homeostasis and signaling have physiological implications for the growth, virulence, and stress responses in fungi (Lange and Peiter, 2020). These effects contributed to a significantly promoted rate of glucose transfer into the fungal biomass in Ca²⁺ stimulates fungi, while this same observation was not made for Mg²⁺ (Pitt and Ugalde, 1984).

Applying the observed results in Figure 1 and Figure 2 to <i>A. oryzae</i>, the symbiotic relationship between CaCO₃ and malic acid could be explained using a calcareous environment as a reference point. <i>A. oryzae</i> is a genus that is distinct from other microbes: they can utilize both a secondary and primary metabolism (Brown et al., 1996). In the primary metabolism, acidic compounds are secreted for nutrient acquisition from the soil (Wu et al., 2018). Specifically, for the extraction of phosphorous where Ca²⁺ sequesters phosphorus, malate improved the efficiency of phosphorus extraction from the soil (Ström et al., 2005). The secondary metabolism can utilize the acidic compounds from the primary metabolism. This allows <i>A. oryzae</i> to produce secondary metabolites that it can utilize to adapt to its current environment and inhibit the primary metabolic pathways (Brown et al., 1996).

MgCO₃ would act similarly when considering a dolomitic soil environment where phosphate would be sequestered by the Mg²⁺ and require a release for phosphorus uptake by the fungi. However, the reduced stimulation of the fungal physiology as compared to the CaCO₃ system resulted in a less pronounced effect of Mg²⁺ on the system. This could be tested in the immobilized bio-reactor using NaOH/Na₂CO₃ to control the pH and spiking with CaCl₂ or MgCl₂.

4. Conclusions

The study presents an investigation of the effect of alkaline earth buffer (CaCO₃ and MgCO₃) on the production of malic acid by <i>A. oryzae</i> in a batch bioreaction system. Based on the results, alkaline-earth metals play a key role in the production rate of malic acid with <i>A. oryzae</i> NRRL 3488. The glucose consumption and utilization were higher when CaCO₃ was used as a buffer compared to MgCO₃, however, similar amounts of malic acid were produced. This study provides invaluable insights into this potentially lucrative yet relatively simple avenue for the improvement of malic acid production by <i>A. oryzae</i>. However, it is clear that the production of malic acid by <i>A. oryzae</i> is a complex, multi-faceted problem requiring understanding of the physiological interactions of the biocatalyst with its environment. The current study is limited to a batch reaction system at laboratory scale and therefore larger scale study is an imperative before real-world application can be realised.

Nomenclature

- C<sub>i</sub><sub>(t)</sub> – time-dependent concentration of component <i>i</i>, g/l
- C<sub>i</sub><sub>min</sub> – minimum concentration of component <i>i</i>, g/l
- C<sub>i</sub><sub>max</sub> – maximum concentration of component <i>i</i>, g/l
- k – parameter describing the shape of the model curve
- t<sub>50</sub> – processing time required to reach the mid-point of the concentration curve, h
References


