CHEMICAL ENGINEERING TRANSACTIONS
VOL. 70, 2018

A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet

Guest Editors: Timothy G. Walmsley, Petar S. Varbanov, Rongxin Su, Jiří J. Klemeš
Copyright © 2018, AIDIC Servizi S.r.l.
**ISBN** 978-88-95608-67-9; **ISSN** 2283-9216
DOI: 10.3303/CET1870301

# Malic Acid Production by *Aspergillus Oryzae*: The Role of $CaCO_3$

Monique Geyer, Faith M. Onyancha, Willie Nicol, Hendrik G. Brink*

Department of Chemical Engineering, University of Pretoria, Lynnwood Road, Hatfield, Private Bag X20, Pretoria 0002, South Africa
deon.brink@up.ac.za

This study investigated the malic acid production capability of *Aspergillus oryzae* NRRL 3488 with three different $CaCO_3$ concentrations, 20 g/L, 80 g/L, and 120 g/L, used as pH buffers and $CO_2$ supply. A black box model was used to determine the distribution of metabolites throughout the fermentation. Comparing the malic acid production for the three different concentrations of $CaCO_3$ after 240 h of fermentation, it was found that 300 % and 400 % more malic acid was produced in the 80 g/L and 120 g/L, in comparison to the 20 g/L experiment. Glucose consumption increased from 65 % for the 20 g/L $CaCO_3$ to 91 % for the 80 g/L $CaCO_3$, and 100 % consumption for the 120 g/L $CaCO_3$ after 240 h. Total available nitrogen measurements indicated incomplete nitrogen consumption for all $CaCO_3$ concentrations. Nitrogen conversions of 68 %, 78 %, and 81 % were measured for the 20 g/L, 80 g/L, and 120 g/L $CaCO_3$. These results indicate that nitrogen limitation is not the determining factor for malic acid production. It is hypothesised that the malic acid production is facilitated by sufficient pH control as well as the availability of $CO_2$. It is unclear whether oxygen limitation in the system is a requirement for malic acid production. Commercial malic acid production by *A. oryzae* NRRL 3448 would require a sufficient pH control and an abundant supply of $CO_2$.

## 1. Introduction

Advancements in developing a sustainable industrial society require greater independence from petroleum and petroleum production-based products. This necessitates a paradigm shift towards the use of renewable resources and bio-refining technologies. The movement has been coupled with an increased interest by customers in products that are natural, biodegradable, and environmentally-friendly, leading to a resurgence in interest and research in this field (Mondala, 2015).
Malic acid is a four-carbon diprotic organic acid and has been identified by the US Department of Energy as one of the top 12 building blocks to produce bio-based chemicals (Werpy and Petersen, 2004). The current worldwide demand for malate is reported to be 200 kt/y (Chi et al., 2016) while the current international supply of L – malic acid is estimated at 40 kt/y (Liu et al., 2018). Malic acid is commercially produced by the catalytic hydration of maleic or fumaric acid, which are both derived from maleic anhydride. Maleic anhydride is, in turn produced from vapour phase oxidation of hydrocarbons, most prominently butane (Hermann and Patel, 2006). 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 utilised as an acidulant (Knuf, 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 genus *Aspergilli* have shown to be 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* being considered the best producers of malic acid (Ochsenreither et al., 2014). Malic acid cultivation generally occurs in a two-step system consisting of a seed culture and an acid production culture under aerobic conditions with a high glucose concentration, a nitrogen source, inorganic salts, and a neutralising agent. $CaCO_3$ is the most frequently used pH regulator in biological research due its abundance and accompanying low cost (Salek et al, 2015). An additional advantage is that the dissolution of $CaCO_3$, during pH regulation, releases $CO_2$ into the fermentation medium. This can then be channelled into the cytosolic based

Please cite this article as: Geyer M., Onyancha F.M., Nicol W., Brink H.G., 2018, Malic acid production by aspergillus oryzae: the role of caco3 , Chemical Engineering Transactions, 70, 1801-1806 DOI:10.3303/CET1870301

reductive TCA pathway where malic acid accumulation occurs (Brown et al., 2013). However, it is not an effective method for controlling the pH since the dissolution of $CaCO_3$ is a kinetically controlled equilibrium reaction and the pH consequently varies throughout the fermentation (Salek et al., 2015). This form of pH control also limits commercial applications due to the processing constraints that coincide with using $CaCO_3$. These include higher broth viscosity which is detrimental to equipment and increases maintenance as well as the formation of calcium malate which requires solubilisation to recover the desired malic acid product resulting in increased downstream processing costs. An improved understanding of the role of $CaCO_3$ in the fermentation process can aid in the economic production of malic acid by *A. oryzae*.
The aim of the study was to determine whether different concentrations of $CaCO_3$ in the medium influence the metabolite distribution, specifically focusing on the production of malic acid, by *A. oryzae*. The study focused specifically on three concentrations of $CaCO_3$, i.e. 20 g/L, 80 g/L and 120 g/L.

## 2. Materials and methods

### 2.1 Organism

The wild-type strain *A. oryzae* NRRL3488 used in this study was acquired from the culture collection (Northern Regional Research Laboratory [NRRL]) of the Agricultural Research Service (ARS), Preoria, IL. The stock cultures were stored in a 50 % w/w glycerol solution at –40 °C.

### 2.2 Preparation of inoculum

Potato dextrose agar (PDA) (Merck KgaA, Darmstadt, Germany) plates were inoculated with the stock solution and incubated at 30 °C for 7 d. Spores were harvested with sterilised distilled water. The inoculum was prepared by adding the spore solution of two agar plates to 10 % w/w glycerol and subsequently stored at −40 °C.

### 2.3 Medium

One-step fermentations were carried out using the culture medium described by Abe et al. (1962) with 120 g/L glucose as the carbon source (Futaste Pharmaceutical Co. Ltd., Shandong, China) and 1.2 g/L $(NH_4)_2SO_4$ as the nitrogen source. Components were autoclaved separately at 121 °C for 1 h. Sterilised and finely powdered $CaCO_3$ was added in 3 different concentrations (20 g/L, 80 g/L, and 120 g/L) at the start of the fermentation. All components were sourced from Merck KgaA (Darmstadt, Germany) unless otherwise specified.

### 2.4 Batch cultivations

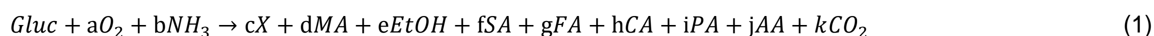
The experiments were carried out with shaker flasks where 100 mL of the fermentation medium was added to 250 mL Erlenmeyer flasks. The medium was then inoculated with 2.4 mL of the inoculum and cultivated at 35 °C and 150 rpm in an incubator shaker for approximately 10 d. Experiments for the three different concentrations of $CaCO_3$ were performed in triplicate. Representative samples were taken periodically, centrifuged, and stored at –40 °C until analysis to ensure cessation of metabolic activity.

### 2.5 Metabolite analysis

The nitrogen concentrations (mg/L) in the samples were determined using the Spectroquant total nitrogen photometric assay (Spectroquant®, Merck KgaA, Darmstadt, Germany). The concentrations of glucose and metabolites in the samples were determined using an Agilent 1260 Infinity HPLC (Agilent Technologies, USA) with a refractive index detector (RID) coupled with an Aminex HPX-87H ion-exchange column (Bio-Rad Laboratories, USA).

## 3. Results and discussion

A black box model (Equation 1), was used to determine the distribution of carbon throughout the fermentation period. The components of the black box model included glucose (Gluc), $O_2$, ammonia ($NH_3$), biomass (X), malic acid (MA), ethanol (EtOH), succinic acid (SA), fumaric acid (FA), citric acid (CA), pyruvic acid (PA), acetic acid (AA), and $CO_2$. The black box model is based on the observed products in the fermentation medium and corresponds well with the metabolic model for *A. oryzae* presented by Knuf et al. (2013). The initial amount of glycerol present from the inoculum was not included since it was found to be negligibly small (<1 g/L). The biomass composition specified by Vongsangnak et al. (2008) was used in the model.



$$Gluc + aO_2 + bNH_3 \rightarrow cX + dMA + eEtOH + fSA + gFA + hCA + iPA + jAA + kCO_2 \quad (1)$$

The model was fully specified using a C–balance, N–balance, degree of reduction (DOR), and the measured concentrations of metabolites, excluding $O_2$, X and $CO_2$. The latter unknowns were predicted by solving the system at each sampling interval and normalizing the data, i.e. the total carbon present at sampling interval

equalled 100 %. It should be noted that Eq(1) does not include the $CO_2$ added to the system by the dissolution of $CaCO_3$; the $CO_2$ calculated in Eq(1) represents the production of $CO_2$ during fermentation.
Figure 1 shows how the carbon was distributed over the fermentation period when using 20 g/L $CaCO_3$. After 240 h, 65 % of the glucose was consumed with the main product being $CO_2$ followed by malic acid. Between 172 h and 240 h, citric acid was formed and there was a decrease in the malic acid fraction. Malic acid could be redirected into the TCA cycle to produce ATP (energy) required by the cell resulting in the subsequent production of citric acid (Liu et al., 2018). The consumption of glucose coupled with the production of large amounts of $CO_2$ during the latter period of the fermentation (172 h – 240 h) further indicate an increased oxidative phosphorylation activity, resulting in an increased production of ATP (requirement for ATP by the organism) during this period. In addition, it was observed that the medium became clear during this period, a result of the depletion of $CaCO_3$ in the system. This resulted in a drop in the pH of the medium to around 4.7 (Table 1), with an accompanying increase in energy required to maintain homeostasis in the organism (García and Torres, 2011). The depletion of $CaCO_3$ signifies a depletion of the $CO_2$ source in the medium; however malic acid production requires the fixing of $CO_2$ to pyruvic acid in the cytosol (Vongsangnak et al., 2008). Consequently, a $CO_2$ limitation would lead to a reduction in malic acid production.

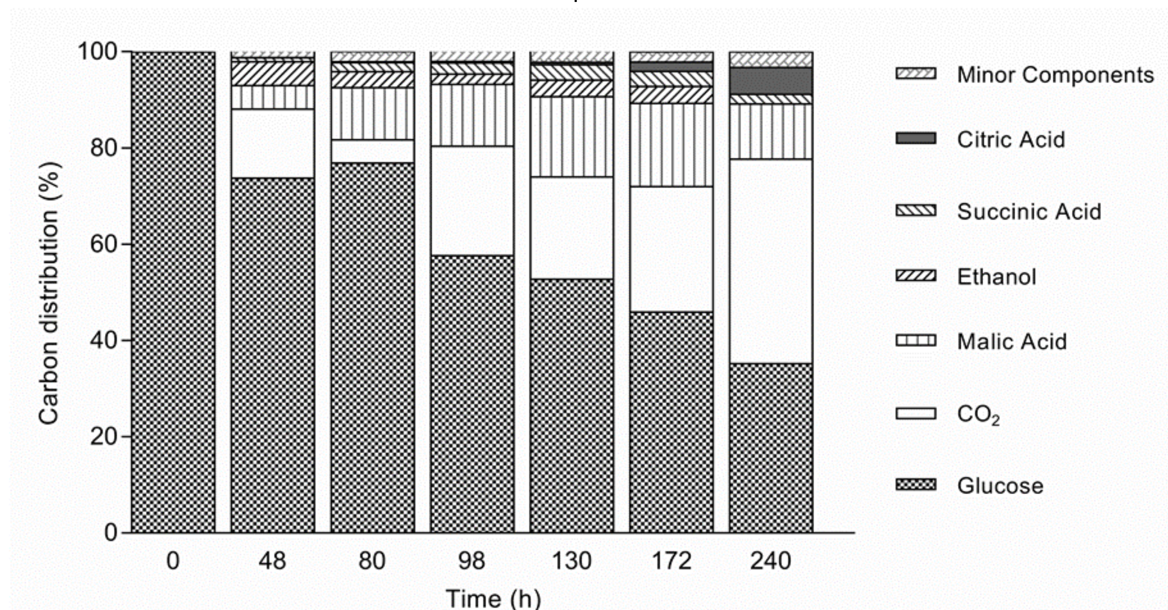



*Figure 1: The distribution of metabolites for A. oryzae NRRL3488 in the presence of 20 g/L $CaCO_3$ with the minor components including biomass, fumaric acid, pyruvic acid and acetic acid.*

Figure 2 shows the distribution of carbon when 80 g/L of $CaCO_3$ was used. After 240 h, more than 90 % of the initial amount of glucose was consumed with the main product being malic acid followed by $CO_2$. This is already a significant improvement in the glucose consumption rate and the yield of malic acid when compared to 20 g/L of $CaCO_3$. Additionally, in contrast to the trend observed in 20 g/L $CaCO_3$, more succinic acid was produced than citric acid.
It is evident from Figure 2 that a smaller percentage of carbon was used to produce $CO_2$ since the higher concentration of $CaCO_3$ resulted in more available $CO_2$ in the medium (in the form of $HCO_3^-$). As mentioned above, the reductive TCA branch requires the fixation of $CO_2$ to produce malic acid; the higher concentration of $CO_2$ available in the medium (due to $CaCO_3$) means more glucose can be directed towards malic acid production. From visual observation it appeared that the medium still contained significant amounts of $CaCO_3$ (white precipitate), which could explain why the pH was maintained above 5.7 (Table 1) throughout the fermentation. This reduced the energy requirements of the organism and therefore a smaller carbon flux to $CO_2$.
It is known that the organism does not produce succinic acid in the cytosol (Vongsangnak et al., 2008), i.e. the succinic acid is not produced by the reduction of malic acid in the cytosol. This necessitates the transport of succinic acid from the mitochondrion to the cytosol resulting in an incomplete TCA cycle in the mitochondrion, with a net redox requirement and a reduced mitochondrionic energy efficiency. The likely reason for this is an oxygen limitation in the system as a result of excess $CO_2$, with a corresponding reduction in oxygen partial

pressure in the headspace of the reactor due to $CO_2$ produced during fermentation and pH regulation.

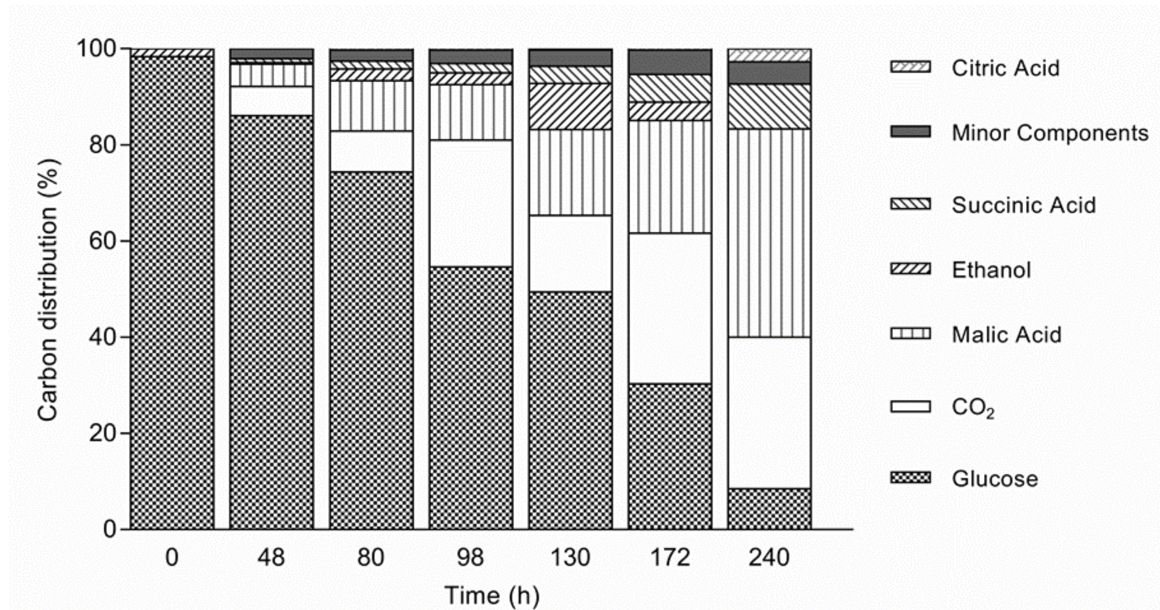



*Figure 2: The distribution of metabolites for A. oryzae NRRL 3488 in the presence of 80 g/L $CaCO_3$ with the minor components including biomass, fumaric acid, pyruvic acid and acetic acid.*

Figure 3 shows the carbon distribution when using 120 g/L $CaCO_3$. Here all the glucose had been consumed after 240 h with malic acid being the main product followed by succinic acid and $CO_2$. Significantly less $CO_2$ and more succinic acid was produced in this experiment, supporting the theory that a reduced energy requirement due to sufficient pH control above 6.1 (Table 1) coupled with an oxygen limitation was present in the system. These findings suggest that sufficient pH control and $CO_2$ supply is required for a maximum flux of glucose to malic acid. It is unclear whether the limited oxygen supply is required for malic acid production, or merely a symptom of the increased presence of $CO_2$ in the system.

Interestingly a decrease in the ethanol concentration was observed between 172 h and 240 h for all three $CaCO_3$ concentrations (final concentrations of 0 g/L). This suggests that given sufficient time *A. oryzae* is capable of consuming ethanol as a substrate.

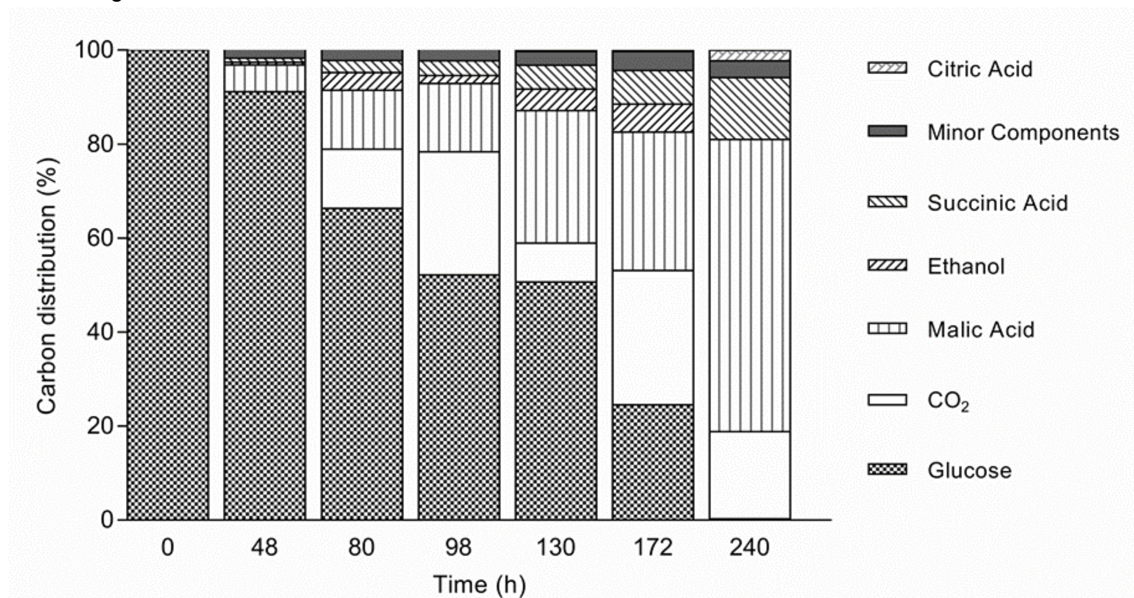



*Figure 3: The distribution of metabolites for A. oryzae NRRL 3488 in the presence of 120 g/L $CaCO_3$ with the minor components including biomass, fumaric acid, pyruvic acid and acetic acid.*

Table 1 below shows the final pH values of the three $CaCO_3$ concentrations, the total amount of nitrogen consumed (measured with the nitrogen assay) with the standard deviation of the triplicates indicated in brackets, the biomass concentrations as predicted by the black box model, and the theoretical $CaCO_3$ concentrations required to neutralise the measured acid concentrations in the medium.

*Table 1: The pH and nitrogen consumption data, final biomass predictions, and theoretical $CaCO_3$ requirement for pH neutralisation at termination of the respective experiments.*

| $CaCO_3$ (g/L) | Final pH | Nitrogen consumed | $X^a$ (g/L) | Total $CaCO_3$ required (g/L) |
|---|---|---|---|---|
| 20 | 4.7 (±0.1) | 62 % (±4 %) | 1.72 | 21.0 |
| 80 | 5.7 (±0.4) | 78 % (±3 %) | 1.75 | 52.9 |
| 120 | 6.1 (±0.3) | 81 % (±5 %) | 1.36 | 70.3 |

[a] Biomass predicted by the black box model

The pH of the three experiments can be attributed to the differences in malic acid yields since the optimum pH for malic acid fermentation is 6.0 (Knuf, 2013). As the fermentations proceeded the dissolution of $CaCO_3$ buffered the pH; 20 g/L $CaCO_3$ fell below the optimum pH after 98 hours, 80 g/L after 172 h and 120 g/L remained above the optimum after 240 h. The total theoretical $CaCO_3$ requirements for the 20 g/L $CaCO_3$ experiment agreed well with the observed decrease in pH, with corresponding decrease in production, as it was observed that a final $CaCO_3$ requirement of approximately 21 g/L was predicted. This means that the pH reduction as well as $CO_2$ limitation can be attributed to the depletion of $CaCO_3$ in the system. In contrast, the theoretical $CaCO_3$ requirements for the 80 g/L and 120 g/L $CaCO_3$ doses were significantly lower than the amount of $CaCO_3$ provided. This indicates that a surplus of $CaCO_3$ should have been present in the system. It is hypothesised that the $CaCO_3$ in the 80 g/L system was depleted through an alternative mechanism, potentially the precipitation of calcium malate during malic acid production which would exhibited as a white precipitate in the system (as was observed during the fermentation). This would increase the solution of $CO_3^{2-}{}_{(aq)}$ and consequently the $HCO_3^-$ present in the system, leading to an increase in the bioavailable $CO_2$ in the system (Vongsangnak et al., 2008). The result would be an increased production of malic acid due to increased $CO_2$ present in the system, but also a higher utilisation of $CaCO_3$ than would be expected through the exclusive use of $CaCO_3$ as pH buffer. This means that the $CaCO_3$ as $CO_2$ source could be elevated by the precipitative removal of calcium in the system.

The nitrogen consumption for the three different $CaCO_3$ concentrations did not vary significantly with the black box model predicting comparable amounts of biomass being produced. In all the experiments, the nitrogen has not been completely consumed which means growth could have continued given sufficient substrate. This is contradictory to literature in which malic acid production by *A. oryzae* NRRL 3488 is attributed to nitrogen starvation (Knuf, 2013).

The final predicted biomass concentrations were remarkably low at termination of the experiments, indicating an almost negligible flux of glucose to biomass when compared to the total glucose converted to metabolites. This suggests a highly desirable condition from a production perspective, as minimal glucose is lost to biomass production.

## 4. Conclusions

The varying concentrations of $CaCO_3$ (20 g/L, 80 g/L and 120 g/L) showed significant differences in the flux of carbon between malic acid and other metabolites in fermentations with *A.* oryzae NRRL 3488. When using 20 g/L of $CaCO_3$, 42.4 % of the total glucose consumed (65 %) was directed towards $CO_2$ production with only 11.5 % used for malic acid production. This is much lower when compared to 80 g/L of $CaCO_3$ where 43.3 % of the total glucose consumed (90 %) was used for malic acid production and 31.5 % used to produce $CO_2$. Using 120 g/L $CaCO_3$ further improved the production of malic acid to 62.2 % of the total glucose consumed, with only 18.5 % used for $CO_2$ production. The theoretically required $CaCO_3$ concentrations for the neutralisation of the pH were significantly lower than the amounts dosed in the 80 g/L and 120 g/L experiments, indicating that an additional $CaCO_3$ depletion mechanism such as calcium malate precipitation could be present. This would lead to an increase in the solution of $CaCO_3$ with a corresponding increase in bioavailable $CO_2$ and subsequent increased malic acid production. These results suggest that the role of $CaCO_3$ is not only as neutralizing agent but also as a source of $CO_2$.

The increased production of succinic acid with increasing $CaCO_3$ concentrations indicated an inefficient TCA cycle in the mitochondria, likely a result of oxygen limitation.

All three experiments evidenced incomplete nitrogen consumption rates. This suggests that nitrogen limitation is not a key factor in the malic acid production capabilities of *A. oryzae* NRRL 3488 but rather sufficient pH

control and availability of $CO_2$ required to activate the reductive TCA branch to malic acid. It is unclear if oxygen limitation is a requirement for malic acid production and should be further explored. Other pH buffering systems should be investigated in conjunction with sources of $CO_2$ to ameliorate the processing constraints experienced by the use of $CaCO_3$.The significantly low production of biomass during the course of all experiments, when compared to the production of metabolites, is highly desirable from a production perspective as minimal glucose is utilised for biomass production

**References**


Abe S., Furuya A., Ken'ichiro T., Saito T., 1962, Method of producing l-malic acid by fermentation, US Patent 3,063,910, assigned to Kyowa Hakko Kogyo Corporation, Tokyo, Japan.

Brown S.H., Bashkirova L., Berka R., Chandler T., Doty T., McCall K., McCulloch M., McFarland S., Thompson Y.D., Berry A., 2013, Metabolic engineering of *Aspergillus Oryzae* NRRL 3488 for increased production of L-malic acid, Applied Microbiology and Biotechnology, 9, 8903–8912.

Chen X., Wang Y., Dong X., Hu G., Liu L., 2017, Engineering rTCA pathway and C4-dicarboxylate transporter for L-malic acid production, Applied Microbiology and Biotechnology, 101, 4041–4052.

Chi Z., Wang Z.P., Wang G.Y., Khan I., Chi Z.M., 2016, Microbial biosynthesis and secretion of l-malic acid and its applications, Critical Reviews in Biotechnology, 36, 99–107.

García, J., Torres, N., 2011, Mathematical modelling and assessment of the pH homeostasis mechanisms in Aspergillus niger while in citric acid producing conditions, Journal of Theoretical Biology, 282(1), 23–35.

Hermann, B.G., Patel, M., 2007, Today's and tomorrow's bio-based bulk chemicals from white biotechnology, Applied Biochemistry and Biotechnology,136, 361–388.

Knuf C., Nookaew I., Brown S.H., McCulloch M., Berry A., Nielsen J., 2013, Investigation of Malic Acid Production in *Aspergillus oryzae* under Nitrogen Starvation Conditions, Applied Environmental Microbiology, 79, 6050–6058.

Knuf C., Nookaew I., Remmers I., Khoomrung S., Brown S., Berry A., Nielsen J., 2014, Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68, Applied Microbiology and Biotechnology, 98, 3517–3527.

Liu J., Li J., Shin H.D., Du G., Chen J., Liu L., 2018, Biological production of L-malate: recent advances and future prospects, World Journal of Microbiology and Biotechnology, 34, 6.

Liu J., Xie Z., Shin H.D., Li J., Du G., Chen J., Liu L., 2017, Rewiring the reductive tricarboxylic acid pathway and L-malate transport pathway of *Aspergillus oryzae* for overproduction of L-malate, Journal of Biotechnology, 253, 1–9.

Melo, A. F., Mauricio, E. F., Salgado, A. M., Pellegrini Pessoa, F. L., Triches Damaso, M. C., Couri, S., 2011, Assessment of catalytic properties in aqueous and media of Aspergillus niger lipase immobilized on supports vitreous, Chemical Engineering Transactions, 24, 973–978.

Mondala A.H., 2015, Direct fungal fermentation of lignocellulosic biomass into itaconic, fumaric and malic acids: current and future prospects, Industrial Microbiology and Biotechnology, 42, 487–506.

Park Y., Kang S., Lee J., Hong S., Kim S., 2002, Xylanase production in solid state fermentation by Aspergillus niger mutant using statistical experimental designs, Applied Microbiology and Biotechnology, 58, 761–766.

Ochsenreither K., Fischer C., Neumann A., Syldatk C., 2014, Process characterization and influence of alternative carbon sources and carbon-to-nitrogen ratio on organic acid production by *Aspergillus oryzae* DSM1863, Applied Microbiology and Biotechnology, 98, 5449–5460.

Salek S.S., van Turnhout A.G., Kleerebezem. van Loosdrecht, 2015, pH Control in Biological Systems Using Calcium Carbonate, Biotechnology and Bioengineering, 112, 905–913.

Vongsangnak W., Olsen P., Hansen K., Krogsgaard S., Nielsen J., 2008, Improved annotation through genome-scale metabolic modelling of *Aspergillus oryzae*, BMC Genomics, 9, 245–258.

Werpy T., Petersen G., 2004, Top Value-Added Chemicals from Biomass Volume I - Results of Screening for Potential Candidates from Sugars and Synthesis Gas, US Department of Energy.


View publication stats