The Influence of Manganese Ions on *Aspergillus niger* Biomass and Citric Acid Biosynthesis in Repeated Fed Batch Fermentation

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Dedicated to Prof. Dr. Đurđa Vasić-Rački on occasion of her 60th birthday

In citric acid technology absence of manganese ions in fermentation substrate is one of the most relevant requests. Presence of manganese ions higher than 1 μgl^{-1} could completely ruin citric acid biosynthesis initiating unproductive filamentous growth morphology and promoting isocitrate dehydrogenase activity in Tricarboxylic Acid Cycle. Running repeated fed batch process it was for the first time found that if fermentation process reach in batch fermentation its stationary growth phase in further continuation, as a repeated fed batch process, even feeding with a such high concentrations of manganese ions up to 200 μgl^{-1} does not effect any changes in fungal morphology either in cumulative biomass X_c or in cumulative citric acid C_c production.

Key words:
Aspergillus niger, manganese ions, repeated fed batch, citric acid fermentation

Introduction

Citric acid is one of the most relevant bulk chemicals produced by fermentation. There have been already proposed many theories to explain the phenomena of citric acid accumulation by *Aspergillus niger*, but still no complete explanation is available.[1–4] Citric acid is accumulated by an induced abnormality in the metabolism of the mould,[5] during the operation of the Tricarboxylic Acid Cycle [TCA] or citric acid cycle, postulated by Krebs in 1937. The traces of heavy metal ions in the fermentation substrate in this process plays the most crucial role.[6]

The requirement for citric acid accumulation requests a deficiency of manganese and iron ions in fermentation substrate that has as a consequence the inhibition of iconitase and isocitrate dehydrogenase activity and reducing of the activity of TCA Cycle and promoting the accumulation of citric acid. Iron ions in higher concentration than 1.5 mgl^{-1} strongly inhibited cellular morphology, by inducing formation of unproductive filamentous growth form.[7,8]

The importance of metal ions, the presence of manganese ions to citric acid fermentation was reported by *Clark et al.*[9] As little as 1 μgl^{-1} of manganese could completely ruined the production yield of and caused organism's morphology to switch from microbial pellets as citric acid productive form, to unproductive filamentous growth.[10,11]

On the other side manganese deficiency lower than 10^{-7} M elevate production of chitin and reduce synthesis of β-glucan. Chitin as relatively unchanged galactose polymer points to the chitin/β-glucan ratio as the most important factor for *A. niger* mycelia shape. Manganese levels also affect lipid synthesis which in turn affects cell membrane composition.[12] It also exhibits effects on DNA synthesis of *A.niger* and under manganese limitation it was observed that DNA formation was not inhibited but RNA synthesis was impaired.[13]

Manganese deficiency in *A.niger* cultivation also results in significantly lower lipid levels due primarily to reduction of triglycerides and with little effect on free acids and sterols.[14] Anyway the influence of manganese ions on *A.niger* is very complex and it obviously represents the most critical metal ion in citric acid fermentation.[8,15]

Trace element nutrition is specially highlighted by the fact that an optimal nutrient medium for citric acid fermentation will not allow high production unless the trace elements content is carefully controlled.[13] On the other hand, if trace element nutrition is correct, other factors (sugar concentration, phosphate and the others) have only less pronounced effects.[13] Anyway, for citric acid production limitation of described trace elements is critical.[16,17]

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In recent references the most attention related to *A.niger* metal ion tolerance was related to action of decreasing manganese ion concentration and to the effects of copper antagonism to iron and manganese[18,19] as well as to various genetic manipulation of *Aspergillus niger* for strain improvement.[20–21]

Although batch techniques in stirred tank or air lift bioreactors are in general use, some promising research results in citric acid biosynthesis were obtained by fed-batch process[22] and even by continuous fermentation.[24,25]

Present article represents new founding in citric acid fermentation as well as in fed batch technology.

Materials and methods

Microorganism: In all of experiments *Aspergillus niger* strain A60 (NRRL 2270) was used. Conidia of 7 day old cultures from worth agar slants incubated at T = 30 °C were used. As inocula for conducting the fermentation process suspension of *Aspergillus niger* conidia in sterile distilled water, in average initial spore concentration of 10^7 conidia ml^{-1} was used through all of the experiments. Inoculum spore concentration was controled by spectroscopic method.[26,27]

Medium: Primary fermentation substrate and control feeding media were consisted of 275 gl^{-1} beet molasses (125 gl^{-1} sucrose), 50 mgl^{-1} H_3PO_4 and 1000 mgl^{-1} K_4 $Fe(CN)_6$. pH was adjusted by H_2SO_4 to 5,8.[27,28] Manganese containing substrate was composed of the same compounds as the control media including addition of 0.1 M $MnSO_4$ in concentration 10 and 200 μgl^{-1}.

Bioreactor: All of the experiments were performed in 10 l Stirred Tank Reactor (Bioengineering AG, Wald, Switzerland) equipped with sterizable pH, pO_2 and redox potential sensors, temperature and foam control unit. Fermentation temperature was 30 °C, agitation N = 400 rpm and aeration 6 l min^{-1} was kept throw all of the experiments.

Cultivation procedure: After establishment of the stationary growth phase at 100 hours of cultivation in batch fermentation, every 24 hours an aliquot of fermentation broth was replaced with the same volume of sterile primary substrate consisted of 275 gl^{-1} beet molasses (125 gl^{-1} sucrose), 50 mgl^{-1} H_3PO_4 and 1000 mgl^{-1} K_4 $Fe(CN)_6$. pH was adjusted by H_2SO_4 to 5,8 without or with addition of manganese ions in concentration 10 and 200 μgl^{-1} 0.1 M $MnSO_4$. Evaporated water was substituted with sterile distilled water to ensure constant broth volume in reactor.

Biomass: Biomass was determined as dry weight after filtering and drying at 105 °C for 24 hours.

Analysis: Citric acid and reducing sugars were analysed by HPLC according to validated methods proposed by BIO-RAD.[27] Samples were filtered through a 0,45 μm membrane and analysed using 300 mm × 7,8 mm Aminex HPX-87H organic acid analysis cationic exchange column (Bio-Ad Laboratories,USA). Elution was performed at 65 °C. The mobile phase was 0,005M H_2SO_4 in bi-distilled water. The pump was operating at a flow rate of 0,5 ml min^{-1} ($8 \cdot 10^{-6}$ ls^{-1}). The injection volume was 20 μl. The eluting compounds were monitored by a fixed wavelength ultraviolet (UV-VIS) detector at 210 nm. Detector was connected in series with a refractive index (RI) detector. Glucose, fructose and ethanol were detected by RI. The peaks were quantified using external standard calibration. The components were identified by a comparison of their retention times with those of the standards.

Results and discussion

The experiments started as a batch fermentation process. After the establishing the stationary growth phase at 100 hours of cultivation in batch mode, fermentation process was turned on repeated fed batch mode. In according to this and to experimental protocol described bellow, every 24 hours the replacement of the adequate volume of the substrate with fresh feed was made. In the total volume of taken sample, biomass and citric acid concentration were determined. The amount of biomass measured in the second sample were summarized with the first amount and the third one was added to this sum and summarized as the third amount ($X_{C2} = X_{C1} + X_{C2}$; $X_{C3} = X_{C2} + X_{C3}$ …..etc). This succession was applied in all of the experiment as the amount of cumulative biomass X_C or as well as the amount of cumulative citric acid C_C .

Repeated fed batch citric acid fermentation proceeded 22 days at constant fermentation temperature T = 30 °C, agitation N = 400 rpm and aeration of Q_g = 6.0 l min^{-1}. In total three runs were made and the most typical results are referred.

According following conditions four experiments were performed in one block of 22 days.

Experiment 1. After the establishing of the stationary growth phase at 100 hours of batch cultivation after each 24 hour 0.5 l of substrate was pumped out of bioreactor, analyzed and replaced by sterile substrate. Substrate feed rate was $F_1 = 0.0147$ l h^{-1}, substrate dilution rate D = 0.0021 h^{-1}. Present run was control experiment, without the addition of $MnSO_4$. Specific growth rate $\mu_{max} = 0.0069$ h^{-1}.

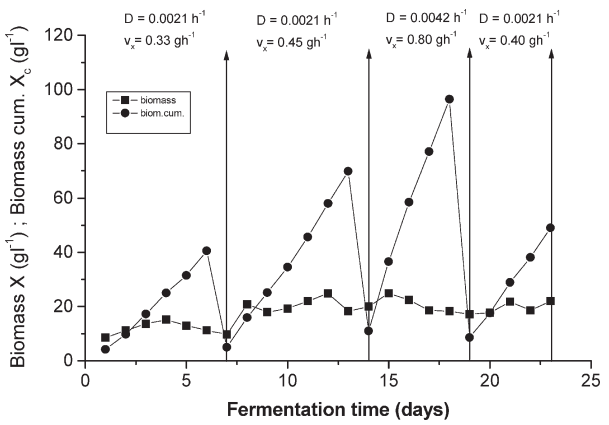
Experiment 2. In seven days run, after each 24 hours, 0.5 l of substrate was pumped out of bioreactor, analysed and replaced by sterile substrate containing 10 μg l^{-1} of $MnSO_4$. Substrate feed rate was F_1 = 0.0147 lh $^{-1}$, substrate dilution rate D = 0.0021 h $^{-1}$. Specific growth rate μ_{max} = 0.0072 h $^{-1}$.

Experiment 3. In four days run, after each 24 hours, 1.0 l of substrate was pumped out of bioreactor, analysed and replaced by sterile substrate. Substrate feed rate was F_1 = 0.0294 lh $^{-1}$, but substrate dilution rate was doubled D = 0.0042 h $^{-1}$. This was control experiment with double dilution rate and double replaced volume of substrate without the addition of $MnSO_4$. Specific growth rate μ_{max} = 0.0145 h $^{-1}$.

Experiment 4. In four days run, after each 24 hours, 0.5 l of substrate was pumped out of bioreactor, analysed and replaced by sterile substrate containing 200 μg l^{-1} $MnSO_4$. Substrate feed rate was F_1 = 0.0147 l h $^{-1}$, substrate dilution rate D = 0.0021 h $^{-1}$. Specific growth rate μ_{max} = 0.0064 h $^{-1}$.

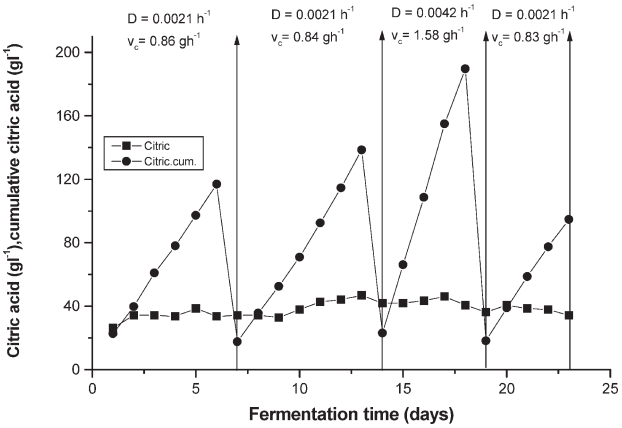
Present results (Fig. 1) indicate that in all three cases feeding with control substrate (Part 1, from 0 to 7 days), substrate with addition of 10 μg l^{-1} of $MnSO_4$ (Part 2, from 7 to 14 days), and 200 μg l^{-1} of $MnSO_4$ (Part 4, from 19 to 23 days), the course of cumulative biomass X_c was not interrupted and it remains the same. In experiments 1,2 and 4 also specific growth rate remained in interval μ_{max} = 0.0064 to 0,0072 h. $^{-1}$

By doubling the dilution rate (D = 0.0021 to 0.0042 h $^{-1}$), the cumulative biomass was significantly increased (Part 3, from 14 to 19 days). In this part also an increasement of specific growth rate μ_{max} = 0.0145 h $^{-1}$ was indicated.



F i g . 1 – *Increase of cumulative biomass X_c production during repeated fed batch fermentation at different dilution rates and different media composition; D dilution rate, v_x cumulative biomass increase. Part 1 (0 to 7 days) , control substrate D = 0.0021 h^{-1}; Part 2 (7 to 14 days), substrate with addition of 10 μg l^{-1} of $MnSO_4$, D = 0.0021 h^{-1}; Part 3 (14 to 19 days) D = 0.0042 h^{-1} and Part 4 (19 to 23 days) 200 μg l^{-1} of $MnSO_4$*

In all the other cases, feeding with control substrate (D = 0.0021 h $^{-1}$, Fig. 2, Part 1, from 0 to 7 days) or substrate with 10 μg l^{-1} (D = 0.0021 h $^{-1}$, Fig. 2, Part 2, from 7 to 14 days), and substrate with addition of 200 μg l^{-1} of $MnSO_4$ (D = 0.0021 h^{-1}, Fig. 2, Part 4, from 19 to 23 days), there were no decrease in cumulative citric acid production indicated.



F i g . 2 – *Increase of cumulative citric acid C_c production during repeated fed batch fermentation at different dilution rates and different media composition; D dilution rate, v_c rate of cumulative citric acid increase. Part 1 (0 to 7 days), control substrate D = 0.0021 h^{-1}; Part 2 (7 to 14 days), substrate with addition of 10 μg l^{-1} of $MnSO_4$, D = 0.0021 h^{-1}; Part 3 (14 to 19 days) D = 0.0042 h^{-1} and Part 4 (19 to 23 days) 200 μg l^{-1} of $MnSO_4$.*

In the same process also the rate of cumulative biomass v_x and the rate of cumulative citric acid v_c were calculated. Analysis showed that the rates of cumulative biomass production in experiments 1,2 and 4, remained in the interval v_x = 0.33 – 0.45 gh^{-1} while the rates of cumulative citric acid v_c = 0.80 – 0.86 gh^{-1}. Even in experiment 4 (Fig. 2, Part 4) feeding with high manganese concentration containing substrate, up to 200 μg l^{-1} $MnSO_4$, did not reduce or did not influence on further cumulative citric acid production at all.

In experiment 3 (Fig. 2, Part 3) with doubling dilution rate (D = 0.0021 to 0.0042 h $^{-1}$), the cumulative rate of biomass production v_x increased from 0.45 to 0.80 gh $^{-1}$, while the rate of citric acid production v_c reased from 0.84 to 1.58 gh $^{-1}$.

Conclusions

In repeated fed batch citric acid biosynthesis the relevance of stationary growth phase for further establishment of repeated feeding batch process was discovered. Although the presence of manganese ions in fermentation substrate during the early exponential growth phase would usually completely

ruin citric acid biosynthesis, by activation of citrate dehydrogenate and promoting the activity of TCA cycle, it was found that after the establishment of the stationary growth phase, at 100 hours of cultivation in batch mode fermentation, the presence of manganese ions even in concentration up to 200 μg l^{-1} (200 times higher than the limit) did not have influence either on cumulative biomass X_c or as on cumulative citric acid production C_c. At constant dilution rate D, the rate of increase of cumulative biomass v_k and rate of cumulative citric acid v_c, remained nearly the same even in a case, if the feeding substrate contains unusually high concentration of highly inhibitory manganese ions. Observing microbial morphology by optical microscopy no remarquable changes in microbial pellet morphology were detected.

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