UDC 582.288:547.477.1:663.11
ISSN 1330-9862

original scientific paper

Aspergillus niger Morphology and Citric Acid Production in Submerged Batch Fermentation: Effects of Culture *pH*, Phosphate and Manganese Levels

Maria Papagianni[1]*, *Michael Mattey, Marin Berovič*[2] *and Bjorn Kristiansen*[3]

Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow, UK

[1]Department of Chemical Engineering, Ohio University, Athens, Ohio 45 701, U.S.A.

[2] Department of Biotechnology, Faculty of Chemistry and Chemical Technology University of Ljubljana, Slovenia

[3] Borregaard Industries, P.O. Box 162, Sarpsborg, Norway

Received: March 18, 1999
Accepted: June 14, 1999

Summary

The effects of culture pH, phosphate and manganese levels on citric acid production and Aspergillus niger morphology were studied under various agitation conditions in submerged batch culture. These factors were found to play important roles in both morphological development and process productivities. In all cases morphology was closely related to productivity and, irrespective of the parameter studied, similar morphological types were obtained. Lower citric acid productivities resulted always from the form of loose clumps with small central cores and a diffuse mass of filaments around them.

Key words: Aspergillus niger, citric acid, morphology, *pH,* phosphate, manganese, agitation

Introduction

A close link between *A. niger* morphology and productivity in the process of citric acid formation has early been identified (*1–4*). Often pellets have been considered as a prerequisite for a successful citric acid fermentation (*1–3*), while, the short, thick and highly branched filaments, which often have a large number of swollen cells are characteristic of the acidogenic mycelium (*5*). This morphology is common for filamentous fungi under intensive agitation conditions (*6,7*). With regard to agitation, morphology has been extensively studied for the citric acid and other filamentous fermentations (*6–8*). However, in a fermentation such as the citric acid process, where many factors may influence the process yields, the role of certain key parameters should be investigated with regard to morphology and productivity.

Culture *pH* can have a profound effect on the production of citric acid by *A. niger*, since certain enzymes within the TCA cycle are *pH* sensitive. The activity of the mitochondrial enzyme $NADP^+$ the inhibition of which during the citric acid fermentation causes citric acid accumulation, is extremely sensitive towards slightly acidic conditions (*9*). The maintenance of a low *pH* during fermentation is vital for a good yield of citric acid and it is generally considered necessary for the *pH* to fall below 2 within a few hours of the initiation of the process in order to obtain high yield (*10*).

Despite the importance of culture *pH* and morphology in citric acid fermentation, information on the effect of *pH* on the morphological development of *A. niger* in the process of citric acid production is limited. Earlier studies involved small glass fermenter or shake flask cultivation (*11,12*) and the characterisation of morphology was qualitative, based on microscopic observations and photographs. The presence of large, swollen, spherical cells at *pH* values below 2 in a number of fungi including *A. niger* has been reported (*13*), while, in the

* Corresponding author; 8 Kamvounion street, Thessaloniki 54 621, Greece; E-mail: mpapagianni@hotmail.com

work of Pirt and Callow (*14*) with *Penicillium chrysogenum*, culture *pH* determined whether the pelleted or the filamentous form predominated. The length of individual filaments was also affected by *pH*. In the more recent work of Carlsen *et al.* (*15*) with *A. oryzae* using a spore inoculum, spore coagulation was determined by *pH* that consequently determined whether the pelleted or the filamentous form would predominate.

Citric acid accumulation is strongly influenced by the composition of the nutrient medium and certain effects have been extensively discussed: Phosphate and /or nitrogen limitation (*16*) and trace metal levels are among the important factors that influence the citric acid fermentation (*17*).

In order to accumulate citric acid, growth must be restricted; however, it is not clear whether phosphate or nitrogen is the necessary limiting factor. According to Shu and Johnson (*18*), phosphate does not have to be limiting, but when trace metal levels are not limiting, additional phosphate results in side reactions and overgrowth. While studies concerning the effect of phosphate levels on citric acid producing *A. niger* morphology are lacking, pellet formation in filamentous fungi has been discussed in many cases and to be induced, among other factors, by the limitation of particular nutrients (*19*). Factors favouring increased growth rates have been shown to reduce pellet formation (*20,21*).

A number of divalent metals have been suggested as being required in limiting amounts for a successful citric acid process and a central role is attributed to Mn^{2+} (*10,17*). Manganese ions are known to be specifically involved in many cellular processes, such as, cell wall synthesis, sporulation and secondary metabolite production (*22–24*). The protein breakdown under Mn^{2+} deficiency results in a high intracellular NH_4^+ concentration and inhibition of phosphofructokinase, which leads to a flux through glycolysis, and the formation of citric acid (*17*). Concerning *A. niger* morphology, omission of Mn^{2+} ions from the nutrient medium resulted in abnormal morphological development with swelled and bulbous hyphae (*25*), while addition of trace amounts (2 ppb) changed the morphological form, from pelleted to filamentous (*2*).

Previous investigations described effects and morphological changes qualitatively. The aim of the present study was to provide quantitative morphological characterisation for *A. niger* over a range of culture *pH*, phosphate and manganese concentrations at different agitation intensities and to investigate the relationship between morphology and productivity for this fermentation. In earlier publications we showed the close link between morphology and citric acid production with regard to agitation (*4,26*). Further examining the effects of the above factors on morphology and citric acid production would make the observations less system specific.

Materials and Methods

Microorganism and process

The microorganism (*A. niger PM 1*, University of Strathclyde), inoculum preparation, fermentation media, sampling procedures and analytical methods, were as described in the previous publication (*4*).

The loop bioreactor used in this work (APV Chemical Machinery) was a propeller loop reactor with hydromechanic flow drive (pump) and total operating volume of 6 L. We have previously shown that the loop reactor behaves much like a stirred tank, with a direct correlation between stirrer speed and pump speed (*26*). The loop consisted of flanged stainless steel sections with a total length of 4 m and internal diameter of 5 cm in the riser, upper transverse and downcomer and 2.5 cm in the lower transverse section. The circulation pump was situated at the lower transverse part of the loop. The *pH* was monitored in the riser and downcomer by steam sterilisable glass reference electrodes (Ingold, Infit 764-50). The riser probe was used for *pH* control. Dissolved oxygen was monitored with a polarographic DO probe (Ingold) placed in the downcomer section of the loop. Fermentations carried out at three different pump settings, which corresponded to broth circulation times, t_c, of 18, 10 and 8 s, respectively.

Process temperature was maintained at 28 °C and the air flow rate at 1 L L^{-1} min^{-1}. *pH* control was by addition of titrants (2 M solution of NaOH and solution of 20 % volume fraction of H_2SO_4). Foam control was achieved by addition of polypropylene glycol (M_r = 2 000).

Quantification of morphology

Morphology was characterised using a semi-automatic image analysis system consisting of a phase contrast microscope (Olympus CH), a CCD camera (Sony XC77CE), a PC with a frame grabber and the image analysis software (Aequitas, Dynamic Data Links, Cambridge, U.K.). The samples were immediately fixed with an equal volume of fixative as described by Tucker *et al.* (*27*) (13 mL 40 % formaldehyde and 5 mL glacial acetic acid added to 200 mL of 50 % ethanol). The fixed sample was further diluted with fixative and the dilution was adjusted to separate the mycelial clumps on the microscope slide. Morphology measurements included the morphology parameters convex perimeter of clumps (*P1* in μm) obtained by measuring the length of a line joining the tips of the filaments protruding from the clump, the perimeter of the cores of clumps (*P2* in μm) and the length (*L* in μm) of filaments and their branches that arose from the cores. Measurements were taken for at least 50 clumps per sample using a magnification of 100x. Mean values of the morphological parameters were calculated for each sample and are presented in this work.

Examination of fresh and fixed samples showed that the fixation method did not affect the morphology and fixed samples remained unchanged for prolonged periods (*28*).

Results and Discussion

Effect of pH

Fermentations were carried out at two pump speeds which corresponded to broth circulation times of 8 and 18 s. Fig. 1 shows the time-course of the fermentation at t_c = 8 s, carried out without *pH* control. The *pH* of 2.9 at

inoculation time dropped to 2 within 48 hours and remained at this value thereafter. The yield of citric acid on glucose was 83 % and it was the highest yield obtained in this bioreactor. Table 1 presents the results (168 h fermentation) for both circulation times (8 and 18 s) and *pH* (2 and 4) tested. Citric acid production was dependent on agitation as was expected, while at each agitation level it was markedly influenced by *pH*. Increasing the *pH* decreased citric acid production. Biomass production appeared to be unaffected by agitation and *pH* changes. Dissolved oxygen concentration levels reduced with increasing the *pH* and the lowest concentrations observed were at t_c = 18 s and *pH* controlled at 4. Increased *pH* conditions caused oxalic acid accumulation while the highest concentration of extracellular polysaccharides and proteins resulted from the combination of low agitation and increased *pH* (Table 1).

In all fermentations, the inoculum consisted of freely dispersed filaments that within 24 hours in the bioreactor aggregated to clumps. The bulk of the mycelium was in the form of clumps, as particles of intertwining filaments around a small core, not having the compact structure of what is usually referred as pellets (*26*) and not macroscopically visible. Pellets were not detected during the course of fermentations.

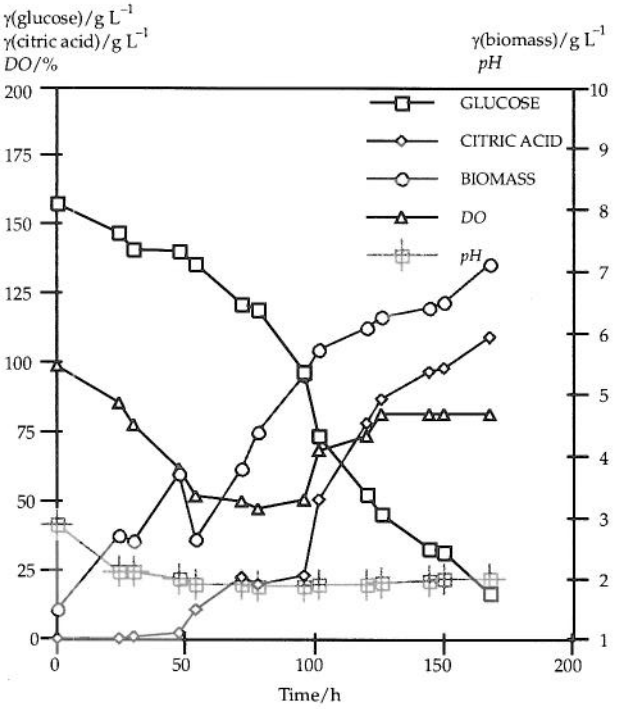

Fig. 1. Time profiles of citric acid, biomass residual glucose, *DO* and *pH* in the loop bioreactor at t_c=8 s and uncontrolled *pH*

Aspergillus niger morphology was greatly influenced by *pH*, as shown in Table 1. Fig. 2 shows the time courses of the convex perimeters of clumps *P1* and the perimeters of cores *P2*, at both circulation times and *pH*. Increased agitation reduced the mean values of the perimeters of clumps as was expected (*4,23,26*), while mean perimeters of cores appeared not to be affected. At each agitation level, increasing the *pH* from 2 to 4 the mean convex perimeters of clumps almost doubled while the perimeters of cores were only slightly increased (Table 1). Changes in the ratio *P1/P2* are characteristic of the change in clumps structure: *P1/P2* increased from 2.79 to 4.39 at t_c = 8 s and from 3.49 to 5.47 at t_c = 18 s, with increasing *pH* from 2 to 4. Considering the unchanged core perimeters and similar biomass concentrations it is obvious that the area occupied by the clumps increased with increasing *pH* on expense of its density. Conditions of increased *pH* transformed the clumped mycelium from a more compact (Fig. 3) to a looser form (Fig. 4) and the change in morphology, along with the increased concentrations of extracellular polysaccharides increased the viscosity of the fermentation and resulted in observed low *DO* levels. Obviously, increased *pH* caused conditions that do not conform to the requirements for successful citric acid fermentation.

Concerning *A. niger* morphology in the citric acid fermentation, the effect of culture *pH* is so far not extensively investigated, and published research mainly concerns the pelleted form. Steel *et al.* (*11*) studying the effect of *pH* on pellet formation of *A. niger* (*pH* values above 5 were tested), showed that increasing the *pH* transformed the mycelium from compact pellets with limited growth around the central core to pellets with dispersed hyphal growth around the core. It is known that at *pH* values above 4.5, cell walls of most microorganisms are negatively charged and electrostatic repulsion tends to cause separation of the aggregated cells (*19*). This seems to be in accordance with the results in the present work, since the same effect was observed with the clumps, although *pH* values below 5 were tested.

Effect of phosphate levels

Phosphorous was provided in the media as KH_2PO_4. Table 2 shows that increasing the KH_2PO_4 concentration in the fermentation medium from 0.1 to 0.5 g/L, at t_c =18 s

Table 1. Summary of the results obtained in the loop bioreactor with *pH* = 2 and 4 and t_c = 8 and 18 s. The effect of *pH* on process productivities and *A. niger* morphology (168 hours)

t_c/s	*pH*	γ(citric acid) g/L	γ(biomass) g/L	γ(pol.+ prot.) * g/L	γ(oxalic) g/L	*P1*/μm	*P2*/μm	*L*/μm
8	2	109.40	7.10	1.17	0.18	944.00	337.70	124.00
8	4	60.52	6.50	1.90	0.10	1805.00	411.20	213.60
18	2	50.00	6.50	4.70	35.00	1238.40	354.90	242.20
18	4	11.70	6.90	33.60	24.80	2216.33	405.10	265.15

* total extracellular polysaccharides and proteines

Table 2. Summary of the results obtained in the loop bioreactor with different potassium phosphate concentrations. Effects on fermentation productivities and *A. niger* morphology (168 hours)

t_c/s	$\gamma(KH_2PO_4)$ g/L	γ(citric acid) g/L	γ(biomass) g/L	*P1*/μm	*P2*/μm	*L*/μm
10	0.10	81.20	10.20	1124.20	508.00	134.40
10	1.00	30.70	11.35	4205.10	603.00	221.20
18	0.10	11.73	6.90	2216.30	405.10	265.10
18	0.50	22.30	11.48	3104.80	420.00	325.00

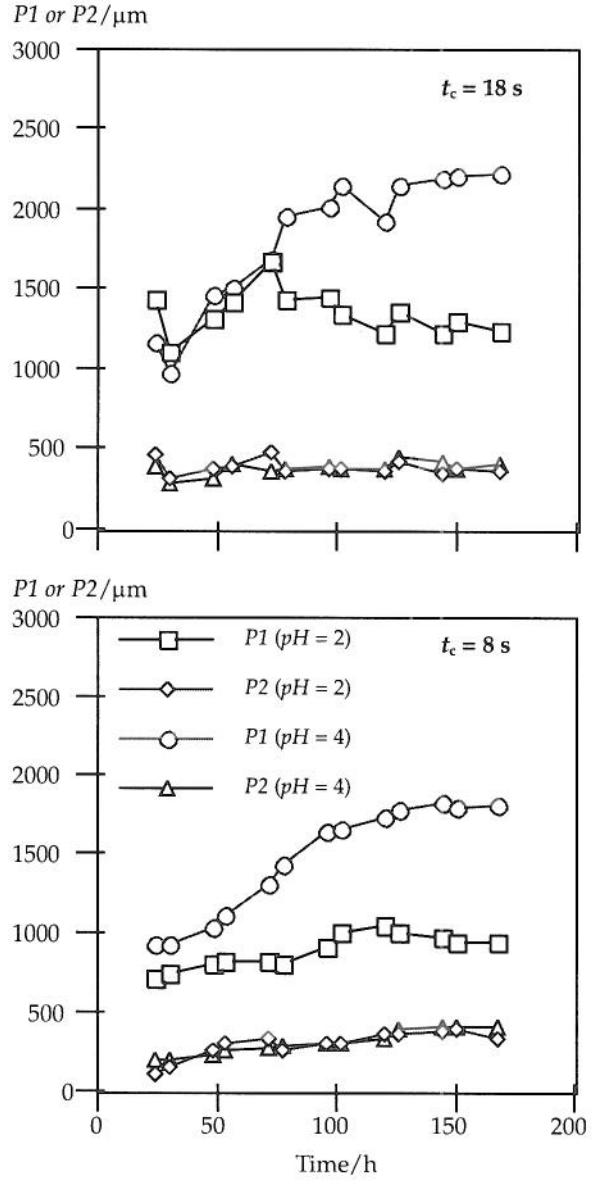

Fig. 2. Effect of *pH* on *A. niger* morphology: Time profiles of mean convex perimeters of clumps (*P1*) and their core (*P2*) under *pH* = 2 and 4, at t_c = 8 s and 18 s

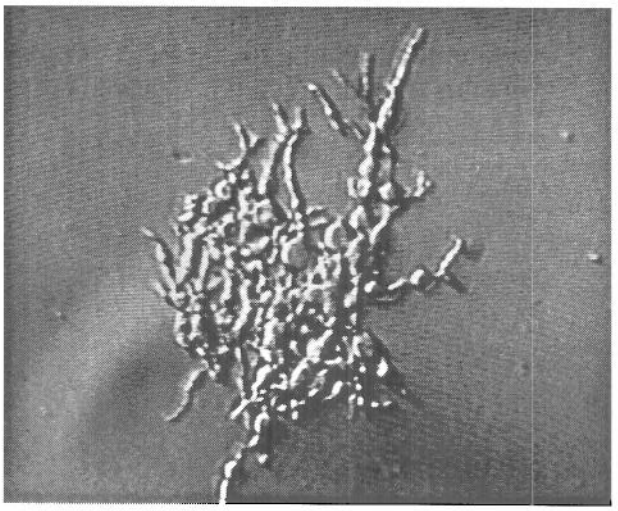

Fig. 3. Typical form of the mycelium under low *pH* conditions (*pH* = 2 and t_c = 8 s) at magnification 100x

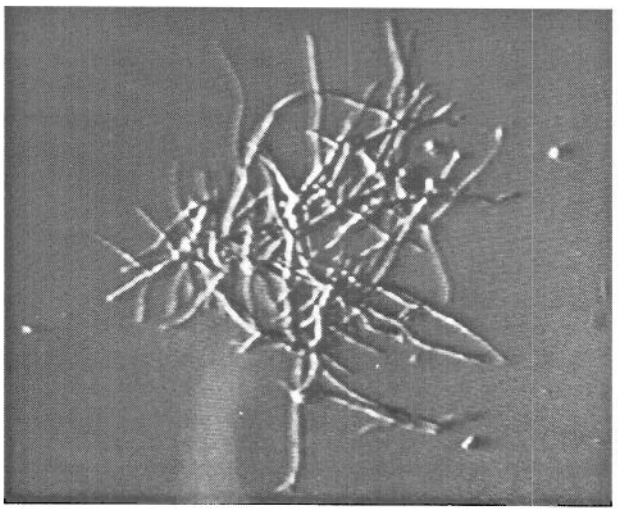

Fig. 4. Typical form of the mycelium under increased *pH* conditions (*pH* = 4 and t_c = 8 s) at magnification 100x

and *pH* controlled at 2, led to a drastic reduction in citric acid production, with the yield on glucose reduced from 70 to 39 %, while biomass concentrations increased from 6.5 to 11.5 g/L. This was reflected in the specific citric acid production rates, which were severely reduced as the phosphate levels increased. A maximum value of 0.04 h^{-1} was noted with KH_2PO_4 0.5 g/L while a value of 0.21 h^{-1} was obtained at 0.1 g/L KH_2PO_4.

A higher KH_2PO_4 concentration of 1 g/L was tested at t_c = 10 s and compared with the standard run (0.1 g/L) at the same circulation time. Again it was found that the high phosphate level stimulates biomass production and

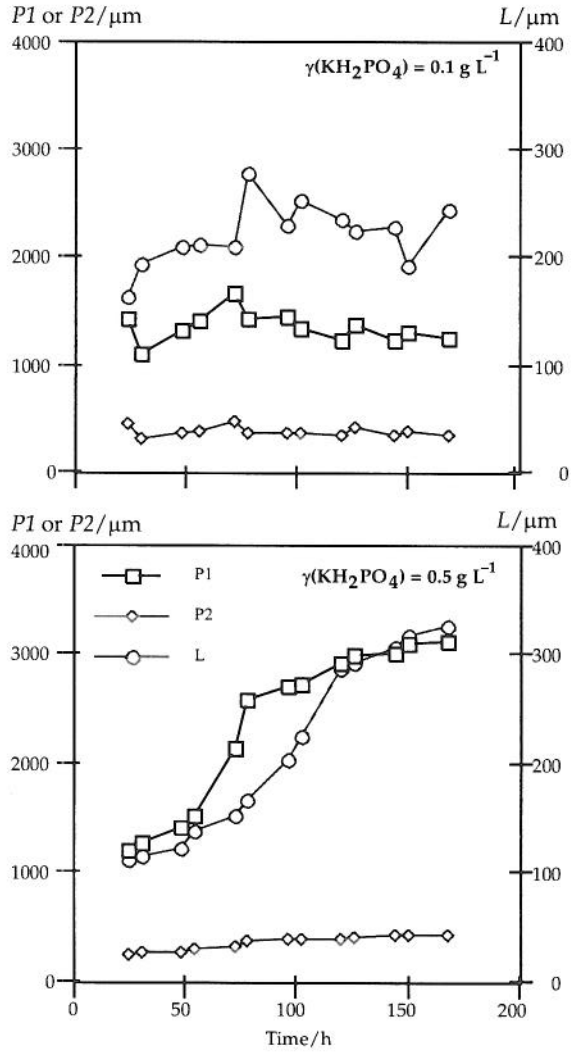

Fig. 5. Effect of phosphate concentration: Time profiles of morphology parameters *P1*, *P2* and *L* with 0.1 and 0.5 g/L KH_2PO_4 in the fermentation media (t_c = 18 s, *pH* = 2)

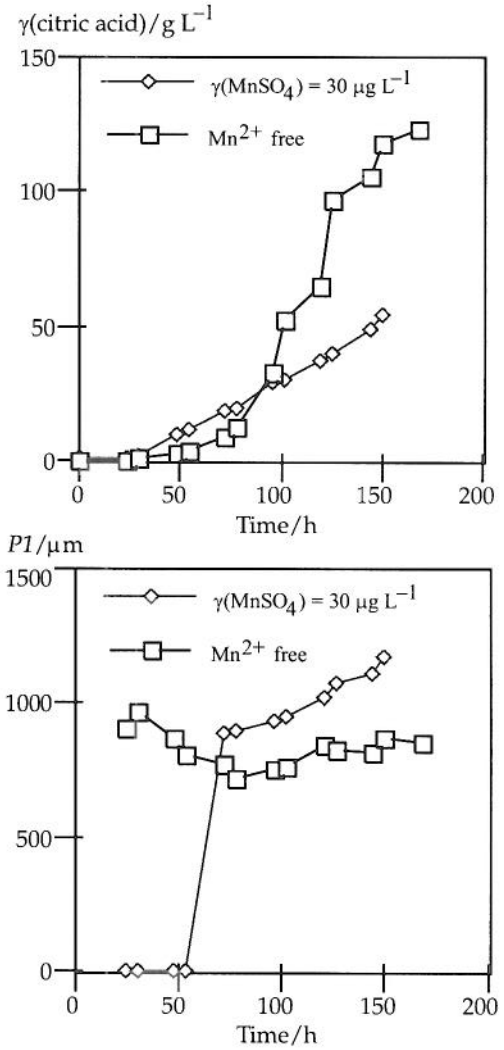

Fig. 6. Time profiles of citric acid concentrations and mean convex perimeters of clumps in the absence and presence of 30 μg/L of manganese ions (t_c = 8 s, *pH* = 2)

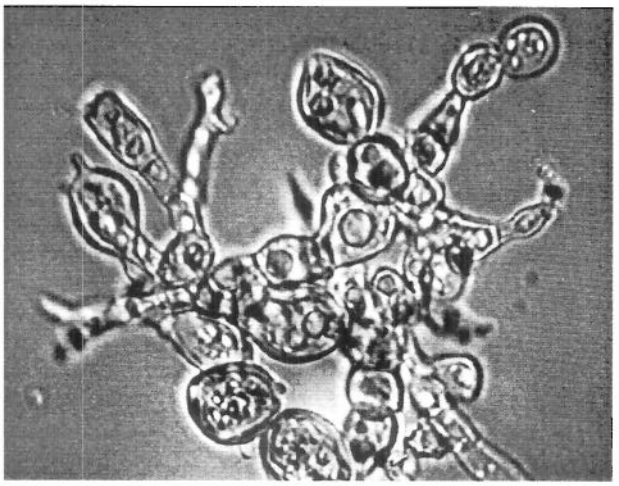

Fig. 7. Typical form of the acidogenic mycelium under manganese deficient conditions: Large, swollen cells and tips. Photograph taken at 92 h of fermentation (t_c = 8s, *pH* = 2) at magnification 1000x

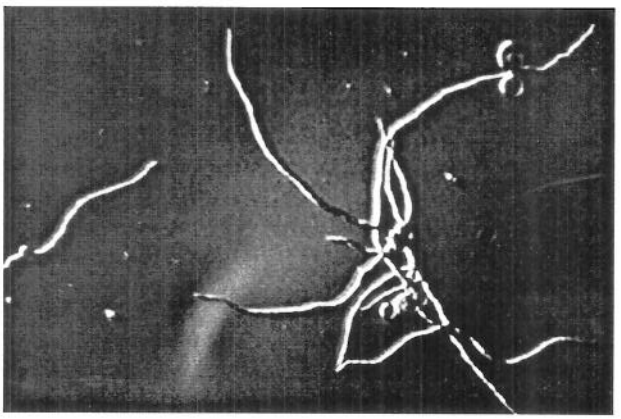

Fig. 8. Absence of morphological abnormalities as a result of the addition of 30 μg/L $MnSO_4$ in the fermentation media. 92 hours of fermentation (t_c = 8 s, *pH* = 2) at magnification 100x

reduces citric acid production. The maximum specific rate of citric acid production obtained was 0.12 h^{-1} as compared with 0.23 h^{-1} in the standard run.

The present results indicate that there should be an optimum phosphate concentration in the fermentation medium and any excess has prohibiting effects on citric acid formation, while it changes the direction of fermentation to biomass overproduction. However, some reports on the necessity of phosphate limitation in citric acid fermentation are contradictory (*10,17*).

Fungal morphology was greatly affected by phosphate levels. A small increase in KH_2PO_4 concentration from 0.1 to 0.5 g/L led to drastic changes in morphology: the convex perimeter of clumps increased by almost three times while the core perimeter remained rather unaffected (Table 2). As with increased *pH* conditions, the clumps lost their compact structure and the looser form with extended growth around a small core predominated (Fig. 5). The KH_2PO_4 concentration of 1 g/L in the fermentation medium increased the mean convex perimeter of clumps by a factor of 3.7 compared to the standard run and counteracted the agitation effect, since *P1* values were higher than those obtained at $t_c = 18$ s and KH_2PO_4 0.5 g/L. Under increased phosphate concentrations mean values of *L* increased during fermentation, while in the standard run they remained unchanged after an early growth phase. However, *L* did not increase to the same extend as *P1* and it remained rather small – although mean values were higher compared to those obtained in all other fermentations. This was attributed to a large number of shorter filaments and increased branching, as a result of enhanced growth, since the measurements included both main filaments and their branches. Increased specific growth rates are associated with increased branching in filamentous fermentations. Also, it has been shown that factors which favour increased specific growth rates and especially media rich in easily assimilated nutrients (e.g. inorganic phosphorous), are responsible for reducing the tendency of the mycelia to agglomerate (*19*). Although the strain used in the present study did not form macroscopic pellets, factors like increased *pH* or phosphate concentrations seem to exert similar controls on different morphological types changing the morphology in a similar way.

Effect of manganese ions concentration

Addition of as little as 30 μg/L of $MnSO_4$ to the fermentation medium ($t_c = 8$ s and $pH = 2$) caused a 20 % reduction in citric acid yield (Fig. 6). The maximum specific production rate was 0.13 h^{-1} while in the absence of manganese ions it was 0.30 h^{-1}. Biomass levels were also lower following manganese addition. A similar effect was observed by Clark *et al.* (2), reporting on a 10 % reduction in yield following the addition of 2 ppb of manganese ions, while higher levels resulted in further sharp decreases in yield. In addition to the yield decrease, the authors noticed an undesirable change in morphology from the pellet-like to the filamentous form with the addition of 2 ppb manganese to beet-molasses media.

Addition of manganese in the fermentation medium in this study prevented the mycelium from clumping. For 72 hours following inoculation no clumps were detected, while later large aggregates formed out of individual mycelial trees and a small number of loose clumps with small central cores (Fig. 6). In contrast to the typical hyphal morphology obtained under intensive agitation conditions in manganese deficient media (Fig. 7), addition of manganese prevented morphological abnormalities and no swollen tips and cells were detected (Fig. 8).

In citric acid fed batch process using high amounts of Mn^{2+} ions up to 6 mg/L in fed media, it was found that when biomass reaches its steady state, even a such concentration of Mn^{2+} did not reduce the citric acid production. The rate of cumulative biomass and rate of cumulative citric acid, at the same dilution rate, remained the same.

Like under increased *pH* and phosphate concentrations, reduced citric acid production due to the presence of manganese ions in the medium was accompanied with morphological changes of the same trend.

Conclusions

Quantitative characterisation of micromorphology of *A. niger* in this study showed that morphology and citric acid production are closely linked in the process of citric acid production. Intensive agitation provides the typical form of the acidogenic mycelium – small clumps with short and swollen filaments – however factors like *pH*, phosphate and manganese levels, all of great importance for a successful citric acid fermentation, play an equally important role in the development of morphology and fermentation productivities. Unfavourable cultivation conditions with regard to the above factors lead to the same morphological type of loose clumps with disproportionally small cores. The importance of these factors to both *A. niger* morphological development and citric acid production becomes more profound in the light of earlier investigations reporting similar effects with strains that formed macroscopic pellets and not microscopic clumps, suggesting that the same factors exert similar controls over fundamentally different morphological forms.

References

1. D. S. Clark, *Can. J. Microbiol. 8* (1962) 133.
2. D. S. Clark, K. Ito, H. Horitsu, *Biotechnol. Bioeng. 8* (1966) 465.
3. D. S. Clark, C. P. Lentz, *Biotechnol. Bioeng. 5* (1963) 193.
4. M. Papagianni, M. Mattey, B. Kristiansen, *Biotechnol. Lett. 16* (1994) 929.
5. L. B. Swaeger, *US Patent 2 970 084* (1964).
6. H. Y. Makagiansar, P. Ayazi Shamlou, C. R. Thomas, M. D. Lilly, *Bioprocess Eng. 9* (1993) 83.
7. M.T. Belmar-Beiny, C. R. Thomas, *Biotechnol. Bioeng. 37* (1991) 456.
8. J. C. van Suijdam, B. Metz, *Biotechnol. Bioeng. 23* (1981) 111.
9. M. Mattey, I. Bowes, *Biochem. Soc. Trans. 6* (1978) 1224.
10. M. Mattey, CRC *Crit. Rev. Biotechnol. 12* (1992) 87.
11. R. Steel, S. M. Martin, C. P. Lentz, *Can. J. Microbiol. 1* (1954) 150.
12. D. S. Clark, *Biotechnol. Bioeng. 4* (1962) 17.

13. J. E. Smith, J. G. Anderson, *Proc. 23rd Soc. General Microbiol. Symposium* (1973) p. 295.
14. S. Pirt, D. S. Callow, *Nature, 184* (1959) 307.
15. M. Carlsen, A. Spohr, J. Nielsen, J. Villadsen, *Biotechnol. Bioeng. 49* (1996) 266.
16. B. Kristiansen, C. G. Sinclair, *Biotechnol. Bioeng. 21* (1979) 297.
17. C. P. Kubicek, M. Roehr, CRC *Crit. Rev. Biotechnol. 3* (1989) 331.
18. P. Shu, M. J. Johnson, *J. Bacteriol. 56* (1948) 577.
19. S. E. Vecht-Lifshitz, S. Magdassi, S. Braun, *Biotechnol. Bioeng. 35* (1990) 890.
20. S. Braun, S. E. Vecht-Lifshitz, *TIBTECH, 6* (1991) 63.
21. H. Hemmersdorfer, A. Leuchtenberger, C. Wadsack, H. Ruttloff, *J. Basic Microbiol. 27* (1987) 309.
22. B. J. M. Zonnevelt, *Arch. Microbiol. 105* (1975) 101.
23. P. R. Mahadevan, E. L. Tatum, *J. Bacteriol. 90* (1965) 1073.
24. H. L. Barnett, V. G. Lilly, *Mycologia, 58* (1966) 585.
25. M. Kisser, C. P. Kubicek, M. Roehr, *Arch. Microbiol. 128* (1980) 26.
26. M. Papagianni, M. Mattey, B. Kristiansen, *J. Biochem. Eng. 2* (1998) 197.
27. K. Tucker, T. Kelly, P. Delgrazia, C. R. Thomas, *Biotechnol. Progr. 8* (1992) 353.
28. M. Papagianni, *Ph.D Thesis*, University of Strathclyde, Glasgow, Scotland (1995).

Morfologija *Aspergillus niger* i proizvodnja limunske kiseline submerznom šaržnom fermentacijom
Utjecaj *pH* te koncentracije fosfata i mangana u podlozi

Sažetak

Proučavan je utjecaj pH te koncentracije fosfata i mangana u podlozi na morfologiju A. niger i proizvodnju limunske kiseline submerznom šaržnom fermentacijom pri različitim brzinama miješanja. Nađeno je da ti faktori bitno utječu na razvoj morfoloških oblika i proces proizvodnje. U svim slučajevima morfologija A. niger neposredno utječe na proizvodnju limunske kiseline i bez obzira na ispitivane parametre, dobiveni su slični morfološki oblici.

Kada je A. niger u obliku rahlih grudica s malom središnjom jezgrom, okružen difuznom masom vlakanaca, uvijek se postiže manja proizvodnja limunske kiseline.