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MORPHOLOGY AND CITRIC ACID PRODUCTION OF *ASPERGILLUS NIGER PM 1*

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SUMMARY

Aspergillus niger PM 1 was grown in a tubular loop and a stirred tank bioreactor. Batch fermentations were performed under various agitation conditions and pH. Citric acid, oxalic acid, extracellular polysaccharides and proteins were assayed. The following morphological parameters were measured: mean perimeter of clumps, mean perimeter of the central core of clumps, mean length of filaments and mean diameter of filaments. Citric acid production and morphology in both reactors were dependent on agitation intensity and pH. The length of the filaments was shown to be the only parameter that could be related to citric acid production in both reactors : the shorter the filaments the more citric acid was produced. However, for the same amount of citric acid produced the morphology of the organism grown in the stirred tank differed considerably from that grown in the loop reactor.

INTRODUCTION

In submerged culture the morphology of filamentous organisms varies between pellets and free filaments depending on culture conditions and the genotype of the strain. Several studies have shown that mechanical forces influence the morphology of filamentous microorganisms and the overall process productivities. Much experimental work concerning the influence of mechanical forces on the morphology is done with filamentous organisms because of their wide use in industrial fermentations and many attempts have been made to relate morphology and differentiation to product synthesis. Metz and co-workers (1,2) in studies with a strain of *Penicillium chrysogenum* showed that the length of the mycelial particles decreased with increasing power input per unit mass in the reactor: agitation caused the hyphae to become shorter, thicker and more highly branched.

The effect of the stirrer speed on the growth and productivity of 3 different strains of *A.niger* was reported by Ujcova et al (3). Higher stirrer speeds resulted in thicker

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and more densely branched filaments. Citric acid production was highly dependent on the agitation intensity.

Gomez et al (4) presented results of *A.niger 110* morphology and citric acid yield as a function of the stirring conditions. At higher stirrer speeds the form of small, compact pellets predominated while at lower speeds a mixture of free filaments and loose pellets existed.

No direct link between morphology and productivity was reported by Belmar-Beiny and Thomas (5). Studying the effect of stirrer speed on *S.clavuligerus ATCC 20764* they found that it was possible to obtain the same biomass and clavulanic acid production with different morphologies. Morphology was characterized by the semi-automatic method described by Packer and Thomas (6).

The aim of the present study is to determine the effect of the agitation intensity and pH on morphology and citric acid production of *A.niger* and to compare the results from the two different bioreactors.

MATERIALS &METHODS

The microorganism used was *Aspergillus niger PM 1.* The medium composition was as follows : D-Glucose, 150 g/l; $CaCl_2.6H_2O$, 0.252 g/l; NH_4NO_3 ,2.5 g/l; $MgSO_4.7H_2O$,0.25 g/l; $ZnSO_4.7H_2O$,4.5 mg/l; KCl ,0.428 g/l; KH_2PO_4 ,0.1 g/l; $FeSO_4.7H_2O$, 0.75 mg/l.

The bioreactors used were a 6 l loop reactor (TLR)-(APV Chemical Machinery ltd-working vol.5 l) and a 10 l stirred tank reactor (STR)-(BIOSTAT, ED-ES 10 Braun Biotech.International- working vol. 8 l). The air flow rate was 1 vvm and the temperature 28^{o}C in both fermenters. The pH was controlled by addition of 3M NaOH.

The fermenter inoculum was 250 ml for the TLR and 300 ml for the STR of a 40 h old shake flask culture.

EXPERIMENTS: TLR fermentations were performed without pH control at the following circulation times (tcir):18, 14, 10.8, 8, 7.1, 6, and 4.5 sec. The pH at inoculation time was about 3 and dropped to 2.1-1.8 after 168 h of fermentation. One experiment on the TLR at tcir 18 sec, was also performed with pH controlled at 4.

STR fermentations were performed at pH 2.1 and at the following stirrer speeds: 100, 200, 300, 500 and 600 rpm, and at pH 4 with the following stirrer speeds: 100, 300, 600 rpm. The pH was not controlled in one run in the STR at 500 rpm.

Fermentations lasted 168 h in both fermenters.

ANALYTICAL TECHNIQUES: The citric acid concentration was determined by the method of Marier and Boulet(7). The glucose and oxalic acid concentrations were assayed using the UV-Methods for the determination of D-Glucose and Oxalate respectively by Boehringer. Extracellular polysaccharides and proteins were determined using the method of precipitation with alcohol.

MORPHOLOGY: The inoculum culture consisted of free(not entangled) mycelial particles. These particles turned into clumps within the first 24 h of fermentation in both fermenters. The bulk of the mycelium was in the form of clumps in all fermentations. Morphology was characterized using an automatic image analysis method (software Aequitas, Dynamic Data Links).

The perimeter of the clumps (P1), the perimeter of the core of the clumps (P2), the length (l) of the filaments and their diameter (d) were the morphological parameters measured and their mean values were estimated.

The perimeters of the clumps were measured by joining the tips of the filaments that arouse from the core of the clump for 15-20 mycelial particles per sample. For the estimation of P2, lines were drawn around the core of the clump and their length was measured. For the estimation of l, the total length of the filaments and their branches that arouse from the core was measured. Because of the high degree of entanglement it was impossible to distinguish the main filaments from their branches. Thus,the parameter l indicates also the degree of branching.The diameter d was measured by joining two opposite points on the hyphal wall and estimating the interpoint distance. The filaments were selected randomly and were always in the periphery of the clumps. For each sample the process was repeated at least 60 times using new positions on the same and on different filaments.

RESULTS & DISCUSSION

EFFECT OF AGITATION INTENSITY: By decreasing the circulation time in the TLR from 18 to 4.5 sec the citric acid production increased as well as the diameter d of the filaments while the perimeter P1 and the length l decreased. The same trend of results was obtained from the STR fermentations. Figures 1,2,3,4,5 and 6 show the effect of agitation on citric acid production and morphology in both reactors.

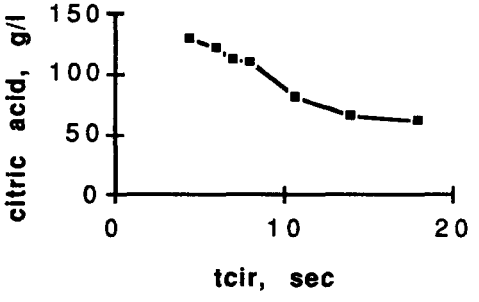

Fig.1: Loop Reactor: citric acid vs circulation time at uncontrolled pH

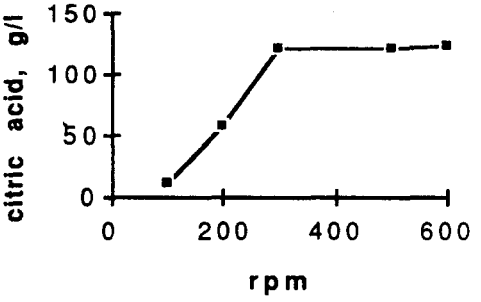

Fig.2: Stirred Tank Reactor: citric acid vs rpm at pH 2.1

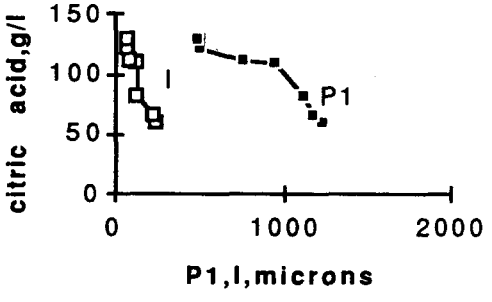

Fig3: Loop Reactor: citric acid vs clump perimeter P1 and filament length l at uncontrolled pH

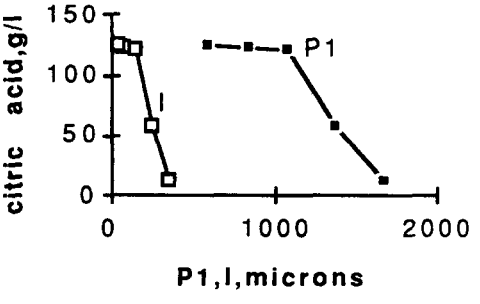

Fig.4: Stirred Tank Reactor: citric acid vs clump perimeter P1 and filament length l at pH 2.1

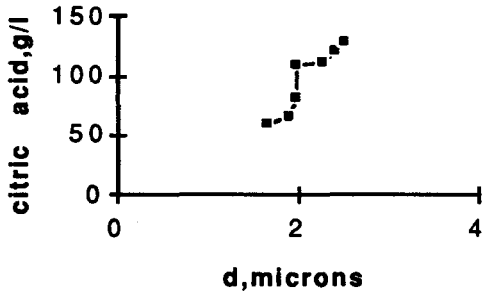

Fig.5: Loop Reactor: citric acid vs filament diameter d at uncontrolled pH

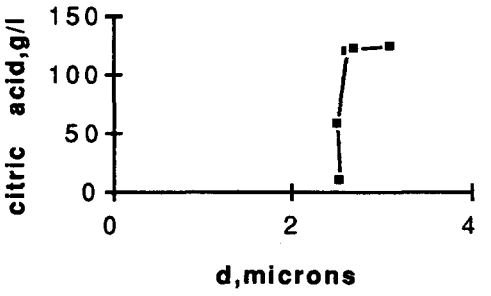

Fig.6: Stirred Tank Reactor: citric acid vs filament diameter d at pH 2.1

In the TLR, citric acid production appears to increase sharply with the hyphal diameter. In the STR this relationship is not as "clear". The very different citric acid concentrations obtained with the same hyphal diameter indicate that there are certain factors which influence the fermentation more strongly. These results are in agreement with those reported by Metz et al (1,2) for P.chrysogenum and by Gomez et al(4) for A.niger.

Intensive agitation conditions transformed the filaments from long, thin and almost unbranched to short and thick with many branches. The volume of the individual filaments changed significantly: the length reduced by a factor of 3 while the thickness increased by a factor of 1.5. Another factor that contributed to the morphological changes is the mycelial fragmentation that took place at high agitation intensity in both fermenters and resulted in the presence of free filaments in the broth. These free filaments gave rise to new, smaller clumps. This cycle of fragmentation and regrowth kept the average size of clumps small. Damage to mycelium due to high agitation intensity was observed by many investigators (1,2,5).

EFFECT OF pH: In both reactors higher pH led to lower citric acid production and the formation of oxalic acid, extracellular polysaccharides and proteins while all morphology parameters increased. The effect of the pH of the fermentation medium is shown on the following tables:

Table 1: TLR- Effect of pH on morphology and product synthesis

tcir	pH	citric acid	pol+pro *	oxalic acid	P1	l	d
sec		g/l	g/l	g/l	microns	microns	microns
18	2.1	60.52	1.9	0.1	1238.4	242.21	1.65
18	4.5	11.73	33.6	24.8	2216.26	265.1	2.54

Table 2: STR- Effect of pH on morphology and product synthesis

rpm	pH	citric acid	pol+pro *	oxalic acid	P1	l	d
		g/l	g/l	g/l	microns	microns	microns
300	2.1	121.19	1.83	0.1	1080	145.7	2.6
300	4	96	19.6	27.8	1649.6	194.34	2.13
500	1.58	64.96	0.2	0.1	375.66	44.48	2.7
500	2.1	122.4	1.1	0.1	591	69.7	2.66

* polysaccharides & proteins

At the higher stirrer speed the different citric acid levels obtained at the same hyphal diameter confirm the view that the latter is not a determining factor for citric acid production. The very low pH in the STR at 500 rpm resulted in irregular shaped filaments and swollen branching points. Swollen cells were not observed when the same run was performed at pH 2.

In figures 7 and 8 citric acid is plotted against P1, P2 and l.

Results from fermentations performed in STR at pH 2.1 and in the TLR were plotted together to show if there is a direct link between morphology and citric acid production irrespective of the bioreactor.

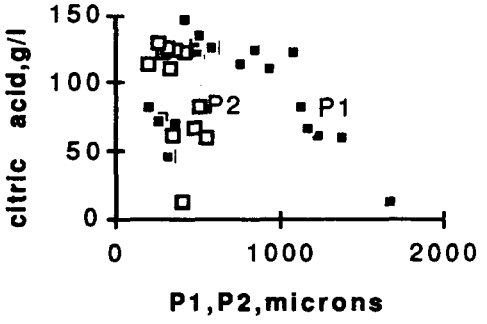

Fig.7: The influence of clump perimeter and clump core perimeter on citric acid production in the TLR and the STR

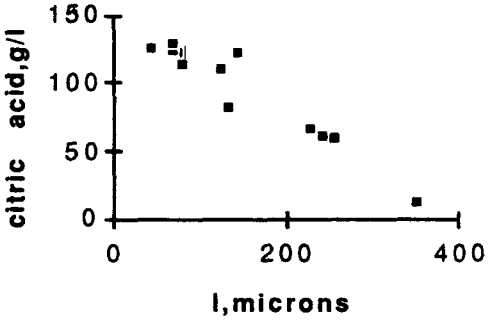

Fig.8: The influence of hyphal length on citric acid production in the TLR and the STR

Figures 7 and 8 show that only P1 and l were proportional to citric acid production. P2 did not relate to citric acid production. The diameter of filaments did not relate to product synthesis since filaments of the same diameter produced very different amounts of citric acid. But since the length of filaments l influenced P1,it is the only parameter that could relate to citric acid production. These results show that morphological changes of *A.niger PM 1*, unlike *S.clavuligerus* in the work of Belmar-Beiny and Thomas (5), were accompanied by changes in productivity and l can be used

as a direct indication of citric acid production. However, as figures 3,4 and 8 show, for the same amount of citric acid produced the morphology of the microorganism grown in the stirred tank differed from that grown in the loop reactor: for 120g/l citric acid produced in both reactors the mean length of filaments was 74 microns in the loop and 145.7 microns in the stirred tank reactor.

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