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Effects of Nitrogen Sources on Cell Growth and Production of Nystatin by *Streptomyces noursei*

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Cell growth and production of nystatin by *Streptomyces noursei* (ATCC 11455) were investigated on the three different nitrogen sources, ammonium sulphate, ammonium nitrate and sodium nitrate. *S. noursei* was able to utilise all of the three tested nitrogen sources for the growth and production of nystatin. High ammonium concentration had a negative effect on production of nystatin when phosphate and glucose was in excess. There was an increased production of nystatin when the cultures became ammonium limited. Cultivation with sodium nitrate as the nitrogen source resulted in a prolonged lag-phase for growth and about 50% lower final nystatin titres compared with cultures grown on nitrogen sources containing ammonium. Nystatin production was shown to be related to the specific growth rate, its production was increased at decreasing specific growth rates.

Nystatin is an industrially important secondary metabolite produced by *Streptomyces noursei* with a world market of more than 250 million US dollars per year. Nystatin belongs to the polyene macrolide antibiotics, which is a subgroup of the polyketides. It has a lactone ring of 37 carbon atoms which consists of a diene-tetraene chromophore. The aminosugar mycosamine is linked to the lactone ring[1]. Nystatin antifungal's activity is expressed through its binding to sterols in cell membranes that affect the permeability and in turn leads to leakage of intracellular compounds[2].

Microbial secondary metabolites are usually produced only at low specific growth rates[3]. Specific growth rate is important in controlling the onset of secondary metabolism, but the mechanisms involved are unknown. Deficiencies in certain nutritional factors are important as well, and many secondary metabolic pathways are negatively affected by nitrogen sources favourable for growth[4]. *Streptomyces venezuelae* grown on a medium containing ammonium and nitrate showed a delayed production of chloramphenicol until ammonium had been consumed, and the growth remained slow until subsequent depletion of nitrate[5]. There have been no reports on the possible nitrogen regulation of the production of nystatin or aminosugars of polyenes.

S. noursei JA 3890b is a spontaneous mutant of *S. noursei* ATCC 11455 and produces a different type of antibiotic, nourseothricin (streptothricin)[6]. This strain was found to have a strong repression of the $NADP^+$-dependent glutamate dehydrogenase and enhanced the capacity to oxidative deamination of alanine *via* the NAD^+-dependent alanine dehydrogenase during excess of ammonium and alanine, where low nourseothricin titres were obtained. A decrease in the intracellular level of ammonium caused by ammonium limitation increased the activity of $NADP^+$-dependent glutamate dehydrogenase and repressed the NAD^+-dependent alanine dehydrogenase, and this was associated with increased production of nourseothricin. *S. noursei* ATCC 11455 failed to demonstrate a similar response on $NADP^+$-dependent glutamate dehydrogenase and NAD^+-dependent alanine dehydrogenase during

Table 1. Media for production fermentation.

Medium / Ingredients	F-NH_4NO_3	F-$NaNO_3$	F-$(NH_4)_2SO_4$	F-$(NH_4)_2SO_4$+(i) F-$(NH_4)_2SO_4$+(ii)
Glucose	45.0 g/L	45.0 g/L	45.0 g/L	45.0 g/L
NH_4NO_3	5.00 g/L	-	-	-
$NaNO_3$	-	10.62 g/L	-	-
$(NH_4)_2SO_4$	-	-	8.26 g/L	10.00 g/L
KH2PO4	1.50 g/L	1.50 g/L	1.50 g/L	1.50 g/L
$MgSO_4 \cdot 7\,H_2O$	1.00 g/L	1.00 g/L	1.00 g/L	1.00 g/L
TMS-1[a]	3.0 ml/L	3.0 ml/L	3.0 ml/L	3.0 ml/L
Pluronic F68	0.5 ml/L	0.5 ml/L	0.5 ml/L	0.5 ml/L

[a] The trace metal solution (TMS-1) contained the following ingredients: $FeSO_4 \cdot 7\,H_2O$ (5.0 g/L), $CuSO_4 \cdot 5\,H_2O$ (390 mg/L), $ZnSO_4 \cdot 7\,H_2O$ (440 mg/L), $MnSO_4 \cdot H_2O$ (150 mg/l), $NaMoO_4 \cdot 2\,H_2O$ (10 mg/L), $CoCl_2 \cdot 2\,H_2O$ (20 mg/L) and HCl (37%) (50 ml/L).

changes of the nitrogen source environment[7].

Nitrogen sources influence the biosynthesis of antibiotics differently for several *Streptomyces* spp. It is important to consider the impact of nitrogen sources in order to develop and improve processes for antibiotic production. This study examines cell growth and production of nystatin by *S. noursei* ATCC 11455 grown on three different industrially relevant nitrogen sources, with a special focus on how the specific growth rate and the nitrogen source affect the biosynthesis of nystatin.

Materials and Methods

Microorganism

Streptomyces noursei ATCC 11455 was obtained from American Type Culture Collection. The culture was inoculated into 50 ml medium containing 37.0 g/liter Tryptic Soy Broth (Difco Lab.) and 7.0 g/liter glucose, and incubated in a 300 ml shake flask with baffles for 30 hours at 30°C and 150 rpm. To this culture 15% (v/v) glycerol was added and kept frozen at −80°C. The frozen stock culture suspensions were used as an inoculum for the seed cultures of fermentation experiments.

Fermentations

The seed culture was prepared by inoculation of 1% (v/v) glycerol culture into 50 ml of the seed medium FD-1. The seed cultures were incubated in 300 ml shake flasks with baffles and 50 glass beads (diameter: 3 mm) for 25 hours at 30°C and 250 rpm. FD-1 is a chemically defined medium containing glucose (10 g/liter), NH_4NO_3 (2.5 g/liter), KH_2PO_4 (2.0 g/liter), $MgSO_4 \cdot 7H_2O$ (1.0 g/liter), $CaCO_3$ (5.0 g/liter) and TMS-1 (trace metal solution) (3 ml/liter). Glucose and TMS-1 were added after sterilisation and the medium was pH adjusted to 7.0 aseptically. TMS-1 included $FeSO_4 \cdot 7H_2O$ (5.0 g/liter), $CuSO_4 \cdot 5H_2O$ (390 mg/liter), $ZnSO_4 \cdot 7H_2O$ (440 mg/liter), $MnSO_4 \cdot H_2O$ (150 mg/liter), $NaMoO_4 \cdot 2H_2O$ (10 mg/liter), $CoCl_2 \cdot 2H_2O$ (20 mg/liter) and 37% HCl (50 ml/liter).

Production fermentations were carried out in bioreactors of 1liter working volume (Biostat B, B Braun Biotech International). The media for the fermentations are given in Table 1. Glucose was added to the cultures 4 times daily during the last part of the fermentation to avoid glucose limitation and to maintain a glucose concentration between 1.0 and 10.0 g/liter. The bioreactors were inoculated with 50 ml seed culture. The pH of the cultures was controlled to 6.5 by addition of 2 M NaOH, and the dissolved oxygen tension was regulated to 40% air saturation by variation of the agitation speed. The temperature was kept at 28°C, and the aeration was at 1.0 v/v/minute.

Analysis

Dry weight of Biomass

For dry weight measurements 5 ml of the culture were filtered through a predried filter (Supor-450, 0.20 μm, Gelman Lab.), washed with 10 ml distilled water and dried to constant weight at 105°C.

Glucose and Nitrate

Glucose and nitrate were measured on an automatic analyser (Cobas Miras Plus, Roche Diagnostic Systems) using standard kits from Roche (Unimate 7 GLUC GDH)

and Boehringer Mannheim (Nitrate).

Ammonium

The concentration of ammonium was determined using ion selective ammonia electrode (Metrohm Ltd.) with ammonium chloride as standard.

Glycogen

Intracellular glycogen was determined from 2 ml of the culture which was centrifuged (10,000×*g*, 5 minutes, 0°C) and washed 4 times with 2 ml 0.9% (w/v) NaCl. The pellets were resuspended in 1 ml 40 mM $NaCH_3CO_2$ (pH 4.8). The samples were immersed in boiling water for 5 minutes and then cooled on ice. One ml of glass beads (diameter: 0.25~0.50 mm) were added to the samples. The cells were then disrupted in a cell disrupter (FP120 Fastprep Cell Disrupter, Savant Instruments) 15 times for 30 seconds each at a speed of 6.5, where the samples were cooled on ice between the runs. The samples were centrifuged (20,000×*g*, 20 minutes, 0°C). 0.75 ml of 2.3 U/ml amyloglycosidase (2200 U/g, Sigma) in 40 mM $NaCH_3CO_2$ (pH 4.8) was added to 0.25 ml supernatant and incubated for 3 hours at 37°C. The concentration of glucose in samples as well as the background was measured by the glucose method previously mentioned.

Nystatin

For HPLC measurements of nystatin a Hewlett Packard instrument (series 1100) with a reversed phase Waters Symmetry column (C-18, 4 μm particles, 4.6×150 mm) and a UV detector (295 nm) was used. The column was kept at 25°C. An Isocratic method was used for the mobile phase at a flow rate of 1.0 ml/minute. The eluent consisted of 0.05 M ammonium acetate (pH 3.8) mixed with acetonitrile in a ratio of 3:2. The injection volume of samples and standards (4670 USP units/mg, Sigma) was 20 μl, and the run time was 10 minutes.

Carbon Dioxide in Exhaust Gas

The partial pressure of CO_2 in the exhaust gas from the bioreactors was measured using a gas analyser (BINOS 100-2M, Rosemount GmbH.).

Results

Cell Growth on Different Nitrogen Sources

S. noursei was able to grow with ammonium nitrate, ammonium sulphate and sodium nitrate respectively as the sole nitrogen source, and growth curves are shown in Fig. 1.

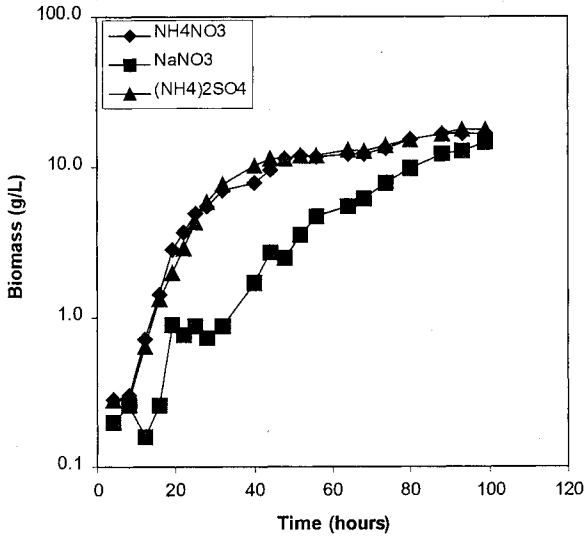


Fig. 1. Cell growth in F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$ with different nitrogen sources.

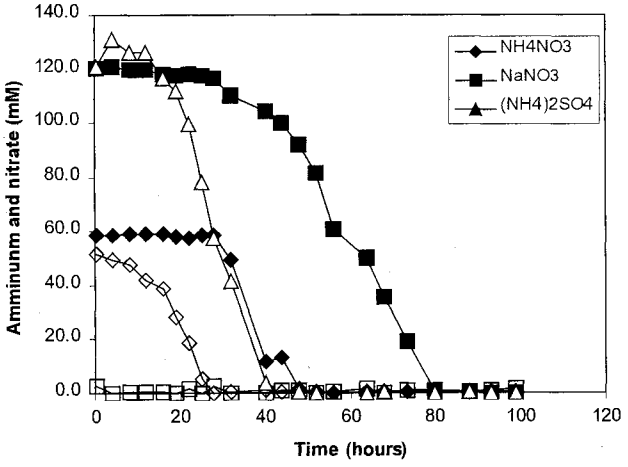
Fig. 1. Respiratory activity by measurements of CO_2 in the exhaust gas (data not shown) corresponded very well to the dry weight measurements of the biomass and verified the estimated specific growth rates. F-NH_4NO_3 and F-$(NH_4)_2SO_4$ followed approximately the same growth pattern. After a lag-phase of 8 hours the cells in these two media entered an exponential growth phase with a specific growth rate of 0.20 h^{-1}. Transition to lower specific growth rates occurred after approximately 25 hours. After 44~48 hours the cultures entered a phase with low specific growth rates in the range of 0.008~0.01 h^{-1} calculated from the biomass measurements, which lasted throughout the remainder of the fermentations. The respiratory activities dropped after 52~56 hours.

Growth on sodium nitrate as the nitrogen source caused a longer lag-phase compared to that on the two other nitrogen sources, and exponential growth did not occur until after 30 hours cultivation. The maximum specific growth rate was only 1/3 of the maximum specific growth rate of F-NH_4NO_3 and F-$(NH_4)_2SO_4$, but F-$NaNO_3$ continued with approximately the same specific growth rate longer before transition to a decreased specific growth rate. During the last 40 hours of the fermentation course the biomass concentration in F-$NaNO_3$ increased twice as much as with the other two nitrogen sources. The respiratory activity in F-$NaNO_3$ remained almost constant from 56 hours throughout the fermentation.

The concentration profiles of ammonium and nitrate during the fermentations are given in Fig. 2. Ammonium was a preferred nitrogen source in F-NH_4NO_3 and was consumed first. Consumption of nitrate in this culture started when all of ammonium was depleted in the medium. Ammonium in F-$(NH_4)_2SO_4$ was depleted after 40 hours, and a large decrease in the specific growth rate was observed 4 hours later. Consumption of nitrate in F-$NaNO_3$ started after a lag-phase in cell growth, and all the nitrate was consumed after 80 hours. Less than 1 mM of ammonium was detected in the fermentation medium during growth on nitrate as the sole nitrogen source. The specific rates of uptake of ammonium and nitrate were calculated during the phases of linear consumption of the nitrogen sources (Table 2). The specific uptake rate of ammonium was approximately twice that of nitrate.

Fig. 2. Concentration of ammonium and nitrate in F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$.

Open symbols represent ammonium and closed symbols nitrate.



Accumulation of Glycogen

Streptomyces spp. are known to accumulate storage compounds such as glycogen, polyhydroxybutyrate and lipids[8,9]. Here glycogen was measured as a representative of the formed storage compounds. Intracellular glycogen accumulation during F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$ are shown in Fig. 3. Accumulation of glycogen differed between the cultures, in a range of 0.3 to 6.9 mg glycogen per dry weight biomass at the end of the fermentations. The highest concentrations of glycogen were found in F-NH_4NO_3 and F-$(NH_4)_2SO_4$, and the intracellular glycogen concentration increased after the cultures became limited by their nitrogen sources. The increase of glycogen accumulation continued during the course of nitrogen limitation. In contrast, no increase in glycogen accumulation was observed when F-$NaNO_3$ became limited by nitrate.

Nystatin Production and Effect of Ammonium

Production curves of nystatin (Fig. 4) showed that the biosynthesis of nystatin took place with all the three tested nitrogen sources. Production of nystatin was initiated during the exponential growth phases. As with the specific growth rate, production of nystatin has an exponential production rate, which remains constant for about 20 hours (log plot is not shown). The linear plot of nystatin production clearly shows that there is a distinct increase in production rates after 28~40 hours of fermentation. The production rate of nystatin in F-$(NH_4)_2SO_4$ increased after 40 hours, which coincides with the time when the culture became ammonium limited. The same phenomenon occurred in F-NH_4NO_3 when all the ammonium was consumed after 28 hours, even though nitrate still remained in the medium. Production of nystatin on nitrate in F-$NaNO_3$ showed an increased production rate after 40 hours while the concentration of nitrate in the medium was above 100 mM. Measurements of glucose and phosphate from the

Table 2. Specific uptake rates (moles/moles·h) of ammonium and nitrate in F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$

Fermentation	F-NH_4NO_3	F-$NaNO_3$	F-$(NH_4)_2SO_4$
$q_{s,\ ammonium}$	0.0346	-	0.0337
$q_{s,\ nitrate}$	0.0167	0.0162	-

Fig. 3. Intracellular concentration of glycogen in F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$ per dry weight cells.

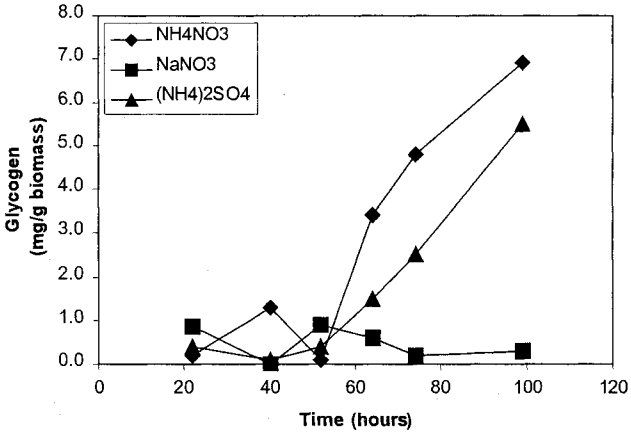


Fig. 4. Production of nystatin in F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$.

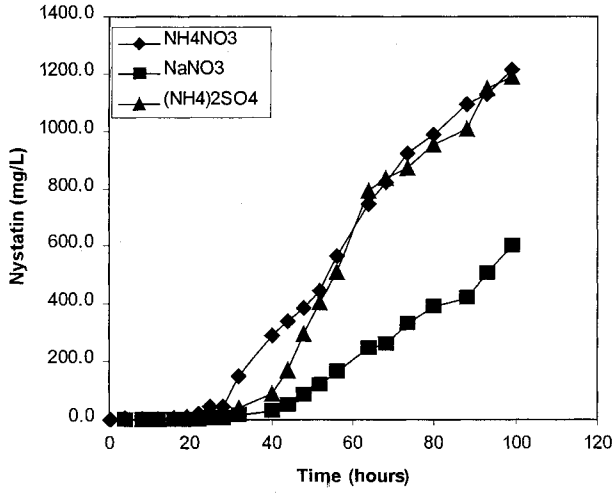


Table 3. Utilisation of the nitrogen sources, specific growth rates, μ (h^{-1}), specific production of nystatin, r_p (C-mmoles/C-moles·h) and comments on effects on nystatin yields on biomass during different phases of the fermentations.

Phase	Time (h)		Fermentation cultures F-NH_4NO_3	F-$NaNO_3$	F-$(NH_4)_2SO_4$	Comments
P1	16-25	N-source	NH_4^+	Lag phase	NH_4^+	$Y_{xp,\ F\text{-}NH4NO3} = 3.5 \cdot Y_{xp,\ F\text{-}(NH4)2SO4}$
		μ	0.138	-	0.098	F-$(NH_4)_2SO_4$: High NH_4^+concentration
		r_p	1.93	-	0.39	
P2	28-40	N-source	NO_3^-	NO_3^-	NH_4^+	$Y_{xp,\ F\text{-}NH4NO3}$ increased 10 fold from P1 to P2
		μ	0.031	0.070	0.045	$Y_{xp,\ F\text{-}(NH4)2SO4}$ increased 5.5 fold from P1 to P2
		r_p	4.28	2.38	0.99	$Y_{xp,\ F\text{-}NH4NO3} = 6.3 \cdot Y_{xp,\ F\text{-}(NH4)2SO4}$
						$Y_{xp,\ F\text{-}NaNO3} = 1.5 \cdot Y_{xp,\ F\text{-}(NH4)2SO4}$
P3	52-80	N-source	Nitrogen limitation	NO_3^-	Nitrogen limitation	$Y_{xp,\ F\text{-}(NH4)2SO4}$ increased 10 fold from P2 to P3
						$Y_{xp,\ F\text{-}NH4NO3} \approx Y_{xp,\ F\text{-}(NH4)2SO4}$
		μ	0.009	0.036	0.009	
		r_p	1.87	2.12	1.96	
P4	80-99	N-source	Nitrogen limitation	Nitrogen limitation	Nitrogen limitation	No increase of $Y_{xp,\ F\text{-}NaNO3}$ from P3 to P4
		μ	0.004	0.022	0.008	
		r_p	0.90	1.21	0.99	

media demonstrated that limitation by these nutrient components did not occur (data not shown).

Production of nystatin proceeded throughout the entire fermentations. In the last 20 hours of the processes the specific production rates in F-$(NH_4)_2SO_4$ and F-NH_4NO_3 dropped more than 3 and 6 folds respectively, from the highest production rates soon after ammonium limitation. F-$NaNO_3$ decreased about 2 fold in the specific production rate of nystatin when the culture became nitrogen limited during the last 20 hours of the fermentation.

The time courses of the fermentations were divided into four phases according to the utilisation of nitrogen components and the achievement of nitrogen limitations in the cultures. Data for cell growth, nystatin production and

Fig. 5. Nystatin yields on biomass (Yxp) in F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$ at different phases of the time courses.

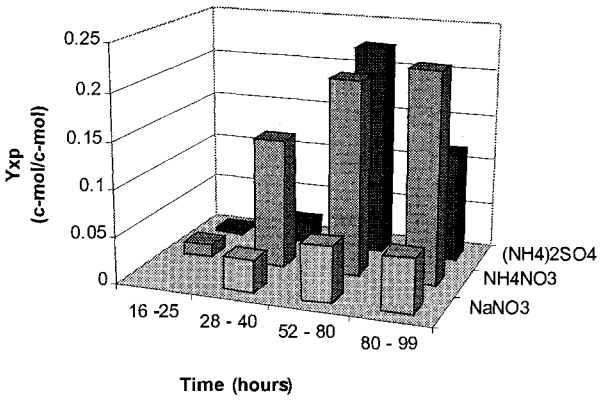
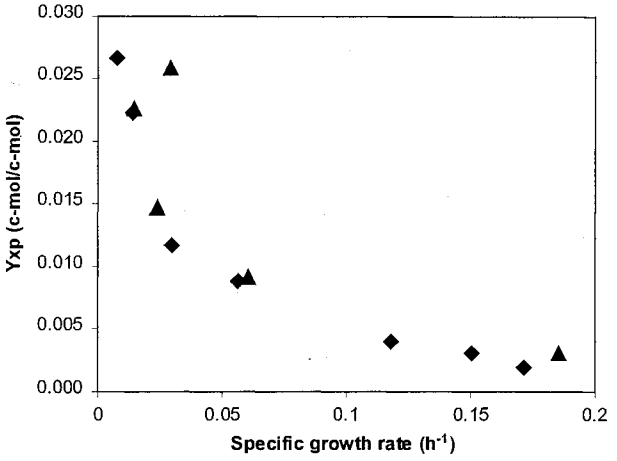


Fig. 6. Nystatin yields on biomass versus specific growth rates in F-$(NH_4)_2SO_4$+(i) (◆) and F-$(NH_4)_2SO_4$+(ii) (▲) before ammonium limitation occurred.



utilisation of nitrogen sources for each of the phases of the cultures are summarised in Table 3. A graphic illustration of nystatin yields from biomass in four phases is given in Fig. 5 (the yield is given as the specific productivity, r_p, divided by the specific growth rate, μ). F-NH_4NO_3 utilised ammonium for cell growth and nystatin production during phase P1 and obtained a higher nystatin yield than F-$(NH_4)_2SO_4$, which contained a considerable higher concentration of ammonium in the medium. The switch from ammonium to nitrate as the nitrogen source from phase P1 to P2 in F-NH_4NO_3 resulted in a 10 fold increase of nystatin yield. The nystatin yield was 6.3 fold higher in F-NH_4NO_3 than in F-$(NH_4)_2SO_4$. F-$(NH_4)_2SO_4$ and F-NH_4NO_3 were limited by their nitrogen sources in phase P3. Ammonium limitation in F-$(NH_4)_2SO_4$ increased the nystatin yield 10 fold compared to the ammonium excess. This increase was in the same range as when F-NH_4NO_3 became ammonium limited in P2. F-$NaNO_3$ was depleted by nitrate in P4, but the depletion did not change the nystatin yield compared to the previous phase where nitrate was in excess.

Impact of Specific Growth Rate *versus* Ammonium for Nystatin Production

Relations between cell growth and nystatin production are shown in Table 3, where specific growth rate and specific production of nystatin yields are given for the four phases in F-NH_4NO_3, F-$NaNO_3$ and F-$(NH_4)_2SO_4$. High nystatin production was obtained in the cultures at low specific growth rates. However, the highest production of nystatin occurred when the cultures grew at ammonium limitation. It was difficult to conclude whether the high nystatin production reflected the low specific growth rate or the ammonium limitation since the fermentation times with low specific growth rate and ammonium excess were too short.

In order to distinguish between the effects of low specific growth rate and ammonium limitation on nystatin production, two fermentations were carried out, F-$(NH_4)_2SO_4$+(i) and F-$(NH_4)_2SO_4$+(ii), where the concentration of ammonium sulphate was increased by 20% compared with F-$(NH_4)_2SO_4$. The two fermentations were cultured in similar medium and under the same culture conditions. The increased concentration of ammonium resulted in a more than 20 hours delay of ammonium limitation compared to the time when cell growth reached low specific growth rates (less than 0.024 h^{-1}). The nystatin yields versus specific growth rates for different phases with ammonium excess during the fermentation in F-$(NH_4)_2SO_4$+(i) and F-$(NH_4)_2SO_4$+(ii) are shown in Fig. 6. A correlation appeared between specific growth rates and nystatin production; there are increasing nystatin yields when the specific growth rates decrease. The cultures F-$(NH_4)_2SO_4$+(i) and F-$(NH_4)_2SO_4$+(ii) were depleted by ammonium after 77 hours and 75 hours respectively. The nystatin yields increased more than 10 fold after ammonium limitation,

and the average nystatin yields for the next 30 hours of fermentation were 0.33 C-moles nystatin per C-moles biomass in F-$(NH_4)_2SO_4$+(i) and 0.26 C-moles nystatin per C-moles biomass in F-$(NH_4)_2SO_4$+(ii).

Discussion

Cell growth by *S. noursei* on media containing ammonium nitrate and ammonium sulphate based on biomass measurements showed two distinct exponential growth phases separated by an approximately 20 hours transition phase. Two-phase exponential growth has earlier been observed in several other *Streptomyces* spp.[10~12] and other actinomycetes[13]. The respiratory activity in F-$(NH_4)_2SO_4$ and F-NH_4NO_3, measured by CO_2 from the exhaust gas, dropped during the last 40 hours of the growth phase indicated that cell growth with cell replication decreased or ended during this phase and that the increase in biomass reflected accumulation of storage compounds. Growth on nitrate as the only nitrogen source also revealed two exponential growth phases. However, no drop of respiratory activity occurred from CO_2 measurements, and the replicatory growth seems to remain constant during the second growth phase. Dry weight measurement without allowance for respiratory activity or DNA measurements is a poor criterion for cell growth. Non replicatory growth from accumulation of storage compounds may account for up to 50~60% of the dry weight at the end of fermentation[3].

In our study we have measured the accumulation of glycogen as a representative of storage compounds. Growth on ammonium sulphate and ammonium nitrate showed an increased intracellular glycogen accumulation when the cultures became nitrogen limited, but no increase in glycogen accumulation was detected when nitrate used as the only nitrogen source was depleted. Accumulation of glycogen has been found in *S. antibioticus* as well during growth on a solid medium in addition to accumulation of trehalose[14]. During growth in submerged culture of *S. venezuelae* accumulation of polyhydroxybutyrate, glycogen and lipids was observed, but no trehalose was detected in mycelium of vegetative cultures[9]. Polyhydroxybutyrate was accumulated during the growth phase but disappeared from the mycelium when the cultures entered the stationary phase. Glycogen was on the other hand, similar to our results, accumulated during the stationary phase after nitrogen depletion. In our study 30~40% of the biomass was obtained after nitrogen limitation in the cultures grown on ammonium sulphate and ammonium nitrate. Accumulation of glycogen contributed to only 1.5~2.5% of the biomass increase after nitrogen limitation, and therefore other storage polymers in addition to glycogen must be responsible for the increase of biomass. These could be neutral lipids like triacylglycerol, which are found intracellularly in *S. lividans*[15].

S. noursei had a more rapid assimilation of ammonium than nitrate. The specific uptake rate of ammonium was twice as high as for nitrate and the rates were the same whether ammonium was added alone or together with nitrate. The calculated specific uptake rate for ammonium was 23% higher than that found in *S. fradiae* with an initial ammonium sulphate addition of 100 mM ammonium[16]. A lower uptake rate for nitrate than ammonium may be due to a requirement of NAD(P)H for the reduction of nitrate to ammonium by nitrate reductase and nitrite reductase. A limited supply NAD(P)H, which is necessary for biosynthesis of nystatin, will reduce the rate of the reduction step from nitrate to ammonium. Measurements of less than 1 mM extracellular ammonium in the cultures that utilise nitrate support this hypothesis. The culture grown on ammonium nitrate showed sequential utilisation of ammonium and nitrate, with consumption of nitrate being initiated when ammonium was depleted in the culture.

Nitrogenous compounds may affect the biosynthesis of antibiotics directly at the level of secondary metabolism, either through their availability as substrates for "antibiotic synthetases" or through modulation of the biosynthesis/activity/stability of these enzymes. Indirect control of biosynthesis of antibiotics may also be exerted "upstream" from secondary metabolism *via* nitrogen regulation of primary metabolism supplying non-nitrogenous precursors of secondary metabolites[17]. Our results suggest that the concentration of ammonium in the medium affects production of nystatin in *S. noursei* when glucose and phosphate were in excess. The nystatin yield in phase P1 (16~25 hours) was 3.5 fold higher in F-NH_4NO_3 than in F-$(NH_4)_2SO_4$ where the concentration of ammonium was more than double. A similar effect was observed in *S. ambofaciens*. Here there was a 60% reduction of spiramycin biosynthesis when the ammonium concentration was increased from 50 to 100 mM[18]. A positive effect of ammonium limitation in *S. noursei* was observed in F-NH_4NO_3 when ammonium was exhausted and the consumption of nitrate took place, which increased the nystatin yield by 10 times. This positive effect was further observed by ammonium limitation in F-$(NH_4)_2SO_4$ that resulted in a 10 times increase of the nystatin yield. Corresponding observations have been made in other *Streptomyces* spp. *S. ambofaciens* grown on ammonium

and nitrate showed a phase of slow growth rate when ammonium was exhausted and the consumption of nitrate began, which led to preceded spiramycin production[19]. Addition of ammonium to a culture of *S. venezuelae* grown on nitrate caused a decrease in the rate of chloramphenicol synthesis, both during and after ammonium consumption[5]. A negative effect of ammonium on production of secondary metabolites has been demonstrated for several other *Streptomyces* spp. such as *S. arenae*[20], *S. avermitilis*[21], *S. clavuligerus*[22~24], *S. flocculus*[25], *S. fradiae*[16,26,27] and *S. lincolnensis*[28]. Other studies showed that ammonium slightly stimulated β-lactam antibiotic production by *S. clavuligerus* under restricted air supply[29] and that maximum yields of avermectin by *S. avermitilis* were achieved with ammonium as the nitrogen source[30].

The control of specific growth rate appears to be important in secondary metabolism and may be the overriding factor in the cases where the nutrient limitation is needed for production of secondary metabolites. Some *Streptomyces* spp. appear to be mainly controlled by specific growth rate but not by nutrient repression. In contrast, both a low specific growth rate and a particular type of nutrient deficiency are needed to support secondary metabolism in other cases[4]. F-NH_4NO_3 and F-$(NH_4)_2SO_4$ produced high titres of nystatin during ammonium limitation. The cultures did not clearly prove that whether ammonium limitation or low specific growth rate caused high nystatin production. An increase of the ammonium sulphate concentration of in the medium in F-$(NH_4)_2SO_4$+(i) and F-$(NH_4)_2SO_4$+(ii) made it possible to obtain phases when the cultures achieved low specific growth rate without ammonium limitation. The results demonstrated significantly higher nystatin yields brought about ammonium limitation in contrast to low specific growth rates. It appears that ammonium limitation may have positive effect on nystatin production either by derepression of genes involved in nystatin biosynthesis or by affecting some essential enzymes. Furthermore there is also a correlation between nystatin production and the specific growth rate; productivity increased at low specific growth rates.

The culture with nitrate as the only nitrogen source had a longer lag phase and a slower specific growth rate compared to when ammonium was supplied either alone or together with to nitrate. Initially during the idiophase, nitrate as the nitrogen source achieved a higher nystatin yield than when ammonium was present. However, when the cultures grown on ammonium became ammonium limited they obtained a large increase in nystatin yield. The final nystatin titres in the cultures with ammonium were doubled compared to those in the cultures where nitrate was used alone. From an industrial point of view it therefore seems important to have a nitrogen source containing ammonium for production of nystatin, but the ammonium concentration should be kept low if no other limitation of nutrients occur.

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