STUDIES ON SUPPORTING MEDIA FOR PROGESTERONE 11α-HYDROXYLATION WITH A PELLETED GROWTH FORM OF *RHIZOPUS NIGRICANS*[#]

Polona Žnidaršič, Katarina Uršič, Aleksander Pavko
Faculty of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva 5, SI-1000 Ljubljana, Slovenia

[#]This paper is dedicated to Professor Dr. Roman Modic at the occasion of his 90th anniversary.

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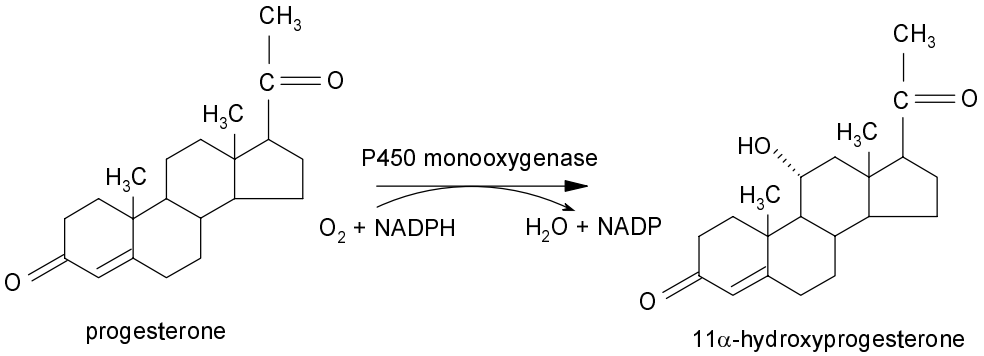
Abstract

Studies on supporting medium composition and its feed rate were performed in order to maintain stationary biomass concentration during the process of progesterone hydroxylation with *Rhizopus nigricans* pellets. Growth kinetic parameters were evaluated for two media with different nitrogen sources. The results indicate the benefit of using complex media at higher feed rates than those used for the maintenance of stationary biomass concentration without exogenous steroid addition.

Introduction

The filamentous fungus *Rhizopus nigricans* ATCC 6227b is a well-known hydroxylator of progesterone at position 11α (Scheme 1), which is one of the key steps in the production of therapeutically useful corticosteroid compounds.[1] The application of a pelleted growth form of this fungus is a promising alternative to the classical batch process with filamentous, freely growing hyphal elements, since it offers the possibility of biomass reuse and thereby continuous operation of the process.[2] Besides, the improved rheological properties of the broth and facilitated product removal lower the costs of bioprocesses employing mycelial pellets.[3]



progesterone

11α-hydroxyprogesterone

Scheme 1: 11α-hydroxylation of progesterone by filamentous fungus *Rhizopus nigricans.*

The maintenance of enzyme activity in living cells requires a combination of appropriate supporting medium composition and a lack of fluctuations in environmental conditions.[4] As the immobilization of *R. nigricans* cells in gel beads resulted in a rapid inactivation of the mycelium, it was suggested that for a long-term operation, maintenance of microbial slow growth, and consequently of general metabolism during biotransformation should be provided.[5]

Since 11α hydroxylase is a NADPH-dependent monooxygenase, it is indirectly regulated by glucose metabolism.[5] We have already shown that the presence of glucose in the transformation medium stimulates the rate of hydroxylation and the final conversion of progesterone with *R. nigricans* pellets in a batch process.[6] Here we report on further studies on medium composition and its feed rate in order to maintain microbial slow growth, giving stationary biomass concentration during the process of progesterone hydroxylation. For this purpose, the maintenance coefficients for two different supporting media were determined in simulated fed-batch experiments.

Materials and methods

Microorganism and growth conditions

Rhizopus nigricans ATCC 6227b, obtained from the National Institute of Chemistry, Ljubljana, Slovenia, was used throughout the experiments. Before inoculation of submerged cultures the fungus was maintained for 14 days on agar slants as previously described.[7] The pelleted growth form of *Rhizopus nigricans* was obtained in 500-mL Erlenmeyer flasks with 100 mL of growth medium 1 (GM1), containing (in g/L): glucose, 10; yeast extract, 2.85; soy flour, 3; NaCl, 4; K_2HPO_4, 1.98; Tween-80, 0.5; the pH adjusted to 5.5. After inoculation with $5 \cdot 10^4$ – $2.5 \cdot 10^5$ spores/L, the cultivation was carried out at 23 °C and 225 rpm for 36-46 h on a rotary shaker with 20 mm amplitude.

Growth in transformation mediums

Pellets from submerged cultures were filtered through gauze and washed for several times with phosphate buffer (0.75 mM Na_3PO_4; 0.21 mM EDTA; 0.04 mM glutation,

red.; pH 5.5). Portions of 3 g of wet mycelia were transferred into 250-mL Erlenmeyer flasks with 50 mL of transformation medium, consisting of the above mentioned buffer and different amounts of supporting media: 5 or 10 g/L of glucose, and 5, 10, 30, 50, 70 or 100 vol.% GM1 or GM2. Growth medium 2 (GM2) was identical to GM1 (see above) with the exception of nitrogen source, which was only yeast extract (5.85 g/L) with no soy flour present. Cultivation was performed in two parallels at 28 °C and 180 rpm for indicated time intervals.

The simulations of fed-batch cultivations were performed in 250-mL Erlenmeyer flasks with periodical addition of 1 mL of GM1 or GM2 to 50 mL of transformation medium with pellets. The initial medium consisted of 2.5 mL (5 vol.%) of GM1 or GM2 and 47.5 mL of phosphate buffer and 1 mL of the same growth media was aseptically added in 1 or 2-hours intervals as described in Results and Discussion.

Progesterone biotransformation

Progesterone, dissolved in *N,N*-DMF (0.1 g/mL) was added to the transformation medium with *R. nigricans* pellets to give the final concentration 0.3 g/L and cultivation proceeded as described above.

Culture analysis

At indicated time intervals the whole content of Erlenmeyer flasks was used for the analyses. Total sugar concentration, expressed as glucose equivalent, was followed spectrofotometrically on the basis of H_2SO_4 hydrolysis of culture filtrate and the reaction with anthrone reagent.[8] Total nitrogen content of biomass-free media was estimated by the Kjeldahl method. Mycelium analysis included standard dry weight determination and pellet size evaluation using image analysis.[2] Chloroform extraction of a known volume of culture filtrate and quantitative analysis of steroids by HPLC using acetonitrile-water gradient elution were described previously.[2]

Determination of growth kinetic parameters

The growth of *R. nigricans* pellets was followed in two growth media with different nitrogen sources (GM1 and GM2), diluted in several ratios with phosphate buffer and cultivated as described above. Maximal specific growth rate, μ_{max} (h^{-1}), was determined from the slope of the linear part of the curve, presenting $\ln(X/X_0)$ *vs*. t (h), where X_0 denotes the biomass concentration at t = 0. Y_{sx} was calculated as the absolute ratio between the biomass growth rate, dX/dt (g/L h) and the total sugar consumption rate, dS/dt (g/L h) in the phase of a constant growth rate. The maintenance coefficients for these two supporting media were determined in simulated fed-batch experiments at the stationary biomass concentration according to Esener *et al.,* 1981.[9]

Results and discussion

Smooth pellets of *R. nigricans*, obtained in shake-flasks cultures on the basis of our previous studies,[2,10] were used as an immobilized living cell system for progesterone biotransformation (Figure 1). Depending on inoculum size, the average pellet diameter varied between 1.8 and 2.5 mm.

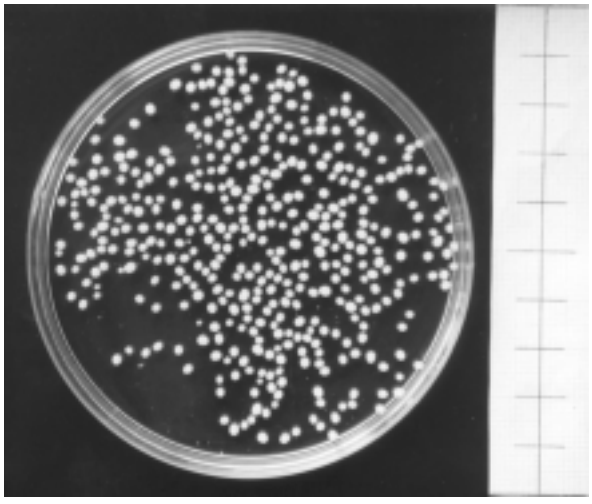


Figure 1: Pelleted growth form of the filamentous fungus *Rhizopus nigricans*.

Studies on biomass and sugar concentration during the process of biotransformation were initially performed in batch experiments with glucose as a sole energy source. The change in culture dry weight at the initial absolute value of 7.1-7.5 g/L is presented in Figure 2.

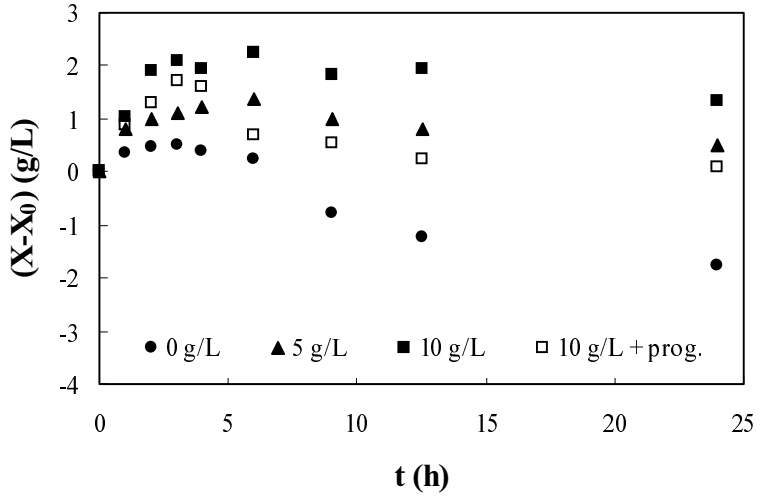


Figure 2: The effect of glucose (0, 5, 10 g/L) and progesterone (0.3 g/L) addition on the growth of *Rhizopus nigricans* pellets in the transformation medium. X and X_0 denote culture dry weight at times t=t and t=0, respectively.

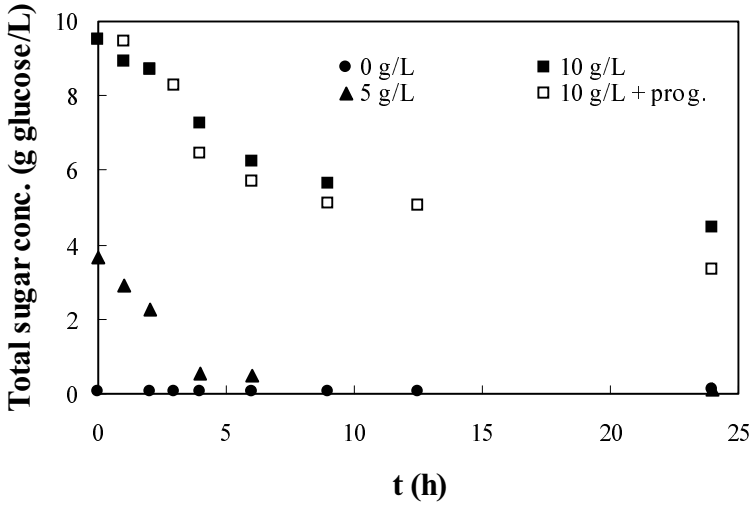


Figure 3: Sugar consumption during *R. nigricans* cultivation in the transformation medium at different starting concentrations of glucose (0, 5, 10 g/L) and progesterone addition (0.3 g/L).

The initial increase in dry weight is in correlation with glucose consumption in the medium, shown in Figure 3. The unexpected slight increase in biomass concentration at cultivation with no glucose addition and after the depletion of glucose at the starting concentration 5 g/L may be a consequence of the presence of residual nutrients from the growth cultivation inside the mycelial networks of pellets. After 3-6 h of cultivation, a decrease in *R. nigricans* biomass concentration is evident at all tested initial conditions. The residuals of glucose at initial concentrations of 10 g/L and the simultaneous moderate decrease in biomass concentrations during the last 10 h of cultivations suggested that the cultures were nitrogen-limited. Furthermore, lower biomass concentrations at progesterone addition indicated inhibition of *R. nigricans* growth, which is in agreement with findings of Breskvar *et al.,* 1995.[11]

To ensure balanced growth, two growth media with different nitrogen sources (GM1 and GM2) were mixed with phosphate buffer in different parts and tested for cultivation of *R. nigricans* pellets. The maximal specific growth rate μ_{max}, and biomass yield on total sugars Y_{sx}, were calculated from the linear growth phase of a batch culture at different initial substrate concentrations. As evident from Table 1, slower growth was obtained on media containing soy flour, which is partially insoluble in water media. In both cases, the maximal growth rate was not reached at the initial concentration 5 vol.% of growth media in the transformation media.

Growth parameter	GM1 (soy flour + yeast extract)	GM2 (yeast extract)
μ_{max} (h^{-1})	0.033 ± 0.0005	0.10 ± 0.01
Y_{sx} (g/g)	0.22 ± 0.02	0.20 ± 0.01
m_s (g/g·h)	0.016	0.015

Table 1: Growth kinetic parameters of *Rhizopus nigricans*, cultivated in transformation media with indicated nitrogen source. The results for μ_{max} and Y_{sx} are the mean of three cultivations with absolute deviations indicated. Y_{sx} and m_s refer to total sugar concentration.

The simulation of growth in the transformation medium using the obtained growth kinetic parameters was made according to the Monod model[12]:

$$\frac{dX}{dt} = \frac{\mu_{max} \cdot S \cdot X}{K_S + S} \quad (1)$$

$$\frac{dS}{dt} = -\frac{\mu_{max} \cdot S \cdot X}{(K_S + S) \cdot Y_{sx}} \quad (2)$$

The saturation constant K_s was set to 0.01 g/L according to the literature data for the growth on glucose[13,14], which is the main carbon source in the transformation medium, since sugars from soy flour and/or yeast extract represent approximately 6% of total sugars. The comparison with experimental data (Figure 4) from cultivations with different amounts of growth media GM1 showed that the proposed model can be used till the beginning of the death phase.

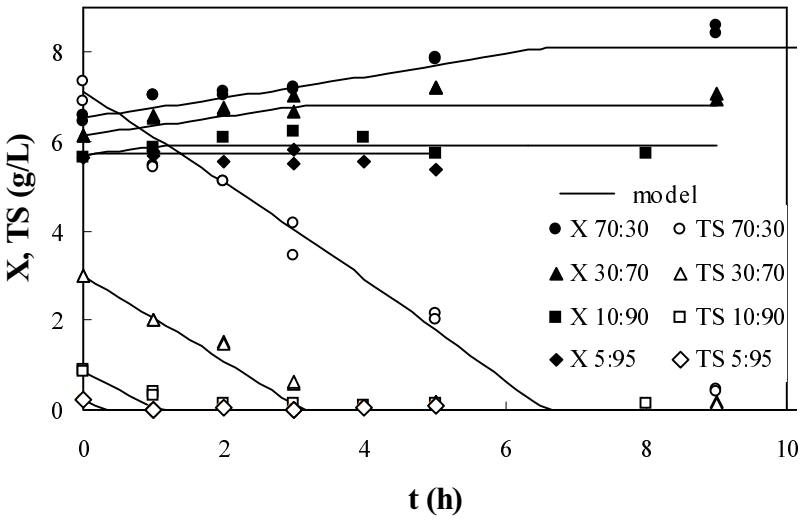


Figure 4: Time-course of biomass (X) and total sugars (TS) concentration at different ratios (v/v) of growth media and phosphate buffer, indicated in the legend, and the simulation using the Monod model with evaluated growth parameters, shown in Table 1.

In simulated fed batch Erlenmeyer flasks experiments, the maintenance coefficients on total sugars (expressed as glucose equivalents) were estimated according to Esener *et al.,* 1981.[9] The method is based on the model of Pirt (1965) assuming a constant rate of substrate consumption for the maintenance metabolism:[15]

$$-\frac{dS}{dt} = \frac{\mu X}{Y_{sx}} + m_s X . \qquad (3)$$

When approaching constant biomass concentration (μ=0 h^{-1}) by using an appropriate substrate feed, the maintenance coefficient could be directly calculated on the basis of substrate consumption. Therefore the growth in the transformation medium was performed with periodical addition of GM1 and GM2 in order to obtain constant biomass concentrations. The calculations of substrate feed, which should lead to constant biomass concentrations, were done on the basis of literature data for the maintenance coefficients of several filamentous fungi.[14,16] In Figures 5 and 6, data for both cultivations are presented.

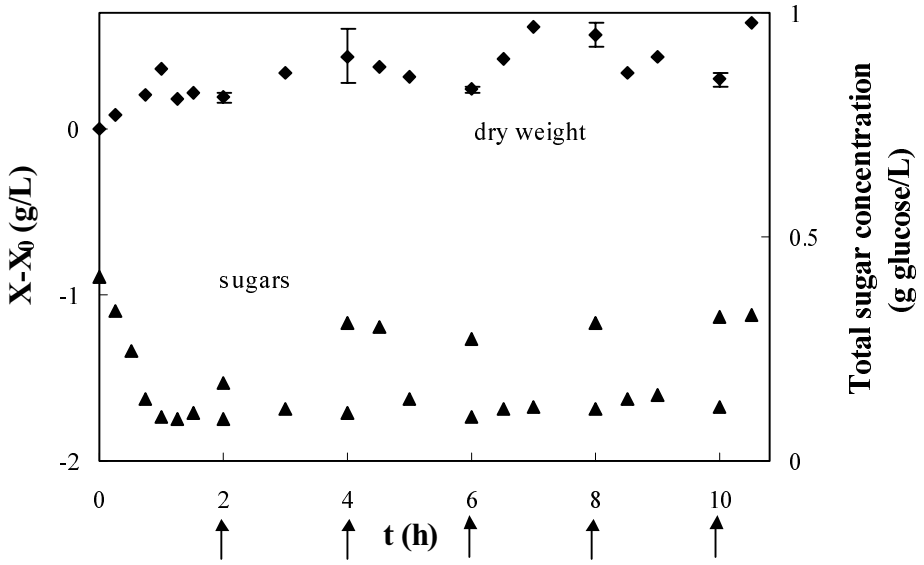


Figure 5: Time-course of a simulated fed-batch growth of *R. nigricans* pellets with indicated times of GM1 addition. The error bars denote the absolute deviation of two parallels from the mean value.

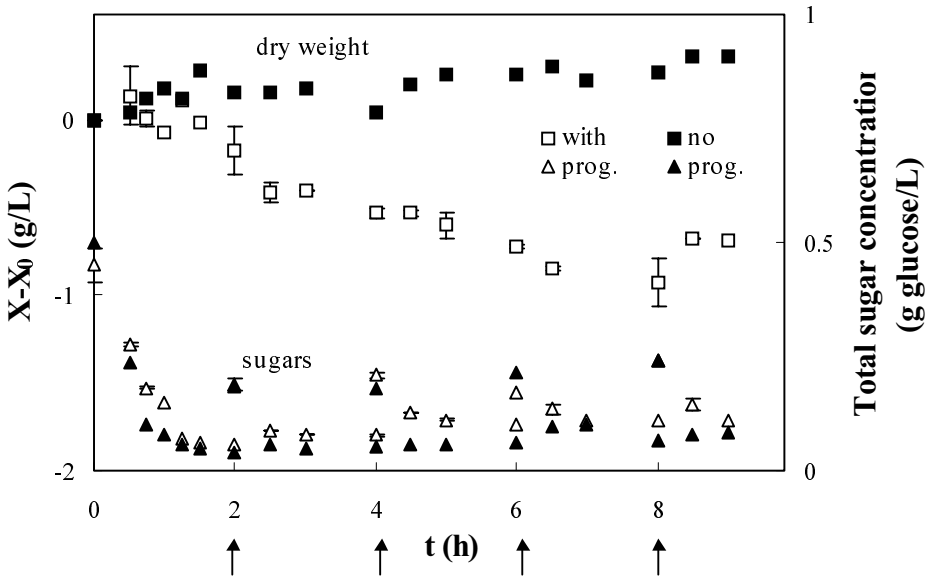


Figure 6: Time-course of a simulated fed-batch growth of *R. nigricans* pellets with GM2 addition every second hour and the comparison with cultivation in the presence of progesterone. The results are the mean of two parallels with absolute error bars noted.

The maintenance coefficients were calculated from substrate consumption after 2 h, when approximately constant dry weight was obtained (for the transformation medium with GM1 5.00±0.14 g/L and with GM2 6.08±0.26 g/L). The values of the coefficients, summarized in Table 1, are almost identical for both media and are comparable with literature data (e.g. for *Penicillium chrysogenum* 0.021 g/g·h and for *Aspergillus awamori* 0.016 g/g·h, both for glucose).[14,16]

While approximately constant dry weight was obtained in both cultivations without adding the steroid, a decrease in biomass concentration in the presence of progesterone was evident (Figure 6). The measurements of total nitrogen concentration revealed an excess of this substrate during both cultivations (data not present), which clearly denies the limiting effect on the growth of *R. nigricans* pellets. This again implies the growth inhibition effect of progesterone and a greater need for a supporting media to counteract the autolysis and to maintain a constant biomass concentration. As the average pellet diameter did not change during cultivation in both cases, we assume that the autolysis of the mycelium primarily occurred in the center of pellets.

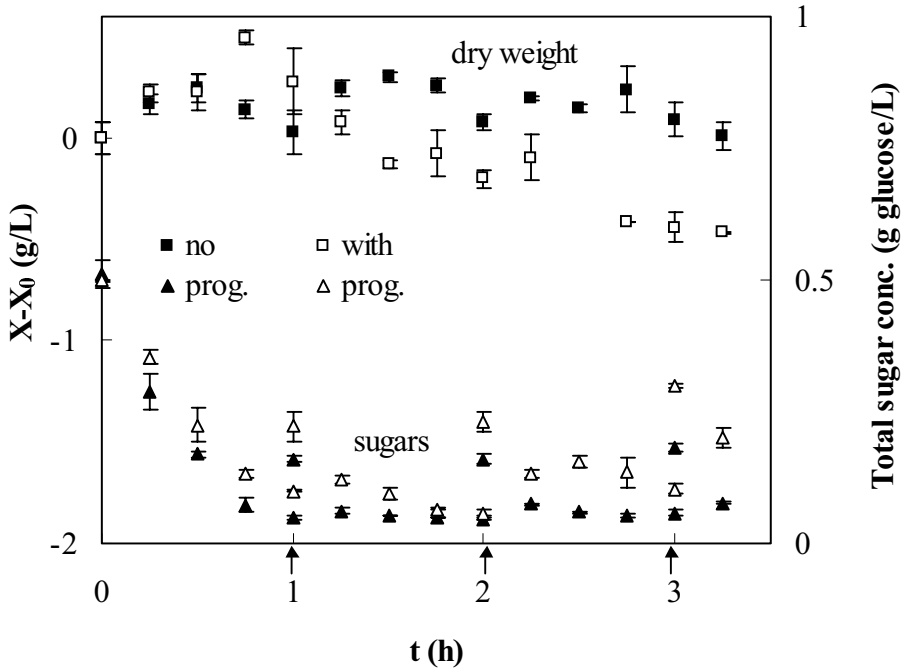


Figure 7: Time-course of a simulated fed-batch growth of *R. nigricans* pellets with GM2 addition every hour and the comparison with cultivation in the presence of progesterone. The results are the mean of two parallels with absolute error bars noted.

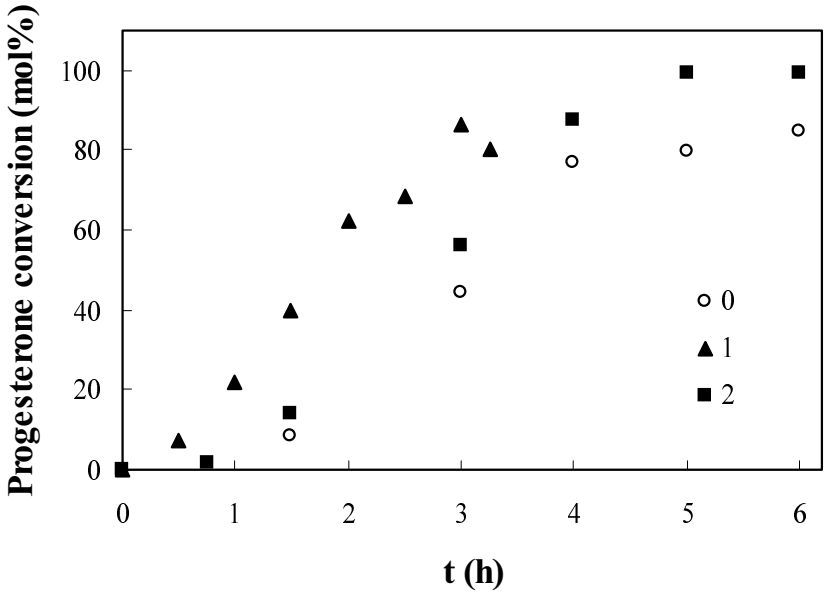


Figure 8: Progesterone biotransformation by *R. nigricans* pellets in transformation media with GM2 addition each hour (1) or every second hour (2), and in a batch process (0) with initial concentration of glucose 10 g/L.

The use of a twice higher feed rate of GM2 in the transformation medium slightly reduced the decrease of dry weight concentration in the presence of progesterone, which was followed in the short cultivation (Figure 7). Again, the autolysis process was faster than the biomass growth.

The comparison of progesterone biotransformation at both feed rates of supporting medium GM2 with the batch transformation in media with glucose as the sole carbon source indicates the benefit of using complex media at higher feed rates (Figure 8). The results will serve for further studies on the fed-batch biotransformation process with *R. nigricans* pellets.

Conclusions

The results indicated the benefit of using complex media as supporting media for progesterone 11α-hydroxylation. The growth inhibition effect of progesterone resulted in a decrease of biomass concentration at the tested conditions, which indicated a higher energy demand to counteract the autolysis and to maintain constant biomass concentration during long-term biotransformation.

Nomenclature

K_s	Saturation constant for total sugars, expressed as glucose equivalents	g glucose/L
m_s	Maintenance coefficient on total sugars	g glucose/g biomass · h
S	Substrate concentration	g/L
t	Time	h
TS	Total sugar concentration, expressed as glucose equivalents	g glucose/L
X	Biomass concentration	g biomass/L
Y_{sx}	Growth yield coefficient	g biomass/ g glucose
μ	Specific growth rate	h^{-1}
μ_{max}	Maximal specific growth rate	h^{-1}

Acknowledgement

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References

1. Hanisch, W.H.; Dunnill, P.; Lilly, M.D. *Biotechnol. Bioeng.* **1980,** *22*, 555-570.
2. Žnidaršič, P.; Komel, R.; Pavko, A. *J. Biotechnol.* **1998,** *60*, 207-216.
3. Gibbs, P.A.; Seviour, R.J.; Schmidt, F. *Crit. Rev. Biot.* **2000,** *20*, 17-48.
4. Dror, Y.; Freeman, A. *Appl. Env. Microbiol.* **1995,** *61*, 855-859.
5. Maddox, I.S.; Dunnill, P.; Lilly, M.D. *Biotechnol. Bioeng.* **1981**, *23*, 345-354.
6. Žnidaršič, P.; Pavko, A. *Proceedings of the First European Congress on Chemical Engineering, Florence, Italy*, **1997,** *Vol. 4*, 2559-2562.
7. Žnidaršič, P.; Pavko, A.; Komel, R. *J. Ind.Microbiol.* **1995,** *15*, 397-400.
8. Žnidaršič, P.; Pavko, A.; Komel, R. *J. Ind.Microbiol.* **1992,** *9*, 115-119.
9. Esener, A.A.; Roels, J.A.; Kossen, N.W. *Biotechnol. Bioeng.* **1981,** *23*, 1851-1871.
10. Žnidaršič, P.; Komel, R.; Pavko, A. *World J.Microb.Biot.* **2000,** *16*, 589-593.
11. Breskvar, K.; Ferenčak, Z.; Hudnik-Plevnik, T. *J. Steroid Molec. Biol.* **1995,** *52 (3)*, 271-275.
12. Monod, J. *Recherches sur la croissance des cultures bacteriennes;* Hermann et C^{ie}, Paris, **1942**.
13. Perdih, A. Izbor in priprava substratov. In: *Biotehnologija* (Raspor, P., Ed.); BIA, d.o.o., Ljubljana, **1996**, pp 367-382.
14. Nielsen, J., Villadsen, J. *Bioreaction Engineering Principles*, Plenum Press, New York, **1994**.
15. Pirt, S.J. *Proc. Royal Soc. London Ser. B* **1965**, 163, 224-231.
16. Heijnen, J.J.; Roels, J.A. *Biotechnol. Bioeng.* **1981,** *16*, 739-763.

Povzetek

Proučevali smo vpliv različnih dohranjevalnih medijev in hitrosti napajanja na koncentracijo biomase med procesom 11α-hidroksilacije progesterona s peletno obliko rasti nitaste glive *Rhizopus nigricans*. Določili smo kinetične parametre rasti za rast peletov v dveh transformacijskih medijih z različnima viroma dušika. Rezultati nakazujejo smiselnost dohranjevanja s kompleksnim gojiščem pri večjih hitrostih napajanja, kot so potrebne za vzdrževanje konstantne koncentracije biomase v procesu brez eksogeno dodanega steroida.