The Influence of Some Engineering Variables Upon the Morphology of *Rhizopus nigricans* in a Stirred Tank Bioreactor

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

Morphological characteristics of submerged cultures of steroid-transforming filamentous fungus *Rhizopus nigricans* were followed in stirred-tank bioreactors at different cultivation conditions. The influence of inoculum concentration and morphology on submerged growth of *Rhizopus nigricans* in the reactor was investigated. The results indicated the benefit of using inocula below 10^3 spores mL^{-1} in order to obtain pelleted growth form and to prevent undesired growth on the broth surface. Furthermore, the effect of energy dissipation rate on the morphology and biomass yield was evaluated by the use of different number and types of impellers at different agitation rates. Our study confirmed the inverse proportional relationship between energy dissipation rate and pellet diameter, which was in correlation with pellet fragmentation at high energy input.

Key words:
Rhizopus nigricans, fungal morphology, pelleted growth, agitation intensity

Introduction

A widespread use of filamentous fungi in several important biotechnological processes, from the production of organic acids, numerous antibiotics, enzymes and nowadays very interesting statins, to biotransformations, included in industrial synthesis of steroid drugs etc., has led to the intensive research on the submerged growth of these microorganisms. Regarding the conditions during submerged cultivation, the polarized growth pattern of molds in the form of filaments results in different macromorphological forms, from freely dispersed mycelium to mostly spherical aggregates referred to as pellets.[1] The later offer several advantages over the filamentous growth, from lower power consumption due to improved rheological characteristics and the possibility of biomass reuse, to the prevention of growth on fermenter walls, wrapping around the impellers or blocking the gas distributors.

The importance of morphological type for the production of specific metabolites and for the energy consumption related with adequate mixing and oxygen supply has now long been established and summarized in several review papers.[1–6] Common ascertainment is that morphology and the related physiology strongly depend on process parameters, and in turn affect the rheological properties of the broth and thereby bioreactor performance. Several factors, from genetic structure and physiological mechanisms, to physico-chemical culturing conditions in the bioreactor, contribute to the complex interrelationship between engineering variables, fungal morphology and process productivity.[1–6] Although some common approaches are suggested, attempts to obtain a desired morphology and biomass activity are still a matter of study for a specific strain and process demand.

Agitation conditions and shear stress in bioreactors, as well as the resulting oxygen level, have a strong influence on microbial morphology. The interaction of pellets or hyphae with eddies of fluid has the potential to cause mechanical damage and fragmentation of cells.[3,4] In the case of pelleted growth form, the fragmentation and break-up of aggregates, caused by shear forces, influence the pellet size and the quantitative relation between pellets and free mycelium in the culture.[2] Besides, the shape of a pellet – whether it is smooth and compact or with fluffy, loose structure strongly depends on the energy input. The pellet diameter d_p was found to be related with the specific energy dissipation rate ε by a correlation

$$d_p = \alpha_1 \cdot \varepsilon^{-\alpha} \quad (1)$$

where the values for coefficient α_1 and the exponent α differ among the studies with different microorganisms and also change with process time.[7–9]

A pelleted growth form of filamentous fungus *Rhizopus nigricans* has been used as a naturally immobilized biocatalyst in a process of progesterone 11α-hydroxylation, enabling a repeated-batch,[10] or

continuous[11] operation mode and, thereby, process optimization. The control of *R. nigricans* submerged growth by means of inoculum concentration, starting pH value, nitrogen and glucose concentration, Ca^{2+} and Tween-80 addition, and aeration conditions, was already attained in a shake-flasks system,[10,12] while here we report on further studies in a laboratory stirred tank bioreactor.

The effect of energy input on the morphology and biomass yield was studied by the use of two types of impellers, a classical Rushton turbine and a propeller operated at different agitation rates. Besides, two different bioreactors with one or two impellers were used. Furthermore, the influence of inoculum type and concentration on *Rhizopus nigricans* submerged growth pattern in the reactor was investigated.

Materials and methods

Microorganism

Filamentous fungus *Rhizopus nigricans* ATCC 6227b (MZKI b1122) was maintained at 26 °C on slants consisting of malt extract 6 °Blg with 20 g L^{-1} of agar (pH 5.5).

Shake flasks culture conditions

14-days old spores of *Rhizopus nigricans* ATCC 6227b from agar slants were transferred into the growth medium according to *Žnidaršič* et al.,[10] containing (in g L^{-1}): glucose, 20; soy flour, 6; yeast extract, 5.7; NaCl, 4; K_2HPO_4, 2. The pH was adjusted to 5.5. Inoculum sizes between 10 and 2.6×10^3 spores mL^{-1} were used and incubations of 100 mL cultures were performed on a rotary shaker at 23 °C and n = 200 min^{-1} for 24 to 35 h.

Laboratory-scale bioreactor cultivations

Fungal pellets, obtained in shake-flasks cultures, were used as inoculum for the submerged cultivations of *R. nigricans* in two different stirred-tank bioreactors:

1. A bioreactor with total volume of 2.6 L (Bia d.o.o., Ljubljana, Slovenia); vessel internal diameter, D, was 0.12 m, broth height, H, was 0.177 m (working volume V_L = 2 L). This bioreactor had one impeller with diameter d = 0.05 m (Rushton turbine) or d = 0.066 m (propeller), which was located in a distance h_a = 0.06 m above the base of the tank. Temperature regulation was performed by means of a heater and cooling coil, set up in configuration which replaces the effect of baffles. The aeration rate of 0.5 – 1.8 L L^{-1} min^{-1} was provided by a ring sparger, placed below the impeller.

2. A bioreactor with total volume of 3.1 L (Bioengineering AG, Wald ZH, Switzerland); D = 0.115 m, H = 0.211 m (V_L = 2.2 L), having two impellers with d = 0.048 m (both Rushton turbines) at h_a = 0.05 m and h_b = 0.145 m. Heating and cooling sticks provided constant working temperature, while aeration at 0.8 L L^{-1} min^{-1} was performed by a porous ceramic sparger, located below the bottom impeller.

0.2–0.22 L of seed cultures were used for the inoculation of 1.8 to 2 L of the same growth medium as used for shake-flasks cultivation with initial pH value 5.5. Batch processes were performed at 26 °C ± 1 °C at stirring speed n = 300 or 400 min^{-1} in the reactor with one impeller, while in the second reactor with two Rushton turbines the agitation speed values between n = 100 and 400 min^{-1} were used. For the regulation of foam, a silicone-based antifoam solution was used. Cultivations were carried out for 24 to 29 h.

Submerged cultures analysis

In bioreactors, on-line measurements of temperature, pH and p_{O_2} were performed by specific sensors. Quantitative analysis of pellet size was done from the photographs of samples by means of commercial image analysis software (Motic Images Plus 2.0). Biomass concentration and culture density were determined at the end of the batch cultivations by measuring wet and dry weight of the whole broth from the bioreactor and the corresponding volume.

Results and discussion

The effect of different inoculum concentration and starting morphology on the submerged cultivation of steroid-transforming filamentous fungus *Rhizopus nigricans* ATCC 6227b was studied in a laboratory-scale bioreactor with 2-L working volume and one Rushton turbine, agitated at n = 300 min^{-1} and aerated at Q_g = 100 L h^{-1}. By varying concentration of spores from slants in shake flasks pre-cultures we have obtained different types of mycelium, which after the inoculation of the bioreactor with the same volume (0.2 L) of the seed culture resulted in different final morphology and biomass concentration, shown in Table 1. The highest inoculum concentration, which in shaken-flasks precultures led to the formation of the highest number of agglomerates with mean diameter of 0.2 mm, resulted in a dispersed filamentous growth form of *Rhizopus nigricans* after 24 h of growth in the bioreactor, which is in agreement with our previous findings in shake-flasks experiments.[10] Furthermore, undesired growth on the broth surface and at the bioreactor wall, which represented the main part

T a b l e 1 – *Average pellet diameter and biomass concentration after 24 h of batch cultivations in a laboratory-scale bioreactor with 2 L working volume and one Rushton turbine, agitated at n = 300 min^{-1} and aerated at the rate of 0.8 L L^{-1} min^{-1} with φ = 10 % of inoculum of different starting pellet diameter and concentration*

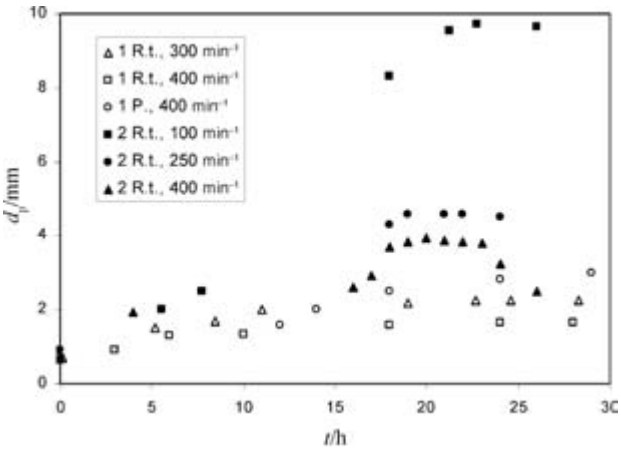
$C_{inoculum}$/spores mL^{-1}	$d_{p,start}$/mm	$d_{p,end}$/mm	γ_X/kg m^{-3}
$2.5 \cdot 10^3$	0.2	filamentous growth	1.9[a]
$1.3 \cdot 10^2$	0.7	2.22	4.5
13	1.1	2.64	4.6

[a] without the part of the biomass, present on the surface of the broth

of the biomass produced, accompanied filamentous submerged growth at these conditions. The biomass above the liquid phase and attached at the reactor wall was mainly aggregated in dense clumps, which was a result of non-sufficient mixing in this regions. The formation of stagnant, non-mixed zones even in laboratory-scale bioreactors was reported as the main problem of bioprocesses with microorganisms in the filamentous growth form, leading to highly viscous fermentation broths. These problems can be especially serious for larger-scale reactors, where mixing times in the order of several minutes have been recorded.[4]

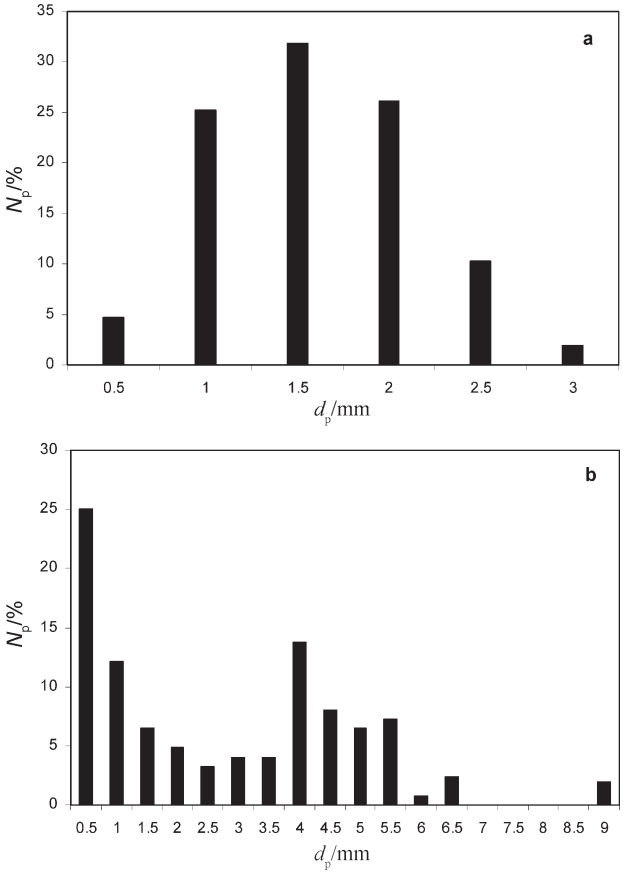
As seen from Table 1, lower concentrations of spores in shake-flasks resulted in the formation of larger pellets, which was already proved for this filamentous fungus with a noncoagulative type of pellet formation.[10] Further cultivations in the bioreactor at the same conditions also revealed the inverse proportionality between the number of pellets and their size, while the biomass concentration after 24 h of growth was the largest in the case of the smallest number of cultivated pellets, which had the highest starting diameter. Besides, the growth on broth surface was practically prevented, which confimed improved rheological characteristics and thereby mixing efficiency in the case of pelleted growth form. Results suggested the benefit of the use of starting inoculum concentrations below 10^3 spores mL^{-1} in order to obtain a desired morphology.

Therefore further cultivations were performed from the same starting inoculum concentration of 10^2 spores mL^{-1} and φ = 10 % of seed cultures were transferred into both bioreactors with one or two Rushton turbines. The influence of various agitation speeds and reactor configurations on the growth of pellets is shown on Fig. 1. Evidently, both cultivations in 2-L bioreactor with one Rushton turbine resulted in a slow increase in pellet diameter, where at higher agitation speed of n = 400 min^{-1} (u_t = 1.05 m s^{-1}) slightly smaller pellets were formed (d_p at 28 h = 1.67 mm) as compared to the



F i g . 1 – *The effect of various agitation speeds on the time course of mean pellet diameter in cultivations with the same starting inoculum, performed in two similar bioreactors with one or two Rushton turbines (R. t.) or with a propeller (P.)*

cultivation with agitation speed of n = 300 min^{-1} (u_t = 0.79 m s^{-1}), where average pellet diameter at 28 h was 2.25 mm. A histogram of a sample, taken at the end of cultivation at n = 400 min^{-1} and shown in Fig. 2a, revealed normal pellet size distribution.



F i g . 2 – *Pellet diameter distributions obtained in cultivations with the same starting inoculum, performed in bioreactors with similar working volume (V_L = 2 or 2.2 L) and dimensions, operated at the same agitation speed of n = 400 min^{-1} and aeration rate of 0.8 L L^{-1} min^{-1}, and with: a) one and b) two Rushton turbines*

On the other hand, in the second reactor with almost identical dimensions (V_L= 2.2 L), but with two Rushton turbines, operated at the same agitation speed of n = 400 min^{-1} (u_t = 1.00 m s^{-1}) and at the same aeration rate of Q = 0.8 L L^{-1} min^{-1}, pellets grew much faster, but they fragmented after 22 h of the process, which resulted in a decrease in average pellet diameter (d_p at 26 h = 2.50 mm) and a bimodal pattern of pellet size distribution at the end of cultivation, which is a consequence of the chip-off of agglomerates due to high local shearing forces, present especially in the regions near the impeller (Fig. 2b). Obviously, local power input around both impellers strongly prevailed over the tensile strength of hyphae, which resulted in huge hyphal fragmentation.

A significant difference in pellet structure between both cultivations with different impeller number can also be observed from Fig. 3. While majority of pellets from the bioreactor with one Rushton turbine (Fig. 3a) have a fluffy surface, agglomerates from the bioreactor operated with two impellers and hereby at higher energy input (Fig. 3b) were very smooth and had much more compact structure. Similar findings were reported also for pellets of *Aspergillus awamori*.[8] For the fragmentation of the submerged culture of *Penicillium chrysogenum*, the physiological state of the hyphae, characterized by the proportions of vacuoles, has been found to have a significant influence on the breakage of mycelial hyphae, which was greater when the hyphae became heavily vacuolated due to nutrient limitation.[13]

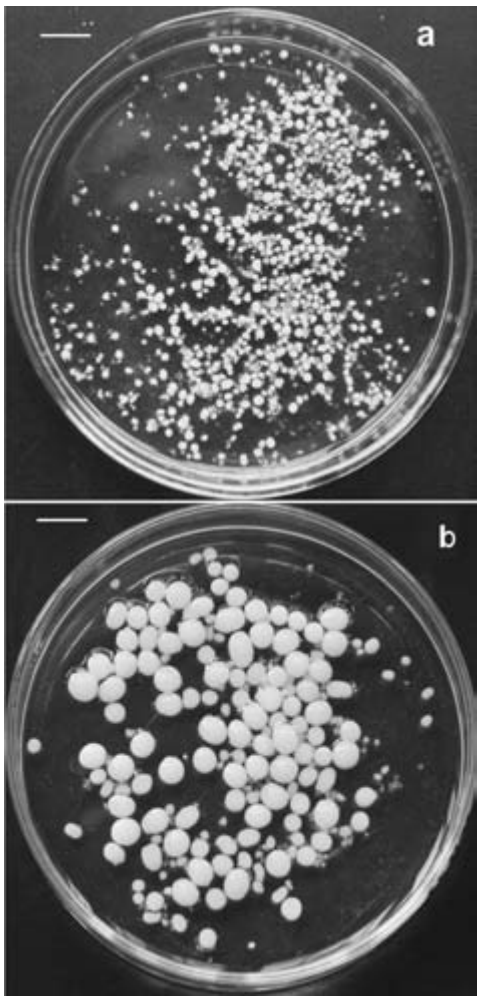
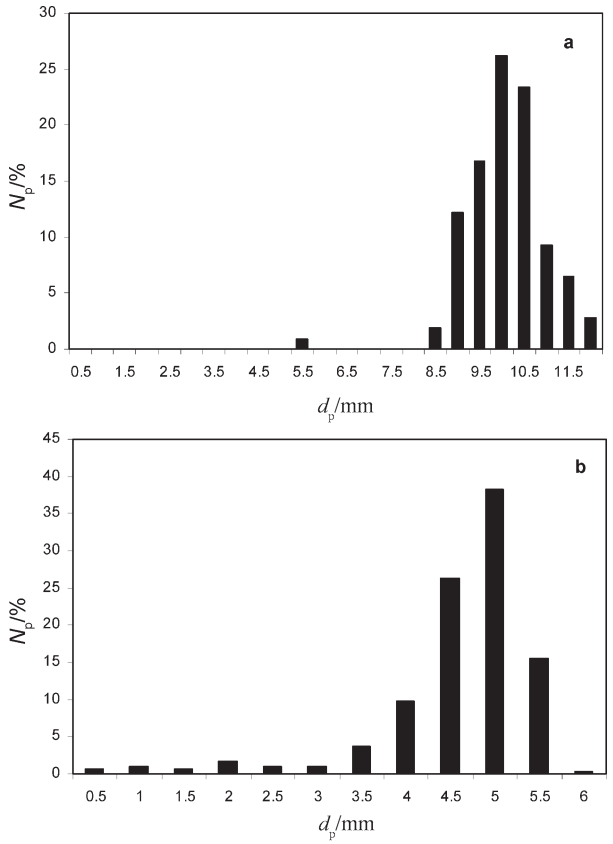


Fig. 3 – *Comparison of pellet morphology at the end of cultivations with a) one and b) two Rushton turbines, agitated at the same speed of n = 400 min^{-1}. Figures correspond to histograms, presented in Fig. 2a and 2b; scale — = 1 cm.*

The same inoculum size, agitation speed and aeration rate were used also in batch process in the first reactor with one propeller, giving an axial flow pattern and lower power input. As seen from Fig. 1, growth pattern was similar to both cultivations in the same reactor with Rushton turbine, except that average pellet diameter was larger and reached 3 mm after 29 h of the process. Pellets had even more loose and hairy structure as compared to the cultivations with Rushton turbine, which confirmed findings of *Cui* et al.,[8] who found that the porosity of pellets showed an inverse relationship with the specific energy dissipation rate. In this experiment it was also demonstrated that axial mixing was not efficient at a top part of the reactor, which resulted in a pronounced surface growth, undesired for the acquirement of pellets to be used in the progesterone biotransformation process.

By lowering agitation speed in the reactor with two Rushton turbines, the growth pattern, shown in Fig. 1, significantly changed compared to cultivations described above and the resulting pellet diameters were again inversely proportional to the energy input. At extremely low agitation intensity of n = 100 min^{-1} (u_t = 0.25 m s^{-1}), the size of pellets was several fold larger (d_p at 26 h = 9.66 mm) than in cultivations with only one impeller, with the same inoculum concentration and morphology, the same aeration rate and an order of magnitude higher specific energy input. As evident from Fig. 4a, pellets grown at the lowest shear rates were of a very uniform size, while at agitation speed of n = 250 min^{-1} (u_t = 0.63 m s^{-1}), a small part of pellets fragmented to yield broader pellet size distribution, shown in Fig. 4b. Pellets from the cultivation at n = 100 min^{-1} had slightly hairy surface, while at n = 250 min^{-1} the agglomerates were smooth and had very regular spherical shape.

The highest final biomass concentration (6.43 kg m^{-3}) was achieved in the reactor with two Rushton turbines at agitation speed of n = 250 min^{-1}, while 4.93 and 3.18 kg m^{-3} of dry cell concentration were reached in the same reactor agitated at n = 400 min^{-1} and 100 min^{-1}, respectively. In the bioreactor with one impeller, the best yield of bio-



F i g . 4 – *Pellet diameter distributions obtained in a 2.2 L bioreactor with two Rushton turbines operated at: a) 100 min^{-1} and b) 250 min^{-1}, and aerated at 0.8 L L^{-1} min^{-1}*

mass (4.72 kg m^{-3}) was attained with Rushton turbine rotated at n = 400 min^{-1}, while at 300 min^{-1} the cultivation with the same impeller resulted in slightly lower biomass concentration (4.51 kg m^{-3}) after the same time of 28 h. In this bioreactor, the growth in the presence of propeller agitated at n = 400 min^{-1} gave only 3.6 kg m^{-3} of biomass. Generally, higher agitation intensity or energy input correlated with higher level of biomass produced during 24 to 29 h of growth in bioreactors, which is in agreement with our previous findings in shake-flasks experiments.[10] This is presumably due to the increased transfer of oxygen, which is stated to be a limiting nutrient for fungal growth in the form of pellets.[1,5] The exception was the cultivation at n = 250 min^{-1} (two Rushton turbines) where the highest biomass concentration was obtained after 24 h, which is a consequence of cca. 15 % higher starting number of pellets from seed cultures, and therefore also initial biomass concentration. A slight increase in biomass yield was found also at increased initial spore concentration in our previous studies.[10]

In order to define the effect of power input on pellet size, energy dissipation rate was calculated according to Cui et al.[14] In the case of the bioreactor with one impeller and for the bottom turbine, the correlation used for the calculation of impeller power input under gassed conditions P_g was:

$$P_g = P\left(1 - \frac{9.9\,Q_g n^{0.25}}{d^2}\right) \qquad (2)$$

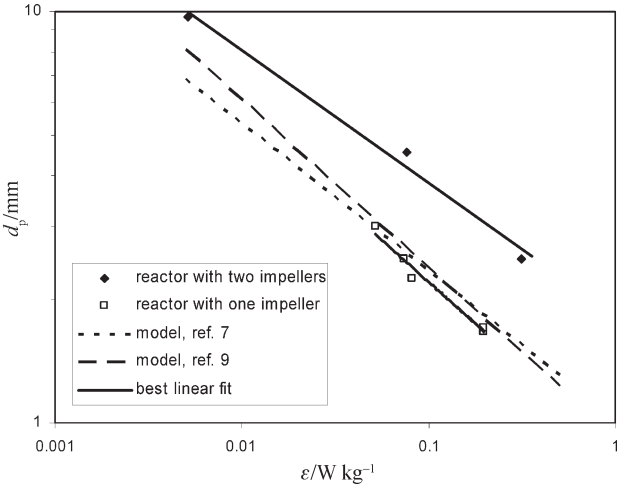
while for the top impeller in the bioreactor with two Rushton turbines, the equation used was:

$$P_g = P(1 - 37.6\,Q_g n) \qquad (3)$$

where power input P was defined as

$$P = Po\,\rho\,n^3 d^5 \qquad (4)$$

Based on the calculated Reynolds numbers for each cultivation, where the viscosity of culture was considered as for the water, while the density was measured to be in the range between 1012 and 1049 kg m^{-3}, power number Po was estimated regarding *Jüsten* et al.[15] and *Casas López* et al.[9] Results of our experiments, presented in Fig. 5, confirmed the proposed correlation between average pellet diameter and energy dissipation rate, although results, obtained in different reactors gave different coefficient for Eq. 1, as revealed from the best fit of the experimental data with the power function. Namely, the relationship in bioreactor with one impeller was d_p (mm) = 0.865 · $\varepsilon^{-0.406}$, which is very similar to findings of *Casas López* et al.[9] for *Aspergillus terreus*, having coefficient α_1 = 0.923 and the exponent α = 0.41, while in the reactor with two Rushton turbines, the correlation was d_p (mm) = 1.826 · $\varepsilon^{-0.322}$, which is closer to results of *Van Suijdam* and *Metz*[7] for *Penicillium chrysogenum*, who got α_1 = 1.02 and α = 0.36. Both models from



F i g . 5 – *The correlation between final pellet diameter and specific energy dissipation rate. Both solid lines represent the best linear fit of experimental data with power function. Dashed lines demonstrate calculations according to models of van Suijdam and Metz[7] and Casas López et al.[9]*

the literature are presented in Fig. 5. Our findings on *Rhizopus nigricans* growth could therefore consent to the literature, which considers morphological data in correlation with energy dissipation rate or energy dissipation/circulation function.[15]

Conclusions

Operating conditions had a significant effect on the size distribution of the pellets and their structure. The use of φ = 10 % of pre-formed pellets from shake flasks with inoculum concentration below 10^3 spores/mL were found to lead to pelleted growth form of *Rhizopus nigricans* in a laboratory-scale bioreactor. In this way and with the use of Rushton turbine instead of propeller, the problems associated with the high tendency of this aerobic fungus to grow on the broth surface were avoided. The study of effect of energy input on pellet size confirmed the inverse proportional relationship, similar to reports for different filamentous fungi. Fragmentation of pellets was also amplified by raising the energy dissipation rate.

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Nomenclature

D – bioreactor diameter, m
d – impeller diameter, m
d_p – pellet diameter, m
H – level of liquid in bioreactor, m
h_a – height of the first impeller, m
h_b – height of the second impeller, m
n – impeller speed, min^{-1}, s^{-1}
N_p – number of pellets
Po – power number
P_g – gassed power input, W
P – power input, W
Q_g – gas flow rate, $m^3\ s^{-1}$
u_t – impeller tip speed, m s^{-1}
V_L – working volume, m^3

Greek letters

γ_x – biomass concentration, kg m^{-3}
α – exponent in Eq. 1
$\alpha 1$ – coefficient in Eq. 1
ε – specific energy dissipation rate, W kg^{-1}
ρ – liquid density, kg m^{-3}
φ – volume fraction, %

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