Efflux of organic acids in *Penicillium simplicissimum* is an energy-spilling process, adjusting the catabolic carbon flow to the nutrient supply and the activity of catabolic pathways

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Continuous cultivation was used to study the effect of glucose, ammonium, nitrate or phosphate limitation on the excretion of tricarboxylic acid (TCA) cycle intermediates by *Penicillium simplicissimum*. Additionally, the effect of benzoic acid, salicylhydroxamic acid (SHAM) and 2,4-dinitrophenol on TCA cycle intermediates was studied. The physiological state of the fungus was characterized by its glucose and O₂ consumption, its CO₂ production, its intra- and extracellular concentrations of TCA cycle intermediates, as well as by its biomass yield, its maintenance coefficient and its respiratory quotient. The excretion of TCA cycle intermediates was observed during ammonium-, nitrate- and phosphate-limited growth. The highest productivity was found with phosphate-limited growth. The respiratory quotient was $\text{RQ} < 3$ under ammonium limitation and $0 < 7$ under phosphate limitation. Citrate was always the main excreted intermediate. This justifies calling this excretion an energy-spilling process, because citrate excretion avoids the synthesis of too much NADH. The addition of benzoic acid further increased the excretion of TCA cycle intermediates by ammonium-limited hyphae. A SHAM-sensitive respiration was constitutively present during ammonium-limited growth of the fungus. The sum of the excreted organic acids was negatively correlated with the biomass yield ($Y_{\text{GlcX}}$).

**Keywords:** overflow metabolism, continuous culture, chemostat, nutrient limitation, biomass yield

INTRODUCTION

Foster (1949) clearly described the physiological state of a fungus that excretes metabolic intermediates and consequently he termed this state 'overflow metabolism'. Although many biochemical details of fungal overflow metabolism have been studied since then, the description of the underlying principle given by Foster is still valid. A prerequisite for overflow metabolism is that the fungus must be able to uncouple catabolism and anabolism. The most probable purpose of such an uncoupling is to maintain a high flux of carbon if the carbon source is in excess and growth is limited by another nutrient.

The most important biotechnological application of fungal overflow metabolism is the production of citric acid by *Aspergillus niger* (Kristiansen et al., 1999). However, the excretion of intermediates of the tricarboxylic acid (TCA) cycle takes place in every culture of a filamentous fungus growing in a medium with a high C:N ratio. Because the main part of these intermediates is excreted during the growth phase and not during the stationary phase [for *A. niger* see Wallrath et al. (1992),...
McIntyre & McNeil (1997) and Krzystek et al. (1996); for *Penicillium simplicissimum* see Burgstaller et al. (1994]), the method of choice to study the physiology of this excretion process is continuous cultivation.

We used continuous cultivation to investigate the excretion of TCA cycle intermediates by *P. simplicissimum*, a filamentous fungus which excretes citrate and which has been used in the leaching of heavy metals from industrial wastes and ores (Burgstaller et al., 1992). Because growth conditions are difficult to control in leaching processes, we aim to understand the overflow metabolism in *P. simplicissimum* in as much detail as possible, to increase the yield of the leaching agent.

In a previous article, we presented results concerning only citrate excretion by *P. simplicissimum* (Gallmetzer & Burgstaller, 2001). To understand the energetic situation as a whole, however, the total excreted carbon must be considered. In this article we report on the situation as a whole, however, the total excreted carbon must be considered. In this article we report on the excretion of all TCA cycle intermediates by *P. simplicissimum* (Gallmetzer & Burgstaller, 2001). We compare our results with the presence of dinitrophenol on the excretion of TCA cycle intermediates, and on the effects of the presence of cissimum excretion of all TCA cycle intermediates by *M. Gallmetzer and W. Burgstaller, 2001*. To understand the energetic metabolism in *P. simplicissimum* in as much detail as possible, to increase the yield of the leaching agent.

METHODS

Organism, inoculum and growth medium. *P. simplicissimum*, isolated from soil contaminated with heavy metals and identified by the Centraalbureau voor Schimmelcultures (Franz et al., 1993), was grown in a minimal medium containing trace elements (Gallmetzer & Burgstaller, 2001). A filamentous inoculum, to start the chemostat culture, was produced by growing *P. simplicissimum* in minimal medium plus 1 M HEPES, pH 7.5. A 100 ml aliquot of medium was decanted into a 500 ml flask and inoculated with $10^4$-$10^5$ spores ml$^{-1}$, and the culture was grown at 400 r.p.m. and 30 °C for 2 days. In the glucose-limited chemostat the glucose concentration was reduced to 10 mM, in the nitrogen-limited chemostat 2.5 mM NH$_4$Cl was used, and in the phosphate-limited chemostat 100 μM KH$_2$PO$_4$ was used.

Chemostat. All chemostat experiments were performed in a Biostat M bioreactor (Braun) at 30 °C, 700–1000 r.p.m. and 1–2 v.v.m. [vol. air (vol. fermentation liquid)]$^{-1}$ min$^{-1}$, with a working volume of 1700 ml. The conditions for the bioreactor runs were as described previously (Gallmetzer & Burgstaller, 2001).

The chemostat was inoculated with 100 ml of the filamentous pre-culture and processed in the batch mode overnight. Then the chemostat mode was started by switching on the medium feed and the alkali pump. Between four and six generation times were allowed to pass for the culture to reach the steady state. Samples were taken every one to four generation times (four samples per steady state).

The results presented in this study are from single chemostat runs.

**RESULTS**

Unlimited growth

The maximum specific growth rate ($\mu_{max}$, h$^{-1}$) of *P. simplicissimum*, when grown in the medium used in this study, was 0.19 h$^{-1}$ [determined in a pH-auxostat according to Simpson et al. (1995)], 0.21 h$^{-1}$ [determined from the critical dilution rate in the chemostat] and 0.19 h$^{-1}$ (determined from the exponential growth phase in shake-flask culture). During growth in the pH-auxostat at $\mu_{max}$, the specific rates of glucose and O$_2$ consumption [q$_{Glc}$ (mmol g$^{-1}$ h$^{-1}$) and q$_{O2}$ (mmol g$^{-1}$ h$^{-1}$), respectively] were independent of the steady-state glucose concentration (this was tested with 5, 13 and 33 mM glucose).

Glucose-limited growth

The elemental composition of the biomass when grown under glucose limitation was CH$_{1.75}$O$_{0.44}$N$_{0.13}$P$_{0.004}$. Very low amounts of TCA cycle intermediates were excreted under glucose limitation. Neither a rise in the extracellular osmolarity nor in the extracellular pH increased the excretion of organic acids, as was observed with ammonium-limited hyphae (Gallmetzer & Burgstaller, 2001). The relationship between $\mu$ and q$_{Glc}$, q$_{O2}$, and the rate of CO$_2$ production [q$_{CO2}$ (mmol g$^{-1}$ h$^{-1}$)] is shown in Fig. 1. With these data we calculated the maintenance coefficients ($m$) and the maximum yield coefficients ($Y$) for glucose, O$_2$ and CO$_2$ (Tables 1 and 2) using the linear

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**Analytical methods.** Sampling and dry weight determination of the mycelium were performed as described previously (Gallmetzer et al., 1998). Extracellular organic acids and glucose were determined by HPLC (Burgstaller et al., 1994). The specific rate of O$_2$ consumption was determined according to Rane & Sims (1994) or measured with a respirometer (BioMetric Systems). CO$_2$ production was also measured with the respirometer. The elemental composition of the biomass was determined by the Austrian Research Centers, Seibersdorf.

**Fig. 1.** Glucose-limited chemostat culture of *P. simplicissimum*. Specific uptake rates of glucose (q$_{Glc}$, ●) and O$_2$ (q$_{O2}$, □), as well as the rate of CO$_2$ formation (q$_{CO2}$, △), are shown as a function of $\mu$ at pH 7.0.
The elemental composition of the biomass when grown under glucose-limited conditions, shows that the values are similar for all fungi tested (Tables 1 and 2). A comparison of *P. simplicissimum* Y and m values with those gained from other filamentous fungi, which were grown under glucose-limited conditions, shows that despite the different media used by the different authors the values are similar for all fungi tested (Tables 1 and 2).

**Glucose-sufficient growth**

The elemental composition of the biomass when grown under ammonium limitation and glucose excess was CH$_1.84$O$_{0.34}$N$_{0.03}$P$_{0.004}$. Results concerning the energetic coefficients, the metabolic rates and the excretion of TCA cycle intermediates are given in Table 3, and Figs 2 and 3. The intracellular concentrations of citrate, 2-oxoglutarate, succinate, fumarate and malate were higher compared to those of glucose-limited growth. An alkaline pH value (tested range pH 2–7) and a higher extracellular osmolarity (tested range 0–2–1.5 osmol kg$^{-1}$) further increased the excretion of TCA cycle intermediates as well as increasing $q_{Glc}$, but decreased $q_O2$ slightly. The addition of an inhibitor of alternative respiration (SHAM) had almost no effect on the intracellular and extracellular concentrations of TCA cycle intermediates, or on $q_{Glc}$ and $q_O2$; however, with SHAM the biomass yield on glucose ($Y_{GlcX}$) changed from 0.47 g g$^{-1}$ to 0.58 g g$^{-1}$. If 5 mM benzoic acid (an uncoupler of the proton gradient at the plasma membrane; Verduyn et al., 1992) was added to ammonium-limited hyphae (at pH 5; no effect was observed at pH 7), the overall rate of metabolism was increased (Fig.

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**Table 1. Biomass yield coefficients of *P. simplicissimum* and other filamentous fungi in glucose-limited continuous cultures**

<table>
<thead>
<tr>
<th>Organism</th>
<th>$Y_{Glc}$ (g g$^{-1}$)</th>
<th>$Y_O2X$ (g mol$^{-1}$)</th>
<th>$Y_CO2X$ (g mol$^{-1}$)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. simplicissimum</em></td>
<td>0.50</td>
<td>48</td>
<td>48</td>
<td>This study</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>0.51</td>
<td>49</td>
<td>48</td>
<td>Christensen et al. (1995)</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>0.40</td>
<td>–</td>
<td>–</td>
<td>Ng et al. (1973)</td>
</tr>
<tr>
<td><em>P. chrysogenum</em></td>
<td>0.45</td>
<td>50</td>
<td>–</td>
<td>Righelato et al. (1968)</td>
</tr>
</tbody>
</table>

**Table 2. Maintenance coefficients of *P. simplicissimum* and other filamentous fungi in glucose-limited continuous cultures**

<table>
<thead>
<tr>
<th>Organism</th>
<th>$m_{GlcX}$ (g g$^{-1}$ h$^{-1}$)</th>
<th>$m_O2X$ (mol g$^{-1}$ h$^{-1}$)</th>
<th>$m_CO2X$ (mol g$^{-1}$ h$^{-1}$)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. simplicissimum</em></td>
<td>0.023</td>
<td>2.4 x 10$^{-4}$</td>
<td>2.0 x 10$^{-4}$</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. chrysogenum</em></td>
<td>0.028</td>
<td>8.5 x 10$^{-4}$</td>
<td>10.1 x 10$^{-4}$</td>
<td>Christensen et al. (1995)</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>0.010</td>
<td>7.8 x 10$^{-4}$</td>
<td>8.8 x 10$^{-4}$</td>
<td>Metwally et al. (1991)</td>
</tr>
<tr>
<td><em>P. chrysogenum</em></td>
<td>0.018</td>
<td>–</td>
<td>–</td>
<td>Ng et al. (1973)</td>
</tr>
</tbody>
</table>

**Table 3. Biomass yield coefficients, maintenance coefficients and RQs of *P. simplicissimum* in glucose-, ammonium-, phosphate- and nitrate-limited chemostat culture**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Growth-limiting nutrient</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>NH$_4$</td>
</tr>
<tr>
<td>$Y_{GlcX}$</td>
<td>0.50</td>
<td>0.48</td>
</tr>
<tr>
<td>$Y_{GlcX}^{\text{max}}$</td>
<td>0.53</td>
<td>0.50</td>
</tr>
<tr>
<td>$Y_O2X$</td>
<td>48</td>
<td>38</td>
</tr>
<tr>
<td>$Y_O2X^{\text{max}}$</td>
<td>54.4</td>
<td>51.2</td>
</tr>
<tr>
<td>$m_{GlcX}$</td>
<td>0.023</td>
<td>0.022</td>
</tr>
<tr>
<td>$m_O2X$</td>
<td>2.4 x 10$^{-4}$</td>
<td>2.0 x 10$^{-4}$</td>
</tr>
<tr>
<td>RQ</td>
<td>0.95</td>
<td>1.30</td>
</tr>
</tbody>
</table>
simultaneously, a higher excretion of total TCA cycle intermediates was observed (Fig. 4b). Another uncoupler of proton gradients, 2,4-dinitrophenol (0.5 mM, added at pH 7), also slightly increased $q_{\text{Glc}}$, $q_{\text{CO}_2}$ and $q_o$ but – opposite to the effect of benzoic acid – decreased the excretion of TCA cycle intermediates by 30% (data not shown).

Under nitrate limitation, a very low $q_o$ was observed. The excretion of TCA cycle intermediates was higher than with glucose-limited hyphae, but lower than with ammonium-limited hyphae (Table 3, Figs 2 and 3).

In phosphate-limited hyphae ($\text{CH}_3\text{O}_7\text{O}_2\text{N}_{0.033}\text{P}_{0.002}$), the overall rate of metabolism was decreased ($q_{\text{Glc}}, q_{\text{CO}_2}$ and $q_o$) but the excretion of TCA cycle intermediates was the highest under all tested conditions (Figs 2 and 3).

The nature of the limiting nutrient determined the relationship between the productivity of TCA cycle intermediate excretion and the specific growth rate (Fig. 5). Under ammonium limitation, this relationship corresponded to the Pirt model of catabolic product synthesis (Pirt, 1993), whereas under phosphate limitation the Luedeking–Piret model (Luedeking & Piret, 1959) was more suitable. Overall, $Y_{\text{GlcX}}$ seemed to be the most important factor, because it was inversely correlated with the degree of overflow metabolism in P. simplicissimum (data not shown).

When the results from all of the conditions tested were compared, the range of intracellular concentrations of TCA cycle intermediates was rather broad (citrate, 5–65 mM; 2-oxoglutarate, 0.5–8 mM; succinate, 20–170 mM; fumarate, 0.5–5 mM; malate, 3–70 mM; and oxalate, 4–50 mM).

**DISCUSSION**

*P. simplicissimum* excreted TCA cycle intermediates under different – and even opposite – states of metabolism (for the purposes of this discussion of the overall physiological state, it is always the sum of the excreted acids that is considered). For instance, an increased excretion was observed together with an increased $q_o$ (after the addition of benzoic acid) and also together with a decreased $q_o$ (under phosphate limitation).

Therefore, it is advisable to discuss each set of conditions separately.

Ammonium-limited hyphae showed a higher $q_{\text{Glc}}$, $q_{\text{CO}_2}$ and $q_o$ compared to glucose-limited hyphae: 0.69 mmol g$^{-1}$ h$^{-1}$ additional carbon was taken up, 0.64 mmol g$^{-1}$ h$^{-1}$ additional CO$_2$ was produced, 0.29 mmol g$^{-1}$ h$^{-1}$ additional O$_2$ was consumed, and 0.36 mmol g$^{-1}$ h$^{-1}$ carbon was excreted in the form of organic acids (at $\mu = 0.1$ h$^{-1}$). The sum of CO$_2$ plus organic acids is higher than the amount of additional consumed carbon. This can be explained as follows. Ammonium-limited hyphae had a lower nitrogen (see biomass formulae) and protein content [27.6% (w/w) versus 43.5% (w/w) in glucose-limited hyphae]. Because of this, less glucose was needed for protein synthesis (precursor formation and energy). In this situation, part of the surplus glucose was oxidized to CO$_2$ and part was ‘spilled’ via the excretion of intermediates.

The low O$_2$ consumption under phosphate limitation was obviously caused by the limitation of phosphate and
Nitrate-limited hyphae showed a high glycolytic flux (i.e. a high \( q_{\text{Glc}} \)) in ammonium-limited hyphae. This bottle-neck, together with a high glycolytic flux led to a high excretion of intermediates, mainly in the form of citrate. Phosphate limitation is also important because a lot of NADPH was needed for the reduction of nitrate to amino-nitrogen. Comparing ammonium- and nitrate-limited growth, we can conclude that a high glycolytic flux is a general prerequisite for a high excretion rate of TCA cycle intermediates. If ammonium-limited hyphae were challenged with benzoic acid at pH 5, \( q_{\text{Glc}} \), \( q_{\text{O}} \), and \( q_{\text{CO}_2} \) increased. This increase must have been due to a higher energy demand, because benzoic acid uncouples the proton gradient at the plasma membrane (Verduyn et al., 1992). Unlike phosphate limitation, it was not a bottle-neck that increased the excretion of intermediates, but the unequal rates of glycolysis, the TCA cycle and the respiratory electron-transport chain. Of the additional consumed carbon, 33% was oxidized to \( \text{CO}_2 \) and less than 5% was excreted in the form of organic acids. Another uncoupler, 2,4-dinitrophenol, decreased the excretion of intermediates (similar to the observation in A. niger; Netik et al., 1997).

That SHAM did not change the \( q_{\text{Glc}} \) and \( q_{\text{O}} \) but increased \( Y_{\text{Glc}X} \) by 23% can be explained as follows. Because more biomass was formed per glucose consumed, more ATP must have been synthesized. Part of the flow of electrons must therefore have been redirected from non-proton-pumping respiration to proton-pumping respiration. This implies that (i) the synthesis of biomass was limited by ATP synthesis, and (ii) during glucose-sufficient growth non-proton-pumping, alternative respiration was a constitutive part of respiration.

Further conclusions can be drawn from the results gained from these experiments with \( P. \text{simplicissimum} \). First, because citrate was always excreted in highest amounts (Fig. 3), we can term this an energy-spilling process, which avoids the synthesis of too much NADH or ATP, respectively. Second, overflow metabolism in \( P. \text{simplicissimum} \) was inversely correlated with \( Y_{\text{Glc}X} \), but not with the biomass yield on \( \text{O}_2 \) (\( Y_{\text{O}_2X} \)). This is in contradiction to Linton (1991), who stated that rapid metabolite production is inversely related to \( Y_{\text{O}_2X} \). If we compare overflow metabolism in \( P. \text{simplicissimum} \) with overflow metabolism in other micro-organisms, we find common themes but different features. One common feature is that no intermediates are excreted if the energy source is the limiting nutrient. Another similarity is that an increase in the concentration of the carbon and energy source increases the excretion of intermediates (Liu (1998); Klebsiella aerogenes (Klebsiella pneumoniae), Hueting & Tempest (1979); A. niger, Papagianni et al. (1999); Methanobac-

**Fig. 4.** Effect of benzoic acid on an ammonium-limited chemostat culture of \( P. \text{simplicissimum} \). (a) Effect of benzoic acid on the specific uptake rates of glucose (■) and \( \text{O}_2 \) (△), as well as on the rate of \( \text{CO}_2 \) formation (▲) and the RQ (○). The arrow indicates the addition of benzoate (5 mM). (b) Effect of benzoic acid on the excretion of organic acids: total acids (■), citrate (□), succinate (▲), malate (○), pyruvate (+) and oxalate (△). The arrow indicates the addition of benzoate (5 mM).

**Fig. 5.** Dependence of the total productivity of excreted TCA cycle intermediates on \( \mu \) in ammonium- (○) and phosphate-limited (■) chemostat cultures of \( P. \text{simplicissimum} \).
terium thermoautotrophicum, Liu et al. (1999)]. We observed the same with P. simplicissimum if we increased the glucose concentration in the medium reservoir from 100 to 400 mM (data not shown).

Nutrient limitations other than glucose increased $q_{\text{Glc}}$ and $q_{\text{CO}_2}$ in K. aerogenes much more than in P. simplicissimum, the $q_{\text{Glc}}$ of K. aerogenes being highest under phosphate limitation (Neijssel & Tempest, 1975). This is a clear difference to the situation in P. simplicissimum. The catabolic pathways seem to be more strictly regulated in P. simplicissimum than in K. aerogenes. With both K. aerogenes (Neijssel & Tempest, 1975) and Escherichia coli (Holms, 1996), pyruvate and/or acetate are the major excreted metabolites, whereas in P. simplicissimum these intermediates did not play an important role.

The most interesting micro-organisms for comparison with P. simplicissimum are clearly other filamentous fungi. If we look at the sum of the excreted acids, the relationship between $\mu$ and the excretion of TCA cycle intermediates is similar in ammonium-limited hyphae of P. simplicissimum, Aspergillus foetidus (Kristiansen & Sinclair, 1979) and A. niger (Kristiansen & Charley, 1981). However, looking at only citrate excretion under either ammonium limitation (Gallmetzer & Burgstaller, 2001) or phosphate limitation (Fig. 5) shows that other relationships between $\mu$ and productivity are possible. In A. niger, the respiratory quotient (RQ) during citrate production in the growth phase is always distinctly lower than 1 (Foster, 1949; Kubicek et al., 1980; McIntyre & McNeil, 1997; all authors used batch cultivation). In P. simplicissimum, an RQ $< 1$ was only observed under phosphate limitation (0.7, Table 3). As is assumed for A. niger, a high activity of CO$_2$-fixing anaplerotic reactions would explain this low RQ. The RQ of 1–3 found under ammonium limitation is obviously not an indication of a fermentative metabolism, but seems to be a consequence of the overflow metabolism. In both fungi, i.e. A. niger and P. simplicissimum, the so-called alternative respiration seems to be constitutive during growth (Kubicek et al., 1980; Wallrath et al., 1992; Kirmira et al., 1987).

The rate of glucose consumption by P. simplicissimum depended strongly on the specific growth rate, but only weakly on the kind of nutrient limitation (Figs 1 and 2). In contrast, $q_{\text{Glc}}$ and $q_{\text{CO}_2}$ varied much more with the kind of nutrient limitation. This means that the hyphae took up as much carbon as the growth rate allowed and then adjusted the activity of catabolic pathways to meet the different demands of the different nutrient limitations. Maintaining a high flux of the carbon and energy source is probably a characteristic feature of soil microorganisms (Teixeira de Mattos & Neijssel, 1997). High metabolic fluxes serve as an adaptation to low nutrient concentrations in order to maximize the growth rate (Westerhoff et al., 1982). A high flux means that the energetic efficiency of the overall metabolism is lowered (Teixeira de Mattos & Neijssel, 1997). Additionally, soil micro-organisms were never forced to develop a control of glucose uptake at high glucose concentrations, because of the low carbon concentration in soil. Both features result in the necessity to spill carbon and energy during growth at a high carbon concentration. The advantage of an overflow metabolism compared to other energy-spilling processes (such as alternative respiration, futile cycles) would be that the synthesis of too much NADH is avoided.

Thermodynamically viewed, a high flux is coupled with a lower efficiency of energy conversion (Teixeira de Mattos & Neijssel, 1997; Westerhoff et al., 1982). This could, in principle, explain an uncoupling of catabolism and anabolism at high rates of carbon flux (Westerhoff et al., 1982). However, it is not clear if this concept – which has been developed from physically coupled flows, such as oxidative phosphorylation (Stucki, 1980) – can be applied to the uncoupling of catabolism and anabolism, where the mechanistic kind of coupling is unclear. It is probably more reasonable to look for a physiological explanation for overflow metabolism than for a thermodynamic explanation.

From the results presented, a general strategy to increase the excretion of intermediates of primary metabolism can be suggested: (i) look for the kind of nutrient limitation which gives the highest excretion rate; (ii) with this nutrient limitation determine the growth rate for maximum productivity; and (iii) maximize the non-growth energy consumption (e.g. by low potassium concentration, high pH, high osmolarity, addition of benzoic acid, etc.). Additionally, if glucose uptake is limiting (which is not the case with P. simplicissimum) an auxiliary carbon source can be added (Babel et al., 1993). This strategy should be valid in general for increasing an overflow metabolism in a filamentous fungus, although it will be modulated by the unique genotype of each strain.

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REFERENCES


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