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GpdA-Promoter-Controlled Production of Glucose Oxidase by Recombinant *Aspergillus niger* Using Nonglucose Carbon Sources

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Abstract

The *gpdA*-promoter-controlled exocellular production of glucose oxidase (GOD) by recombinant *Aspergillus niger* NRRL-3 (GOD3-18) during growth on glucose and nonglucose carbon sources was investigated. Screening of various carbon substrates in shake-flask cultures revealed that exocellular GOD activities were not only obtained on glucose but also during growth on mannose, fructose, and xylose. The performance of *A. niger* NRRL-3 (GOD3-18) using glucose, fructose, or xylose as carbon substrate was compared in more detail in bioreactor cultures. These studies revealed that *gpdA*-promoter-controlled GOD synthesis was strictly coupled to cell growth. The *gpdA*-promoter was most active during growth on glucose. However, the unfavorable rapid GOD-catalyzed transformation of glucose into gluconic acid, a carbon source not supporting further cell growth and GOD production, resulted in low biomass yields and, therefore, reduced the advantageous properties of glucose. The total (endo- and exocellular) specific GOD activities were lowest when growth occurred on fructose (only a third of the activity that was obtained on glucose), whereas utilization of xylose resulted in total specific GOD activities nearly as high as reached during growth on glucose. Also, the portion of GOD excreted into the culture fluid reached similar high levels (≅ 90%) by using either glucose or xylose as substrate,

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whereas growth on fructose resulted in a more pelleted morphology with more than half the total GOD activity retained in the fungal biomass. Finally, growth on xylose resulted in the highest biomass yield and, consequently, the highest total volumetric GOD activity. These results show that xylose is the most favorable carbon substrate for *gpdA*-promoter-controlled production of exocellular GOD.

Index Entries: *Aspergillus niger*; glucose oxidase; *gpdA*-promoter.

Introduction

Glucose oxidase (GOD); (β-D-glucose: oxygen, 1-oxidoreductase; EC 1.1.3.4) is an enzyme of considerable industrial importance. Among other applications, it is used for the enzymatic determination of glucose in commercial glucose analysis kits and as immobilized enzyme in biosensors.

GOD is most commonly produced by the filamentous fungus *Aspergillus niger*. The synthesis of this enzyme by wild-type *A. niger* is observed only when cell growth occurs in excess of glucose and at high concentrations of molecular oxygen *(1)*. GOD is a flavoprotein that catalyzes the oxidation of glucose to glucono-δ-lactone with a concomitant reduction of molecular oxygen to hydrogen peroxide. In *A. niger*, the cell-toxic hydrogen peroxide is subsequently degraded by different catalases, and glucono-δ-lactone is transformed into gluconic acid either spontaneously or catalyzed by lactonase *(1)*.

In the past, several attempts have been made to increase protein production by *A. niger* using genetic engineering techniques. The expression signals of homologous genes controlled by strong promoters have been used to direct the synthesis of proteins of interest. For example, the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpdA*), a key enzyme in glycolysis and gluconeogenesis, is an extremely efficiently expressed constitutive gene in *Aspergillus nidulans (2)* whose expression signal has been used for homologous and heterologous protein production by recombinant *Aspergilli* with the utilization of glucose as supporting carbon source for cell growth and product formation *(3,4)*.

In addition, a recombinant strain of *A. niger* has been generated that allows *gpdA*-promoter-controlled production of GOD *(5)*. The recombinant strain *A. niger* NRRL-3 (GOD3-18), carrying multiple copies of the *god* gene fused to the α-amylase signal sequence and controlled by the *gpdA*-promoter, can produce four times more exocellular GOD compared to the respective wild-type strain grown under identical culture conditions with glucose as the carbon source. However, GOD being synthesized by the fungal cells during growth on glucose then rapidly catalyzes the transformation of the remaining glucose into gluconic acid, a carbon substrate that is not favorable for further cell growth and GOD production. Therefore, substitution of glucose by nonglucose carbon sources could be advantageous for further improvement of exocellular GOD production. In the present study, we have examined various nonglucose carbon substrates

with regard to their suitability to replace glucose as the carbon source during *gpdA*-promoter-controlled production of GOD by recombinant *A. niger* NRRL-3 (GOD3-18).

Materials and Methods

Microorganism and Cultivation Medium

The construction of recombinant *A. niger* NRRL-3 (GOD 3-18), carrying multiple copies of the *god* gene fused to the α-amylase signal sequence and controlled by the *gpdA*-promoter, has been described before *(5)*. CM-agar plates for sporulation and GOD indicator pates were prepared as described previously *(5)*. The composition of the standard medium for shake-flask and bioreactor cultivations was as follows: 3 g/L of $NaNO_3$, 1 g/L of K_2HPO_4, 0.5 g/L of $MgSO_4$, 0.5 g/L of KCl, 0.01 g/L of $FeSO_4$, 2 g/L of yeast extract (Difco), and 80 g/L of glucose. When indicated, glucose was replaced by other carbon sources (2.7 mol/L based on the number of carbon atoms for comparability, i.e., equivalent to 80 g/L of glucose).

Shake-Flask Cultivations

Cultivations were carried out in 100-mL Erlenmeyer flasks with a 20-mL culture volume. The flasks were inoculated with 1×10^7 spores/mL and incubated in a rotary shaker (Pilot Shake Rc 6SR; B. Braun Diesel Biotech GmbH, Melsungen, Germany) at 125 rpm and 30°C. Cultivations were always carried out in duplicate.

Bioreactor Cultivations

Bioreactor cultivations were carried out in a 5-L stirred-tank bioreactor Biostat MD (B. Braun Diesel Biotech GmbH) with a working volume of 3 L essentially as described previously *(5)*, except that the airflow rate was adjusted at 0.75 vvm during the first 5 h and increased to 1.5 vvm for the rest of the cultivation. The concentrations of oxygen and carbon dioxide in the exhaust gas of bioreactor cultures were determined by paramagnetic and infrared gas analysis systems, respectively (Maihak, Germany). Dissolved oxygen concentrations were analyzed by a polarographic electrode (Ingold, Germany).

Off-Line Analysis

Cell disruption and sample preparation for analysis of endo- and exocellular GOD activities were carried out as described previously *(6)*. Determination of GOD activities and cell dry wt measurements were performed as before *(5)*. Sugars, organic acids, and xylitol were analyzed by high-performance liquid chromatography as described previously *(5)*. Nitrate was determined using an ion-sensitive electrode model Orion 93-07 (reference electrode model Orion 90-02; both from Orion Research, Boston,

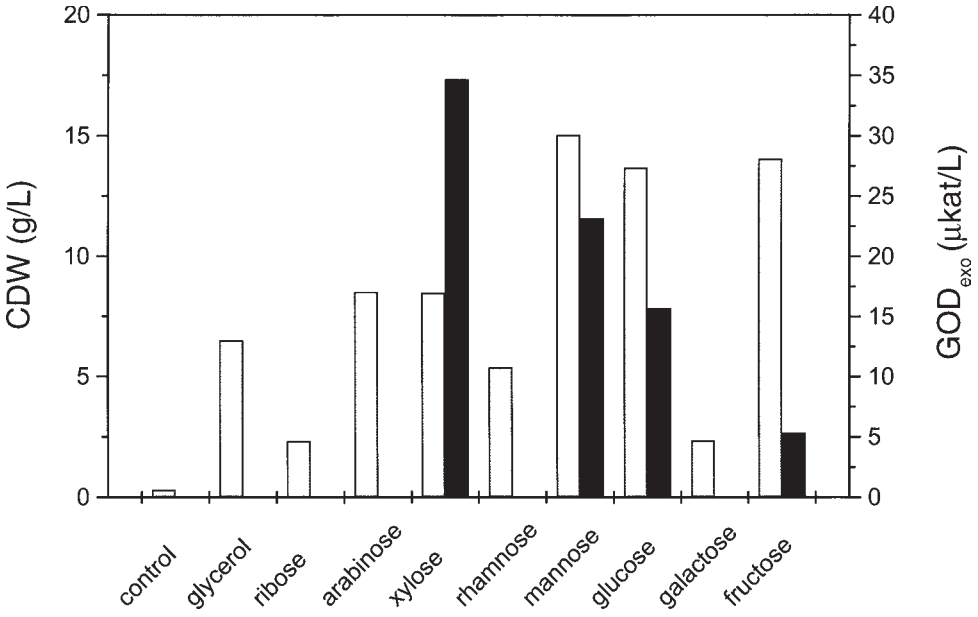

Fig. 1. Exocellular GOD production by recombinant *A. niger* NRRL-3 (GOD3-18) during growth on different carbon sources. Cell dry wt (shaded columns) and exocellular GOD activity (solid columns) were determined after 48 h of growth in shake flask cultures. CDW, cell dry wt.

MA). Phosphate concentrations were analyzed according to the German Standard Methods *(7)*.

Results and Discussion

Cell Growth and Exocellular GOD Production in Shake-Flask Cultures Using Glucose and Nonglucose Carbon Sources

Preliminary comparative studies of GOD production with recombinant *A. niger* NRRL-3 (GOD3-18) using glucose and nonglucose carbon sources were carried out in shake-flask cultures. Cell growth and GOD formation were investigated using polyols (glycerol) and pentose (ribose, arabinose, and xylose) and hexose monosugars (rhamnose, mannose, glucose, galactose, and fructose) as carbon substrates. Cell growth occurred on all carbon sources tested (Fig. 1). However, exocellular GOD activities were detected only during growth on glucose, fructose, xylose, and mannose (Fig. 1). Because of the high price, mannose was not considered an appropriate carbon source for larger-scale cultivations. Therefore, more detailed studies on the cellular performance with respect to exocellular GOD production on nonglucose carbon substrates were restricted to the utilization of fructose and xylose.

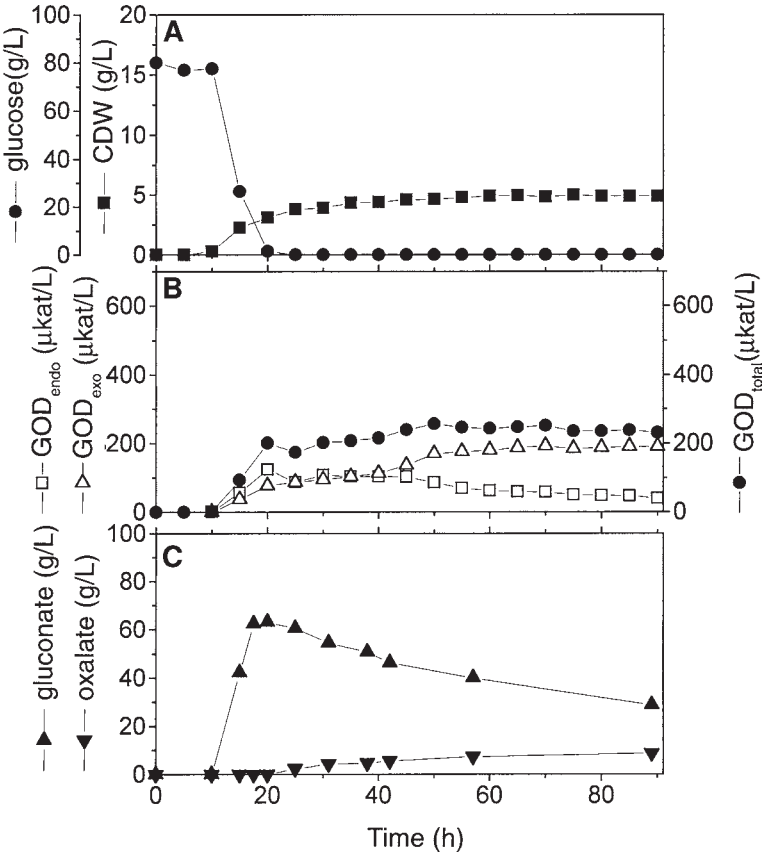

Fig. 2. Performance of recombinant *A. niger* NRRL-3 (GOD3-18) during growth on glucose in bioreactor culture. The time course data of **(A)** cell dry wt (■) and glucose concentrations (●), **(B)** the endo- (□) and exocellular (△) and the total volumetric GOD activities (●), and **(C)** the gluconic (▲) and oxalic acid concentrations (▼) are shown. CDW, cell dry wt.

Cell Growth and Exocellular GOD Production in Bioreactor Cultures Using Glucose and Nonglucose Carbon Sources

The performance of recombinant *A. niger* NRRL-3 (GOD3-18) during growth on glucose, fructose, and xylose was compared in more detail in bioreactor cultures. Except for the carbon substrate, cells were grown under identical conditions.

Bioreactor Performance During Growth on Glucose

Figure 2 summarizes the experimental results from a bioreactor culture of *A. niger* NRRL-3 (GOD3-18) grown on glucose. Cell growth and GOD synthesis were observed simultaneously during glucose consumption (Fig. 2A,B). The resulting GOD catalyzed a rapid oxidative transformation of glucose into gluconic acid and, thus, reduced the effective carbon

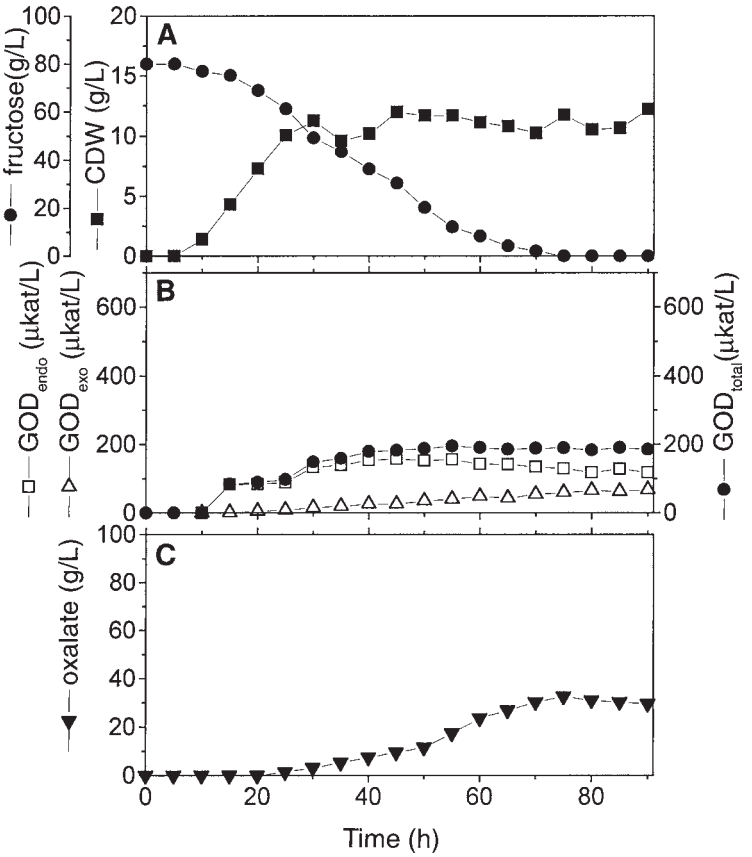

Fig. 3. Performance of recombinant *A. niger* NRRL-3 (GOD3-18) during growth on fructose in bioreactor culture. The time course data of **(A)** cell dry wt (■) and fructose concentrations (●), **(B)** the endo- (□) and exocellular (△) and the total volumetric GOD activities (●), and **(C)** the concentration of oxalic acid (▼) are shown. CDW, cell dry wt.

substrate suitable for further cell growth and GOD production (Fig. 2C). Accordingly, after exhaustion of glucose, no significant cell growth and synthesis of GOD occurred. The analysis of phosphate and nitrate concentrations in the culture broth did not show that growth-limiting concentrations of these substrates were reached (data not shown), additionally supporting the conclusion that cell growth and GOD synthesis were limited by the presence of glucose. GOD accumulated first as cell-bound (endocellular) enzyme and was subsequently slowly released into the culture fluid (Fig. 2B), as has been reported previously *(6)*. Because of the slow excretion kinetics, exocellular GOD appeared to be produced in a non-growth-associated manner. However, summing up the endo- and exocellular GOD activities clearly revealed that synthesis of GOD was restricted to the time period of cell growth (Fig. 2A,B). After depletion of glucose, gluconic acid was further converted into oxalic acid (Fig. 2C) and carbon dioxide (data not shown). Other acidic byproducts were not detected in significant amounts (data not shown).

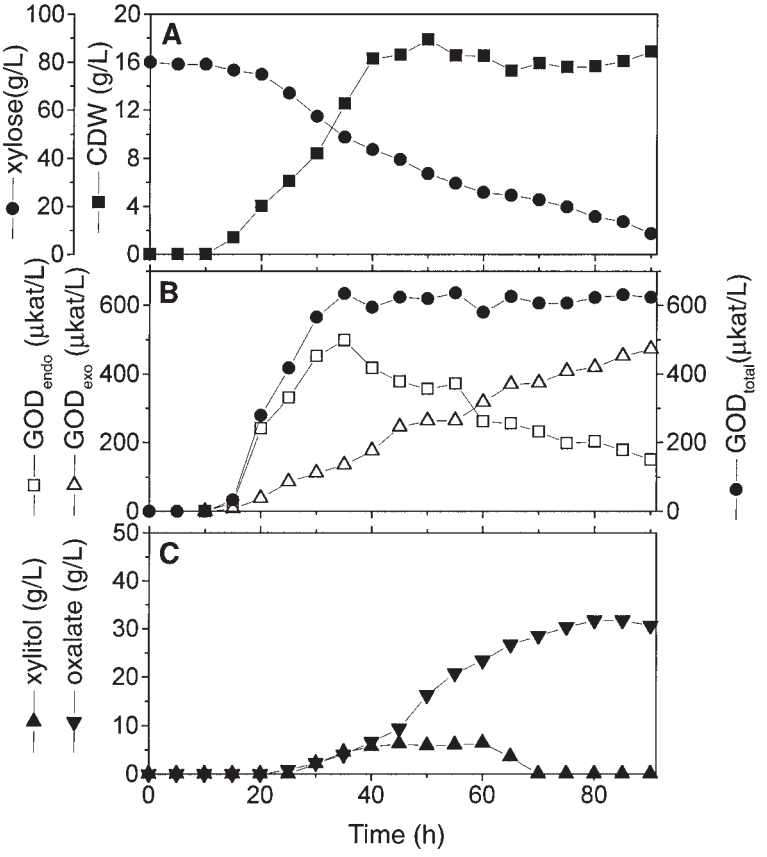

Fig. 4. Performance of recombinant *A. niger* NRRL-3 (GOD3-18) during growth on xylose in bioreactor culture. The time course data of **(A)** cell dry wt (■) and xylose concentrations (●), **(B)** the endo- (□) and exocellular (△) and the total volumetric GOD activities (●), and **(C)** the concentrations of xylitol (▲) and oxalic acid (▼) are shown. CDW, cell dry wt.

Bioreactor Performance During Growth on Fructose

Figure 3 presents the experimental results from a bioreactor culture of *A. niger* NRRL-3 (GOD3-18) grown on fructose. As observed during growth on glucose, GOD was synthesized in a growth-associated manner, accumulated first as cell-bound enzyme and subsequently was released slowly into the culture fluid (Fig. 3A,B). Cessation of cell growth and GOD production could be attributed neither to limiting concentrations of fructose (Fig. 3A) nor to limiting concentrations of phosphate and nitrate (data not shown). And, as expected, no gluconic acid was formed, and oxalic acid accumulated as the only acidic byproduct (Fig. 3C).

Bioreactor Performance During Growth on Xylose

Figure 4 presents the performance of *A. niger* NRRL-3 (GOD3-18) during growth on xylose. Again, synthesis of GOD was observed only during the time period of cell growth (Fig. 4A,B). Also, as observed during

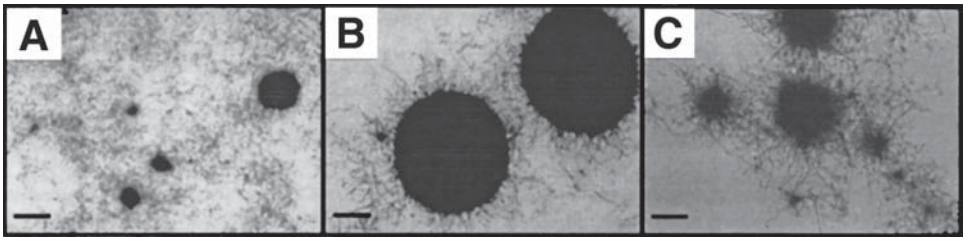

Fig. 5. Fungal morphology in bioreactor cultures during growth on **(A)** glucose, **(B)** fructose, and **(C)** xylose. Samples were taken 50 h postinoculation. Bars = 300 µm.

growth on glucose and fructose, GOD accumulated first as cell-bound enzyme and subsequently was released slowly into the culture broth (Fig. 4B). The phosphate and nitrate concentrations did not reach growth-limiting concentrations (data not shown). As expected, no formation of gluconic acid was observed when xylose was used as the carbon substrate. Instead, growth on xylose was accompanied by the transient accumulation of xylitol (Fig. 4C). As observed during growth on glucose and fructose, oxalic acid was the only acidic byproduct (Fig. 4C).

GOD Excretion and Fungal Morphology During Growth on Glucose, Fructose, and Xylose

Comparison of the time course of endo- and exocellular GOD activities during growth on the different sugar carbon substrates in bioreactor cultures revealed that growth on glucose resulted in the fastest release of cell-bound GOD into the culture fluid (Fig. 2B). The release of cell-bound GOD into the culture medium occurred most slowly during growth on fructose (Fig. 3B). Also, during growth on fructose nearly half the GOD remained cell bound, even after prolonged cultivation. Intermediate excretion kinetics were observed when xylose was used as carbon substrate (Fig. 4B). Studies on the morphology of the fungus during growth on the different sugar substrates revealed a more filamentous morphology with small pellets when glucose was used as the carbon substrate (Fig. 5A). More pelleted growth was observed using fructose as the carbon source (Fig. 5B), and the fungus exhibited an intermediate morphology during growth on xylose (Fig. 5C). These observations indicate that a more pelleted growth form results in a more severe delay of GOD release into the culture fluid and, moreover, decreases the portion of the enzyme that can be excreted. These findings are in agreement with previously reported results that culture conditions that cause the formation of bigger pellets also result in lower exocellular GOD activities *(8)*.

Conclusion

Table 1 summarizes the bioreactor performance of *A. niger* NRRL-3 (GOD3-18) with respect to GOD production. The highest total specific GOD

Table 1
Comparison of Performance of Recombinant *A. niger* NRRL-3 (GOD3-18) After 120 h of Growth in Bioreactor Cultures Using Glucose, Fructose, and Xylose as Carbon Substrates

	Glucose	Fructose	Xylose
Cell dry wt (g/L)	4.9	10.2	14.9
Volumetric total GOD activity (μkat/L)	234.0	167.0	626.0
Specific total GOD activity (μkat/g of cell dry wt)	48.0	16.0	42.0
Exocellular GOD activity (%)	89.0	47.0	87.0

activity was reached when glucose was used as the carbon substrate, indicating that the *gpdA*-promoter is most active during growth on this carbon source. However, the unfavorable rapid GOD-catalyzed transformation of glucose into gluconic acid, a carbon substrate that is not favorable for further cell growth and GOD production, reduces biomass and product yields and, therefore, reduces the usefulness of glucose as the carbon source for GOD production. The total specific GOD activity was lowest when growth occurred on fructose, whereas utilization of xylose resulted in a total specific GOD activity nearly as high as obtained during growth on glucose. In addition, the portion of GOD excreted into the culture fluid reached similar high levels by using either glucose or xylose as substrate, whereas growth on fructose resulted in a more pelleted morphology with more than half the total GOD activity retained in the fungal biomass. Finally, growth on xylose resulted in the highest biomass yield and, consequently, in the highest total volumetric GOD activity. Altogether, these results show that xylose is the most favorable carbon substrate for *gpdA*-promoter-controlled production of exocellular GOD.

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