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In-depth analysis of the *Aspergillus niger* glucoamylase (*glaA*) promoter performance using high-throughput screening and controlled bioreactor cultivation techniques

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Abstract

An in-depth characterization of the *A. niger* glucoamylase (*glaA*) promoter performance was carried out on defined medium employing multi-well high-throughput screening as well as controlled batch and fed-batch bioreactor culture techniques with GFP as a fluorescent reporter protein. A variety of metabolizable carbon substrates and non-metabolizable analogs were screened with regard to their effect on the *glaA* expression system. The results clearly demonstrate that only starch and its hydrolytic products, including glucose, act as inducers. However, induction of the *glaA* expression system through the monosaccharide glucose is significantly lower compared to starch and the higher molecular weight starch degradation products. All other 26 carbon substrates tested do not induce, or even, as in the case of the easily metabolizable monosaccharide xylose, repress *glaA*-promoter controlled gene expression in the presence of the inducing disaccharide maltose with an increase of repression strength by increasing xylose concentrations. The complex effect of glucose on *glaA*-promoter controlled expression was also analyzed using non-metabolizable glucose analogs, namely 5-thio-glucose and 2-deoxyglucose, which were identified as novel and potent inducers of the *glaA* expression system. The results show that the induction strength depends on the inducer concentration with a maximum at defined concentrations and lower induction or even repression at concentrations above. Moreover, controlled fed-batch cultivations using a high maltose feed rate with concomitant extracellular accumulation of glucose resulted in lower levels of the reporter protein compared to cultures with a low maltose feed rate without extracellular glucose accumulation, thus supporting the conclusion that increasing the glucose concentration beyond a critical point reduces the induction strength or may even cause repression. This way, the speed of polymer hydrolysis, glucose uptake and intracellular breakdown can be fine-tuned for optimal fungal growth and the metabolic burden for glucoamylase synthesis can be limited adequately in response to nutrient availability.

1. Introduction

The *Aspergillus niger* glucoamylase (GlaA) is an enzyme of considerable industrial importance used in the sugar, baking and alcohol industry for starch hydrolysis (Crabb and Mitchinson, 1997). Moreover, glucoamylase is the most efficiently secreted protein of *A. niger*, thus, the homologous *glaA*-promoter as well as the glucoamylase signal sequence are widely used for heterologous protein production. Regulation of *glaA*-promoter controlled protein synthesis is not fully understood, though it seems clear that *glaA* expression is regulated on the level of carbon catabolite repression in a CreA-dependent way (Verdoes et al., 1994b) as the expression of many other genes encoding hydrolytic enzymes involved in the degradation of polysaccharides (Ruijter and Visser, 1997). It is well established, that *glaA*-promoter controlled protein synthesis occurs when the growth medium contains starch or its degradation products maltodextrin, maltose or glucose, and that synthesis is not observed when growth occurs on xylose as carbon source (Carrez et al., 1990; Fowler et al., 1990; Ganzlin et al., 2007; Nunberg et al., 1984; Santerre Henriksen et al., 1999; Schrickx et al., 1993; Siedenberg et al., 1999; van den Hondel et al., 1991; Verdoes et al., 1994b; Withers et al., 1998).

Here, we present an in-depth characterization of the *glaA*-promoter performance of *A. niger* using 33 different metabolizable and non-metabolizable carbon substrates and combinations thereof with a mutant GFP as the fluorescent reporter protein. Experiments were performed on defined medium employing a novel high-throughput screening technique based on 24-well plates. Moreover, results obtained from high-throughput screening were additionally verified in controlled batch and fed-batch cultivations.

2. Materials and methods

2.1. Recombinant strain, medium, analytical methods and spore preparation

The GFP-expressing recombinant *A. niger* strain (AB6.4Δ*pepE*[pAN52-10S65TGFPn/s]Ac5) was generated by introducing a synthetic gene encoding red-shifted GFP (Sheen et al., 1995) under control of the *glaA*-promoter into *A. niger* AB6.4Δ*pepE* (Siedenberg et al., 1999), which is a fawncoloured (*fwnA*) glucoamylase (Δ*glaA*)- and vacuolar protease-deficient (Δ*pepE*) derivative of *A. niger* AB4.1 (Verdoes et al., 1994c; Verdoes et al., 1994a). *A. niger* AB4.1 is a *pyrG* mutant (requiring uridine or uracil for growth) of *A. niger* N402 (van Hartingsveldt et al., 1987), which is a *cspA*-derivative (short conidiophores) of *A. niger* ATCC9029 (=NRRL3) (Bos et al., 1988).

A defined growth medium (modified Vogel's medium) without addition of vitamins and with ammonium as sole nitrogen source was used (Vogel, 1956). The basic composition was as follows: Na_3·Citrate·$2H_2O$, 2.85 g·L^{-1}; KH_2PO_4, 5 g·L^{-1}; $(NH_4)_2SO_4$, 7 g·L^{-1}; $CaCl_2$·$2H_2O$, 0.1 g·L^{-1}; $MgSO_4$·$7H_2O$, 0.2 g·L^{-1}; and trace elements (citric acid·H_2O, 5 mg·L^{-1}; $ZnSO_4$·$7H_2O$, 5 mg·L^{-1}; $Fe(NH_4)_2(SO_4)_2$·$6H_2O$, 1 mg·L^{-1}; $CuSO_4$, 0.16 mg·L^{-1}; $MnSO_4$·H_2O, 0.037 mg·L^{-1}; H_3BO_3, 0.05 mg·L^{-1}; and Na_2MoO_4·$2H_2O$, 0.05 mg·L^{-1}). The trace element solution was stored as 10,000-fold concentrated sterile-filtered stock solution. The salt solution (Na_3·Citrate·$2H_2O$, KH_2PO_4, NH_4NO_3, and $CaCl_2$·$2H_2O$) was stored as 50-fold concentrated sterile-filtered stock solution. The sugar carbon source (initial concentration 10 g·L^{-1}), uridine (initial concentration 2.44 g·L^{-1}), and for bioreactor cultivations the antifoam reagent Ucolub N115 (0.1 ml·L^{-1}) were sterilized for 30 min at 121°C prior to inoculation. The composition of the feed medium used during fed-batch cultivation was as follows:·maltose, 50 or 170 g·kg^{-1} for low-maltose or high-maltose feeding conditions, respectively; uridine 6 g·L^{-1}, Na_3·citrate·$2H_2O$, 5.7 g·L^{-1}; KH_2PO_4, 10 g·L^{-1}; $(NH_4)_2SO_4$,

14 $g \cdot L^{-1}$; $CaCl_2 \cdot 2H_2O$, 0.2 $g \cdot L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.4 $g \cdot L^{-1}$; and trace elements (citric acid·H_2O, 10 $mg \cdot L^{-1}$; $ZnSO_4 \cdot 7H_2O$, 10 $mg \cdot L^{-1}$; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2 $mg \cdot L^{-1}$; $CuSO_4$, 0.32 $mg \cdot L^{-1}$; $MnSO_4 \cdot H_2O$, 0.074 $mg \cdot L^{-1}$; H_3BO_3, 0.1 $mg \cdot L^{-1}$; and $Na_2MoO_4 \cdot 2H_2O$, 0.1 $mg \cdot L^{-1}$).

Quantification of S65TGFP, cell dry mass, and sugar concentrations were carried out as described previously (Siedenberg et al., 1999).

Spores for inoculation were obtained from 3.8% ($w \cdot v^{-1}$) potato-dextrose-agar plates, suspended in 0.9% ($w \cdot v^{-1}$) NaCl solution, and stored at -70°C prior to inoculation.

2.2. Cultivation in 24-well culture plates

For high-throughput screening of fungal promoter performance, a novel procedure for reproducibly growing *A. niger* AB6.4Δ*pepE*[pAN52-10S65TGFPn/s]Ac5 as confluent mycelium layer on the bottom of 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, USA) was developed. A stock culture of spores frozen at -70°C was used for inoculation (basic modified Vogel's medium supplemented with 10 $g \cdot L^{-1}$ xylose, $4 \cdot 10^4$ spores·mL^{-1}, final volume 1.6 mL per well). These conditions warrented that a confluent mycelium layer firmly attached to the bottom of the well of low relative fluorescence intensity (RFI < 10%) and a mycelium free supernatant were reached after 96 h at 100 rpm and 30°C in a multi-well plate reader (Type CytoFluor™ 4000, PerSeptive Biosystems, Farmingham, USA). Subsequently, the mycelium free supernatant was removed and, after rinsing the firmly attached mycelium with basic modified Vogel's medium, 1.5 mL basic modified Vogel's medium additionally containing carbon substrates (10 $g \cdot L^{-1}$) as specified in the Results and Discussion section were added. As positive control, maltose (10 $g \cdot L^{-1}$) was used for *glaA*-promoter induction in 5 wells of each plate. The mean of fluorescence intensities in these wells were taken as 100% relative fluorescence intensity (RFI). In another well growth on xylose (10 $g \cdot L^{-1}$) was used as negative control (RFI < 15%). The remaining 18 wells were used to test three different carbon

substrates per plate (6 repetitions/wells per carbon substrate, 10 $g \cdot L^{-1}$). The reading of each well was normalized against the mean of the positive control. Two plates were always loaded for each combination, thus, 12 repetitions/wells per carbon substrate were obtained. From these measurements, the mean and the standard error of the mean were determined. When testing non-metabolizable inducers, citrate (10 $g \cdot L^{-1}$) was added to the basic modified Vogel's medium. Incubation was carried out for 24 h at 100 rpm and 25°C followed by automatic measurement of the GFP fluorescence intensity (excitation: 485 - 505 nm; emission: 530 - 560 nm; sensitivity: 90; average of 30 measurements) using the multi-well plate reader (Type CytoFluor^{TM} 4000, PerSeptive Biosystems, Farmingham, USA). Final pH values, determined by a small glas electrode, differed only by 0.5 pH units using different carbon substrates.

2.3. Bioreactor cultivations

Batch cultivation was carried out in a 5-L bioreactor with three six-bladed Rushton turbine impellers (Type Biostat MD; B. Braun Diessel Biotech GmbH / Sartorius AG, Melsungen / Göttingen, Germany) at 25°C, pH 5.5, stirrer speed 300 rpm and aeration with 1 vvm air using xylose as carbon substrate (Inoculum: final spore concentration of 10^6 spores$\cdot mL^{-1}$). After xylose depletion (65 h), four identical small-scale bioreactors (1-L working volume with two four-bladed Rushton turbine impellers, Type Biostat Q; B. Braun Diessel Biotech GmbH / Sartorius AG, Melsungen / Göttingen, Germany) were each filled with 700 mL of the above culture broth and growth was continued at 25°C, pH 5.5, 300 rpm and 1 vvm air by addition of 300 mL concentrated medium (3.33 times concentrated modified Vogel's medium) containing either xylose, fructose, glucose or maltose as carbon substrate. Wall growth was prevented in these small scale bioreactors by increasing the stirrer speed in 15 min intervals for one minute to 345 rpm.

Fed-batch cultivations were carried out essentially as described previously (Siedenberg et al., 1999). Briefly, a 50-L bioreactor (Type U50K; B. Braun Diessel Biotech GmbH / Sartorius AG, Melsungen / Göttingen, Germany) was inoculated using a final spore concentration of 10^6 spores·mL^{-1}. The pH was kept constant at pH 5.5 using NaOH (5.0 mol·L^{-1}). The stirrer speed (400 rpm) and aeration rate (13 L·min^{-1} air) were kept constant. After a batch phase with identical conditions using xylose as carbon substrate, two different maltose feeding strategies were applied to induce *glaA*-promoter controlled GFP production. In order to reach maltose-excess conditions a high initial pulse of maltose (5.0 g·L^{-1}) followed by a continuously increasing maltose feeding rate (0.091 g·min^{-1}·h^{-1}) was used. To establish maltose-limiting conditions a low pulse of maltose (0.5 g·L^{-1}) followed by a lower continuously increasing maltose feeding rate (0.028 g·min^{-1}·h^{-1}) was applied which resulted in maltose concentrations below the detection limit. In both types of fed-batch cultures, feeding was initiated after depletion of xylose. No growth limiting concentrations of uridine and ammonium were detected during the course of all fed-batch cultivations.

3. Results and Discussion

3.1. High throughput screening of different metabolizable carbon substrates on glaA-*promoter performance using GFP as reporter protein*

A variety of different soluble high- and low-molecular weight carbon substrates were screened and compared with respect to their effect on *glaA*-promoter controlled GFP production (Fig. 1). As expected, the starch-derived polysaccharides maltodextrin and amylose induced high levels of GFP fluorescence with the highest level obtained for the starch hydrolysate maltodextrin (Fig.1). Maltose induced the second highest level of GFP fluorescence. Strong induction of *glaA*-promoter controlled gene expression by maltose has been described before for glucoamylase production (Barton et al., 1972; Chiquetto et al., 1992; Fowler et al., 1990) as well as for *glaA*-promoter controlled production of heterologous proteins (Faus et al., 1998; Verdoes et al., 1994b; Ward et al., 1997; Wiebe et al., 2001). Maltotriose induced equally high levels of GFP fluorescence as maltose. High-level induction of *glaA*-promoter controlled gene expression by maltotriose was previously also reported for *A. oryzae* (Hata et al., 1992) and *A. terreus* (Ghosh et al., 1990) but not yet for *A. niger*. Moreover, glucose also induced GFP fluorescence up to 75% of the level reached by maltose corroborating previous results that glucose is inducing strongly - but not as strongly as maltose - *glaA*-promoter controlled gene expression (Barton et al., 1972; Fowler et al., 1990; Verdoes et al., 1994b).

The other carbon substrates tested did not induce significant GFP fluorescence and thus, cannot be considered as inducers of the *glaA* expression system in *A. niger*. For example, the disaccharides trehalose, sucrose and lactose, the monosaccharides xylose, fructose, mannose, rhamnose, galactose and ribose and the polyols mannitol, *myo*-inositol and sorbitol did not induce significant GFP fluorescence in agreement with the findings by (Barton et al., 1972) on carbohydrate effects on glucoamylase formation by *A. niger*. Also, absence of *glaA*-promoter controlled gene expression during growth on xylose has been confirmed by many authors

(Fowler et al., 1990; MacKenzie et al., 2000; Nunberg et al., 1984; Schrickx et al., 1993; Verdoes et al., 1994b; Withers et al., 1998). Moreover, glycerol did not induce significant GFP fluorescence consistent with the report by (Nunberg et al., 1984) on absence of glucoamylase formation by *A. niger* during growth on glycerol. The monosaccharide arabinose, the polyols arabitol, erythritol, and xylitol, the organic acids glucuronic acid, galacturonic acid, 2-ketoglutarate, succinate, oxalate, pyruvate, fumarate and citrate and glucosamin did not induce *glaA*-promoter controlled gene expression, findings that have not been reported before.

The results clearly demonstrate that only starch and its hydrolytic products, including glucose, act as inducers of *glaA*-promoter controlled gene expression. None of the other carbon substrates tested were able to induce or derepress *glaA*-promoter controlled gene expression but resulted in basal expression levels. This includes also the disaccharides trehalose, sucrose, and lactose, which also contain glucose moieties, and glucosamin, an amino sugar analog of glucose.

3.2. Repression of glaA-*promoter controlled gene expression by xylose*

It is well established that xylose does not induce *glaA*-promoter controlled gene expression. However, there are contradicting results concerning repression of *glaA*-promoter controlled protein synthesis by xylose. For example, (Verdoes et al., 1994b) reported reduced levels of expression when xylose was added to a maltose containing growth medium. On the other hand, (Fowler et al., 1990) did not observe a negative effect of xylose addition on glucoamylase synthesis in a culture growing on maltose.

To clarify the effect of xylose on *glaA* controlled gene expression screens for GFP expression were performed in 24-well culture plates where the maltose containing growth medium was additionally supplemented with various concentrations of xylose (Fig. 1B, insert). All cultures showed significant GFP expression, however, increasing xylose concentrations caused

decreasing GFP fluorescence, thus, clearly proving that xylose is not only a non-inducer of *glaA*-promoter controlled gene expression but represses expression in the presence of the inducing carbon substrate maltose with an increase of repression by increasing xylose concentrations. In contrast, increasing concentrations of citrate, a non-inducing carbon substrate, to a maltose grown culture did not reduce *glaA*-promoter controlled gene expression, and, thus citrate can not be considered as a repressing carbon substrate (Fig. 1B, insert).

3.3. Induction and repression of glaA-*promoter controlled gene expression by non-metabolizable analogs of D-glucose*

Reports on the effect of glucose on *glaA*-promoter function have revealed conflicting results when glucose was supplied in addition with other carbon substrates. As glucose will not only act as an inducer of *glaA*-promoter controlled gene expression but is also readily metabolized two different types of non-metabolizable glucose analogs (5-thio-glucose and 2-deoxyglucose) were used to study their concentration-dependent effects on *glaA*-promoter controlled gene expression in the presence of the non-inducing and non-repressing carbon substrate citrate. Both analogs exhibit growth-inhibiting effects at higher concentrations and have been widely used to isolate glucose derepressed yeasts and filamentous fungi after chemical or UV mutagenesis (Egilsson et al., 1986; Fiedurek et al., 1987; Ghosh et al., 1991; Matosic et al., 1996).

5-Thio-glucose as well as 2-deoxyglucose induced the production of GFP in a concentration-dependent manner (Fig. 2). 2-Deoxyglucose was identified as the strongest inducer of *glaA*-promoter controlled gene expression, the maximum fluorescence was reached at 20 $mmol \cdot L^{-1}$ 2-deoxyglucose and corresponded to 175% of the fluorescence intensity measured after growth on maltose. A further increase in the 2-deoxyglucose concentration caused a steady decrease of GFP fluorescence clearly showing that 2-deoxyglucose is a strong inducer of the *glaA* expression system at low concentration (< 20 $mmol \cdot L^{-1}$; for reference 20 $mmol \cdot L^{-1}$

glucose corresponds to 3.6 $g \cdot L^{-1}$) but exhibits decreasing induction strength with increasing concentrations. With 5-thio-glucose maximum expression was reached at 64-fold lower concentrations compared to 2-deoxyglucose (0.25 versus 20 $mmol \cdot L^{-1}$, respectively). Also, the concentration range for maximum expression was very narrow with a dramatic decrease in inductions strength at concentrations above 20 $mmol \cdot L^{-1}$ 5-thio-glucose to basal levels which are obtained on non-inducing substrates (Fig. 2). Thus, both analogs induce *glaA*-promoter controlled gene expression at low concentrations while higher concentrations lead to lower induction efficiency or might even cause repression of the *glaA* expression system. Although the molecular basis of the variation in the concentration-dependent induction/repression efficiency of 5-thio-glucose and 2-deoxyglucose, or derived metabolites, has not yet been disclosed, their different molecular structures should convey different interactions with corresponding regulatory proteins. Finally, as these analogs are potent inducers of *glaA*-promoter controlled gene expression at low concentration but do not promote growth they may have interesting applicabilities for induced protein production as they could be added while cells are growing on non-inducing carbon substrates.

3.4. Examination of glaA-*promoter performance in controlled batch and fed-batch cultivations*

The reliability of the high-throughput screening technique was additionally verified by analyzing *glaA*-promoter performance in controlled batch cultivations. For this purpose, cells were first grown in a 5-L bioreactor on xylose to stationary phase (depletion of xylose). Following, 700-mL aliquots of the obtained culture broth were transferred to four identical small-scale bioreactors (1-L working volume) and growth was continued with the addition of either maltose, glucose, fructose, or xylose, respectively (Fig. 3). The time-dependent amounts of intracellular GFP were quantified. The obtained results clearly comply with those obtained from high-throughput screening. Again, the highest amount of GFP was obtained

with maltose as carbon substrate, intermediate levels with glucose, and insignificant levels with fructose and xylose (Fig. 3A and B). Analysis of carbon substrate concentrations revealed a similar slow decline for glucose, fructose and xylose (Fig. 3C). In contrast, maltose concentrations declined more rapidly concomitant to an increase of the extracellular glucose concentration (Fig. 3C) caused by the hydrolysis of the maltose α-1,4-glucosidic bond [e.g. through α-glucosidase (Kita et al., 2007)].

As glucose analogs only act as strong inducers at low concentrations but exhibit reduced induction efficiency or might even cause repression at higher concentrations, it is possible that high glucose concentrations also negatively effect *glaA*-promoter controlled gene expression. For example, glucose accumulation through hydrolytic cleavage of maltose might reduce the induction efficiency of this inducing carbon substrate. To test this hypothesis, two different types of fed-batch cultivations both with continuous addition of maltose were performed. In the first case, feeding was performed to keep maltose concentrations above 2 $g \cdot L^{-1}$, thus maintaining high inducer concentration but accepting a potentially negative effect of accumulating glucose through maltose hydrolysis ("high-maltose feeding"). In the second case, maltose feeding was performed in such a way that accumulation of glucose was prevented ("low-maltose feeding").

In both fed-batch cultures, cells were first grown under identical conditions in batch culture with xylose as carbon substrate (Fig. 4). After depletion of xylose, either high- or low-maltose feeding was initiated as described in the Materials and Methods section. In both cultures dispersed mycelial growth was observed. With high-maltose feeding, concentrations of maltose stayed above 2 $g \cdot L^{-1}$ while glucose levels continued to rise (Fig. 4B). When the low-maltose feeding protocol was applied, maltose was completely metabolized and maltose as well as glucose concentrations were below the detection limit throughout the entire fed-batch phase (Fig. 4B). In this case, final GFP concentrations reached almost 30 mg per gram cell dry mass (Fig. 4C). In contrast, high-maltose feeding resulted in only 10 mg GFP per gram

cell dry mass (Fig. 4C). Moreover, specific GFP production rates during the first nine hours after the onset of induction reached 2.5 and 0.4 mg GFP per gram cell dry mass and hour using the low- and high-maltose feeding strategy, respectively, clearly proving that excess inducer combined with increasing glucose concentration conveys lower induction efficiency. Nevertheless, more biomass accumulated using the high-maltose feeding strategy compared to the low-maltose feeding protocol (Fig. 4A), thus, final volumetric GFP concentrations were similar in both cultures.

These findings are in accordance with a general scheme proposed for induction of polymer degrading enzymes in fungi (Hondmann and Visser, 1994). According to this scheme polymer degrading enzymes are secreted at low constitutive level and produce 'signal molecules' when the respective polymer is present. These molecules are taken up by the cell, where they may be converted into or act directly as inducer for the synthesis of a subset of enzymes required for a rapid breakdown of the polymer. In the presence of more favorable, easily to metabolize low molecular weight carbon substrates, the synthesis of polymer-degrading enzymes is not advantageous and thus, their synthesis is prevented by carbon catabolite repression (Ruijter and Visser, 1997). In this context, glucose might have a dual role. On the one hand, as a product of starch hydrolysis, it might be directly or indirectly involved in inducing *glaA*-promoter controlled genes, and, on the other hand, as an easily metabolizable carbon substrate it might convey carbon catabolite repression of the *glaA* expression system. Moreover, as cleavage of maltose into glucose units occurs in the extracellular environment it might be possible that not maltose but glucose or glucose-derived metabolites are more directly involved in the regulation of *glaA*-promoter controlled gene expression. Thus, glucose or glucose-derived metabolite(s) may control induction efficiency of *glaA* expression; up to a certain threshold concentration induction efficiency increases, but beyond this level induction efficiency decreases or a repressive effect might even prevail. This way, the speed of polymer hydrolysis, glucose uptake and intracellular breakdown can be fine-tuned for optimal fungal

growth. Moreover, the metabolic burden for glucoamylase synthesis can be limited adequately in response to nutrient availability. A dual role of glucose or glucose-derived metabolites as inducer and repressor can also explain apparent contradicting observations of the "glucose effect" on the synthesis of polymer degrading enzymes releasing glucose moieties, e.g. cellulases in *Trichoderma reseei* (Ilmén et al., 1997) and glucoamylases in *Aspergillus terreus* (Ghosh et al., 1990).

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Captions to figures

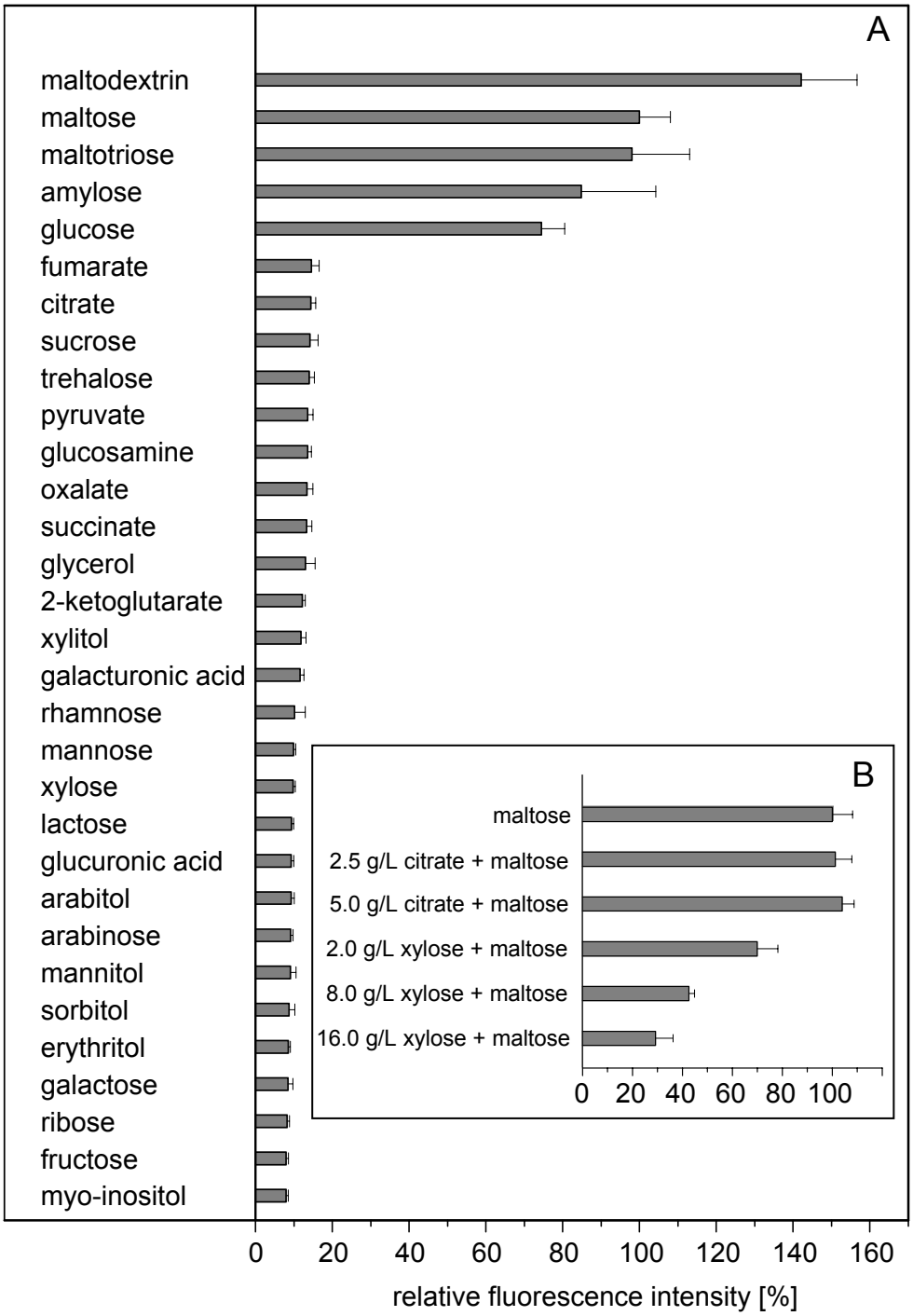
Figure 1: High-throughput screening of *glaA* promoter performance on different soluble high- and low-molecular weight carbon substrates and substrate combinations using GFP as the fluorescent reporter protein. (A) GFP fluorescence was determined on basic modified Vogel's medium supplemented with the carbon substrates (10 $g \cdot L^{-1}$) indicated. (B, insert) GFP fluorescence on basic modified Vogel's medium supplemented with 10 $g \cdot L^{-1}$ maltose and citrate or xylose as indicated. The average fluorescence intensity reported for each substrate or substrate combination is related to the fluorescence intensity reached on basic modified Vogel's medium supplemented with 10 $g \cdot L^{-1}$ maltose (relative fluorescence intensity = 100%). The error bars represent the standard error of the mean.

Figure 2: Concentration-dependent performance of the *glaA*-promoter using non-metabolizable analogs of D-glucose, namely 2-deoxyglucose (full squares, left insert shows only the data at 2-deoxyglucose concentrations up to 20 mmol L^{-1}) and 5-thioglucose (open squares, right insert shows only the data at 5-thioglucose concentrations up to 0.6 mmol L^{-1}), as inducer (always 10 $g \cdot L^{-1}$ citrate added to basic modified Vogel's medium). The average fluorescence intensity reported is related to the fluorescence intensity reached on basic modified Vogel's medium supplemented with 10 $g \cdot L^{-1}$ maltose (relative fluorescence intensity = 100%). The error bars represent the standard error of the mean.

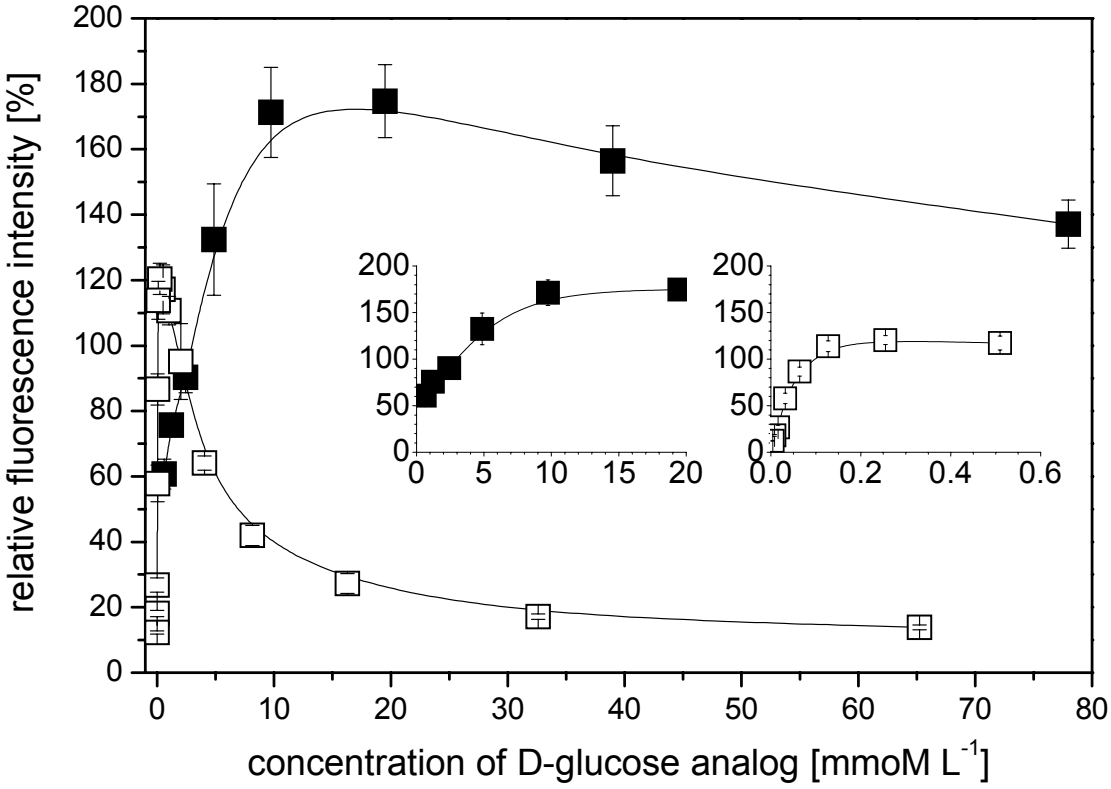
Figure 3: *GlaA*-promoter performance during growth of *A. niger* in batch culture on either maltose, glucose, fructose or xylose as carbon substrate. Time-course data of (A) the volumetric and (B) specific GFP concentrations and (C) the concentrations of the sugar substrates are shown.

Figure 4: *GlaA*-promoter performance during growth of *A. niger* in fed-batch culture using either a high-maltose (full symbols) or low-maltose feeding protocol (open symbols). Time-course data of (A) cell dry mass, (B) sugar substrates (triangles: maltose; circles: glucose), and (C) specific GFP concentrations are shown. The batch phase was performed under identical conditions using xylose as carbon substrate. The vertical line indicates the onset of maltose feeding, started after the depletion of xylose.

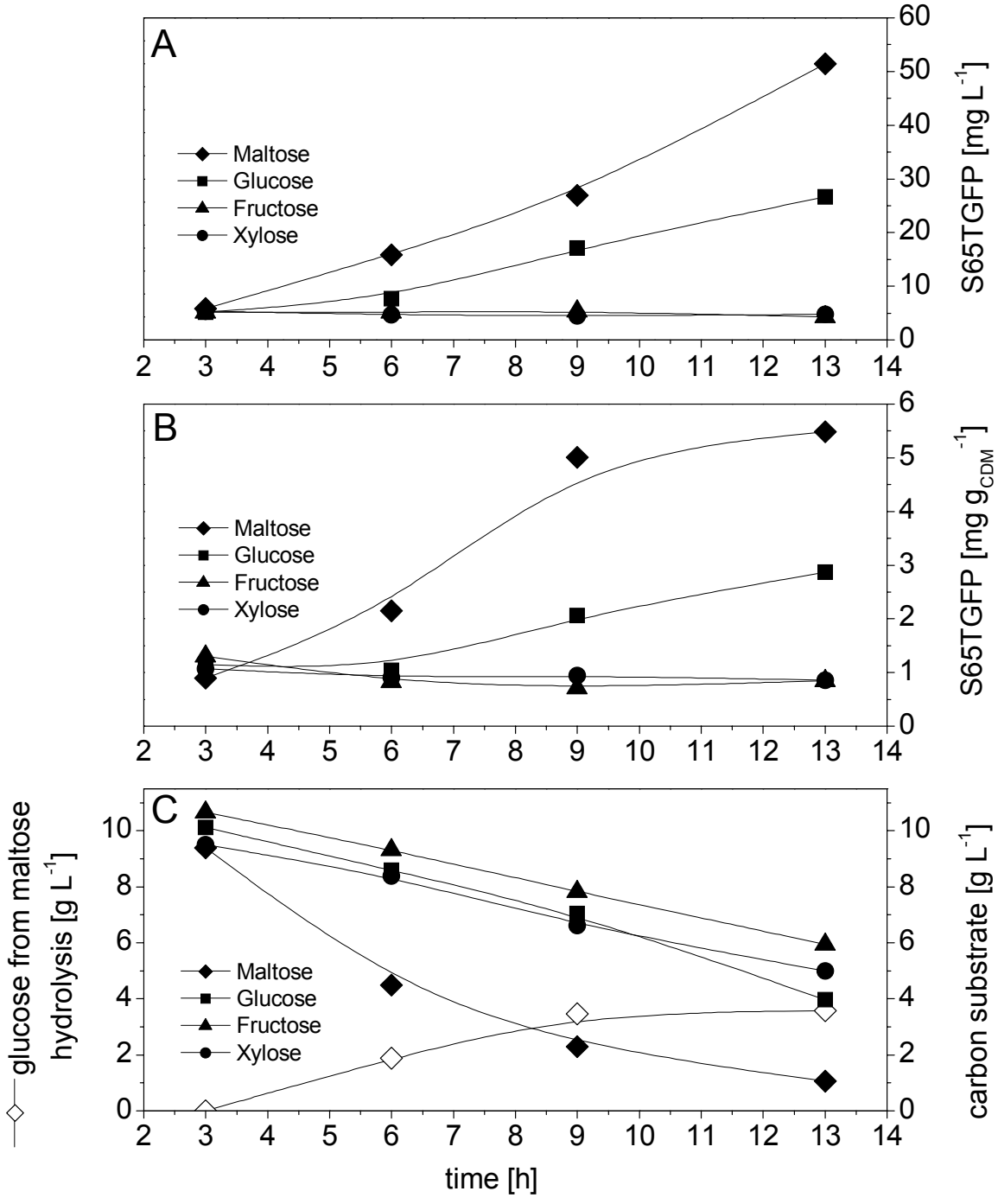
Ganzlin and Rinas, Figure 1



Ganzlin and Rinas, Figure 2



Ganzlin and Rinas, Figure 3



Ganzlin and Rinas, Figure 4