Green fluorescent protein in Saccharomyces cerevisiae: real-time studies of the GAL1 promoter

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Green fluorescent protein (GFP) was used to study the regulation of the galactose-inducible GAL1 promoter in yeast Saccharomyces cerevisiae strains. GFP was cloned into the pGAL110 vector and transformed into the yeast strains. Time course studies comparing culture fluorescence intensity and GFP concentration were conducted along with on-line monitoring of GFP expression. Our results demonstrated that GFP fluorescence could be used as a quantifiable on-line reporter gene in yeast strains. The effect of an integrated GAL10p-GAL4 transcription cassette was investigated. Induction time studies showed that there was no significant difference in GFP expression level by adding galactose at different culture times. A wide range of galactose concentrations was used to study the initial galactose concentration effect on GFP expression kinetics. A minimum of 0.05 g/L galactose doubled the GFP fluorescence signal as compared to the control, whereas 0.1 g/L gave the highest specific GFP yield. A simple analytical model was proposed to describe GFP expression kinetics based on the experimental results. In addition, this GFP-based approach was shown to have potential use for high-throughput studies. The use of GFP as a generic tool provided important insights to the GAL expression system and has great potential for further process optimization applications. © 2000 John Wiley & Sons, Inc.

INTRODUCTION

Since recombinant DNA technology emerged in the 1970s, overexpression of a large number of biologically active products has been successful in strains varying from E. coli to yeast and to mammalian cells. Yeast as a host strain has its own advantages: it is nonpathogenic, does not produce endotoxin and is considered by the FDA as a generally safe organism. As with any recombinant system, two common goals are desired: one is to obtain high cell densities and another is to achieve high product yield per single cell (specific productivity) or per unit volume of medium (volumetric productivity). Among the factors affecting specific productivity in yeast are promoter strength, plasmid copy number, and the 5’ untranslated leader sequences. The GAL1, GAL10, and GAL7 gene promoters in the GAL gene system are among the strongest in yeast Saccharomyces cerevisiae (St. John et al., 1981) and have been widely used for recombinant protein production. The GAL system is one of the most studied systems in yeast strains (for review, see Johnston, 1987; Lohr et al., 1995). It is repressed by the presence of glucose and induced when galactose is available and glucose is not present. Repression of the GAL system is not well understood and the Mig1 gene complex is believed to play an important role (Nehlin et al., 1991; Gancedo, 1998). Derepression of the GAL genes has been possible through specific gene mutations (Nehlin and Ronne, 1990).

Although extensively studied (Johnston, 1987), many details remain unclear about the GAL system. For instance, the chemical nature of the actual inducer for the GAL genes has not been identified. The specific chemical species is be-
lieved to be one of the intermediates in the galactose pathway and is likely catalyzed by the GAL3 gene product (Johnston, 1987). Moreover, the multiple roles of galactose as inducer / carbon source / energy source have not been well assigned. Galactose is an expensive reagent (30 times more than glucose in bulk) and a significant amount is often needed for optimal protein expression. Therefore, a clear understanding of the effect of galactose concentration on protein expression would not only benefit feeding strategy optimization but would also be useful for practical purposes in a manufacturing setting. However, little information is available on its effects over a wide range of galactose concentrations, particularly the economically significant low range.

Since its first discovery in 1962 (Shimomura et al., 1962), and more so after it was cloned in 1992 (Prasher et al., 1992), green fluorescent protein (GFP) has drawn tremendous interest (for review, see Chalfie and Kain, 1998). While GFP as a reporter gene has mostly been used for qualitative studies, its great potential as a quantitative, real-time, and on-line indicator for protein production has been realized in recent years. Albano et al. (1996, 1998) first demonstrated that GFP fluorescence could be used to quantitatively monitor GFP-fusion protein production in E. coli. Other work using GFP for monitoring and controlling in E. coli were also successful (Poppenborg et al., 1997; DeLisa et al., 1999; Cha et al., 2000). Other than E. coli, GFP has also been used to monitor protein production in insect cells (Cha et al., 1997). Meanwhile, various methods and devices for quantitative measurements of GFP were developed (Randers-Eichhorn et al., 1997; Craig et al., 1997; Korf et al., 1997; Endow and Piston, 1998; Knight et al., 1999).

In this article, we report on overexpression and monitoring of GFP in S. cerevisiae strains and its use as a tool to study the galactose-inducible GAL1 promoter, particularly the effect of galactose concentration on cloned GFP expression. There are several studies in the literature on GAL1 promoter induction/dynamics (Da Silva and Bailey, 1989; Eitzman and Siren, 1991; Napp and Da Silva, 1994). A similar study was carried out using β-galactosidase as a reporter (Da Silva and Bailey, 1989). Specifically, they used the GAL10-CYC1 hybrid promoter and the strains had a reg1 mutation that released glucose repression. However, they examined a narrow range of galactose concentrations and protein expression was not studied in detail, since the focus for their studies was quite different.

MATERIALS AND METHODS

Yeast Strains

Saccharomyces cerevisiae strain CF36 (MATa, leu2-04, ade1, cirb) and DMY11 (provided by Merck & Co., Inc.) were both used in this study. Strains CF36 and MDY11 are isogenic except that strain DMY11 also contains an integrated copy of the GAL10p-GAL4 transcription cassette in the His3 chromosomal region (Schultz et al., 1987). The GAL10p-GAL4 cassette allows for overproduction of the GAL4 protein.

pGAL110-GFP Construct and Transformation

The vector used for overexpression is pGAL110 (provided by Merck & Co. Inc.). This is a shuttle vector and is described elsewhere in detail (Hofmann et al., 1995). The Bam HI site was used for cloning the GFP gene under the GAL1 promoter (Fig. 1). The plasmid pGAL110 was digested with Bam HI (BRL). The 720 bp GFP gene was amplified by PCR (model AmpliTeron II, Barnstead Thermolyne Co. Dubuque, IA) from pBAD-GFP plasmid (Affymax Research Institute, Palo Alto, CA) using the following synthetic oligonucleotide primers which contained flanking Bgl II sites (bold): sense primer 5’-GGC-TCA-GAT-CTC-ACA-AAA-CAA-AAT-GGC-TAG-CAA-AGG-AGA-AGA-AGA-AGA-AGA-ACT-3’; antisense primer 5’-GGG-AGA-GAT-CTT-ATT-TGT-AGA-GCT-CAT-3’. The sense primer introduces a yeast nontranslated leader sequence (Kniskern et al., 1986) immediately upstream to the GFP initiating codon (highlighted in italic bold). The PCR fragment was digested with Bgl II (BRL), purified, and ligated with the Bam HI digested pGAL110 plasmid. The plasmid was then transformed to E. coli strain DH5 (BRL) with the provided protocol. Restriction digest with Nco I (BRL) was carried out to confirm the right orientation of the GFP gene. Finally, the pGAL110-GFP construct was transformed to the yeast strains (Hinnen et al., 1978) and selected with leucine minus medium plates.

Media Composition

The plasmid-free strains were grown in YEHD rich medium (2% yeast extract, 1% soy peptone, and 2% glucose) and
stored at -79°C. YEHD medium was also used in preparing for yeast transformation. The selective top agar used for yeast transformation was leucine-minus (designated 1x LEU; Schulman et al., 1991) agar containing 1 M sorbitol. It contains (w/v) 2% bacto-agar, 2% glucose, 0.67% yeast nitrogen base without amino acids, and 40 mg adenine L⁻¹, 50 mg L-tyrosine L⁻¹, 40 mg uracil L⁻¹, 20 mg arginine L⁻¹, 10 mg histidine L⁻¹, 60 mg isoleucine L⁻¹, 40 mg lysine L⁻¹, 10 mg methionine L⁻¹, 60 mg phenylalanine L⁻¹, 40 mg tryptophan L⁻¹. The medium (designated 5x LEU; Schulman et al., 1991) used for shake flasks, batch, and fed-batch studies contains 4% glucose, 0.864% yeast nitrogen base without amino acids, and ammonium sulfate, 1% succinic acid, 0.5% ammonium sulfate, and 0.25 g tyrosine L⁻¹, 0.2 g adenine L⁻¹, 0.2 g uracil L⁻¹, 0.1 g arginine L⁻¹, 0.05 g histidine L⁻¹, 0.3 g isoleucine L⁻¹, 0.2 g lysine L⁻¹, 0.05 g methionine L⁻¹, 0.3 g phenylalanine L⁻¹, 0.2 g tryptophan L⁻¹. This medium was adjusted to pH 5.2 with NaOH.

**LB medium** contains (w/v) 2% bacto-agar, 2% glucose, 0.67% yeast extract, 0.5% peptone, 0.5% sodium chloride, 50 mg L-tyrosine L⁻¹, 40 mg uracil L⁻¹, 20 mg arginine L⁻¹, 1420 Multilabel Counter (Wallac Oy, Finland), with excitation and emission wavelengths of 380 and 510, respectively. When final GFP expression levels were to be compared, samples were taken every few hours until fluorescence intensities leveled off or decreased, and the highest readings were recorded as final expression levels. For studies of galactose concentration effects, samples were centrifuged and the cell pellets were resuspended with 1x PBS buffer in order to remove the autofluorescence background of the culture medium; otherwise, whole culture broth was used for off-line fluorescence measurement. For batch fermentations, on-line fluorescence intensities were also measured using a prototype GFP sensor (Randers-Eichborn et al., 1997).

**Protein Analysis, SDS-PAGE, and Western Blot**

For time course studies, 3 ml of samples were centrifuged and the cell pellets stored at -79°C. When ready for protein extraction, the cell pellets were resuspended with Y-PER yeast extraction solution (Pierce Chemical Co., Rockford, IL) in a ratio of 150 μl Y-PER per ml culture, incubated at room temperature for 30 min with gentle shaking, then centrifuged. The supernatant was used for subsequent analyses. Total protein of each sample time point was calculated with a Micro Protein Determination Kit (Sigma Chemical Co., St. Louis, MO). For gel electrophoresis and Western analysis, different volumes of protein samples were loaded from different time points in order to get clear and nonsaturated GFP bands. Samples were run on SDS-PAGE at 170 V for 1 h in a Mini-Protein II unit (Bio-Rad, Hercules, CA) and transferred to nitrocellulose (Schleidher and Schuell, Keene, NH) with a Bio-Rad Mini-Trans Blot cell in Towbin transfer buffer (25 mM Tris, 193 mM glycine, 20% methanol) for 2 h at 100 V. The nitrocellulose was probed with a 1:2,000 dilution of polyclonal anti-GFP (Clontech Laboratories, Palo Alto, CA) and a 1:30,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase and developed with a Bio-Rad alkaline phosphatase conjugate substrate kit. Samples were calibrated to known concentrations of pure GFP generated by this lab on the Western blot. Image analysis was carried out with an Alpha Imager 2000 documentation and analysis system with multimage light cabinet (Alpha Innotech Corp.). The band intensity of each sample was calculated according to a standard curve generated by known amounts of pure protein also loaded on each gel or Western blot.
RESULTS AND DISCUSSION

Time Course Studies and On-Line Fluorescence Intensity Monitoring

A time course study was conducted comparing fluorescence intensity with GFP concentration. The experiments were carried out using shake flasks. Figure 2a shows that fluorescence intensity can quantitatively indicate GFP concentration. The same conclusion was reached earlier with an E. coli strain by Albano et al. (1996). Note here that in yeast strains the 95-min time lag for GFP's chromophore formation (Albano et al., 1998) has much less impact on real-time protein production estimation because yeast fermentations last considerably longer than E. coli. Our results suggest that GFP fluorescence intensity might be used to quantitatively estimate recombinant protein production in yeast strains.

On-line fluorescence intensity monitoring was also used for batch fermentations. Figure 2b shows that the on-line GFP sensor developed in this laboratory could be used to monitor culture fluorescence intensity on-line and in real time. This, together with the time course studies results, shows great potential for using GFP to monitor protein production in yeast strains.

Cell Growth, GFP Expression, and Glucose, Galactose, and Ethanol Metabolism

Experiments were carried out in a batch fermentation mode, with on-line measurements of GFP fluorescence intensity as well as cell mass. 10 g/L galactose was added in the beginning of each fermentation. For the control, all the conditions were the same except that no galactose was added.

As shown in Figure 3, the cell growth followed two distinct phases. After the lag phase, the cells grew exponentially in the presence of glucose. Ethanol accumulated during this period due to the Crabtree effect (De Deken, 1966). As glucose was depleted, the cell growth entered a much slower, but continuous growth phase. During this phase, the ethanol accumulated in the first phase, along with galactose were used as carbon sources. Although galactose was added in the beginning of the fermentation, it was not consumed while glucose was present due to the well-known glucose repression effect (Entian, 1986). When glucose was depleted (about 18 h after fermentation started), the cells began to utilize galactose and thus turned on the induction system. Galactose uptake was slow in the first few hours.
after glucose was consumed and then it was consumed at an almost constant rate (about 0.45 g/L/h). On-line GFP fluorescence intensity started to increase at an almost constant rate after glucose was depleted for approximately 4 h, accounting for the transient induction period and the 95 min required for GFP’s chromophores to fold correctly (Heim et al., 1994).

The cell growth profiles of the induced and uninduced cultures are noteworthy. Overexpression of some foreign proteins has been widely reported to impose an adverse effect on host cell growth due to metabolic burden and other reasons (Lee et al., 1985; Bentley et al., 1990). However, in our system the induction of GFP expression actually triggered a slightly higher growth rate after glucose depletion (average 0.023 vs. 0.014 h⁻¹), as seen in Figure 3. This is probably due to galactose being utilized as a carbon source in addition to serving as an inducer. Meanwhile, the growth curve of the induced culture reached its plateau earlier than the uninduced culture and then declined slowly. This earlier plateau is probably due to earlier depletion of some other nutrient in the induced culture caused by the slightly higher growth rate.

Effect of GAL10p-GAL4 Integrated Cassette

The utilization of the GAL system for recombinant protein production has been somewhat limited due to the relatively low expression level of the GAL4 protein in wild-type S. cerevisiae strains. Researchers have studied the effect of regulatable overexpression of GAL4 protein on recombinant protein production and different conclusions were drawn in different cases (enhanced production level by Schultz et al., 1987, and decreased production level by Choi et al., 1994). In our studies, we found that the overexpression of GAL4 protein by the integrated GAL10p-GAL4 cassette clearly enhanced the production of GFP (Table I). Experiments were carried out using shake flasks under the same culture conditions. Fluorescence intensities were measured using whole culture broth without any sample treatment. Table I shows that both CF36[pGAL110-GFP] and DMY11[pGAL110-GFP] strains have very tight control over the GAL1 promoters. Without induction, both strains show minimal increase in fluorescence intensity compared to strains that do not contain GFP gene (CF36[pGAL110] and DMY11[pGAL110], respectively). The fluorescence signals from strains CF36[pGAL110] and DMY11[pGAL110] were due to the background fluorescence from culture media. Under the same culture/induction conditions, strain DMY11[pGAL110-GFP] was able to express twice the amount of GFP that strain CF36[pGAL110-GFP] could express. Since the two strains are isogenic except for the integrated GAL10p-GAL4 cassette, these results clearly demonstrate the enhancement of GFP production with overproduction of GAL4 protein.

Moreover, it is interesting to note that under the same growth and induction conditions, the growth mode and final cell mass was almost identical between the strains without and with the GFP gene (CF36[pGAL110] vs. CF36[pGAL110-GFP], and DMY11[pGAL110] vs. DMY11[pGAL110-GFP], respectively) (Table I). Moreover, the same observation was made between CF36[pGAL110-GFP] and DMY11[pGAL110-GFP] strains, where the latter produced twice the amount of GFP (Table I). This suggests that the overexpression of GFP does not affect host cell physiology, at least in terms of cell growth.

Induction Time Effect

The induction time effect on GFP expression was examined. Experiments were carried out using shake flasks. Since galactose would not be utilized while glucose was present, the term “induction time” here actually means galactose addition time. Our results show that the timing of galactose addition did not affect final GFP expression levels significantly (Table II). It is interesting to note that even when galactose was added (about 13 h) long after glucose depletion (glucose is depleted after about 23 h of cultivation in Table I.

### Table I. Effect of GAL4 protein overexpression on GFP production.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Induction mode</th>
<th>Final GFP expression level (AU/OD)</th>
<th>Final OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF36</td>
<td>pGAL110</td>
<td>20 g/L</td>
<td>15</td>
<td>7.60</td>
</tr>
<tr>
<td></td>
<td>pGAL110-GFP</td>
<td>Not induced</td>
<td>19</td>
<td>7.42</td>
</tr>
<tr>
<td>DMY11</td>
<td>pGAL110</td>
<td>20 g/L</td>
<td>778</td>
<td>7.56</td>
</tr>
<tr>
<td></td>
<td>pGAL110-GFP</td>
<td>Not induced</td>
<td>20</td>
<td>7.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 g/L</td>
<td>1,662</td>
<td>7.65</td>
</tr>
</tbody>
</table>

*Data are average of two independent experiments. The GAL4 protein is overexpressed by the GAL10p-GAL4 integrating cassette in the DMY11 strain. CF36 strain is isogenic to DMY11 except for the integrating cassette.

**Table II.** Induction time effect on GFP expression.*

<table>
<thead>
<tr>
<th>Induction time (h)</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final GFP expression level (AU/OD)</td>
<td>CF36[pGAL110-GFP] strain</td>
<td>770 ± 35</td>
<td>800 ± 55</td>
<td>760 ± 50</td>
</tr>
<tr>
<td></td>
<td>DMY11[pGAL110-GFP] strain</td>
<td>1,580 ± 50</td>
<td>1,560 ± 45</td>
<td>1,600 ± 35</td>
</tr>
</tbody>
</table>

*Data are average of three independent experiments. 20 g/L of galactose were used.
shake flasks), the final GFP expression levels were not affected. This is probably due to the availability of ethanol as the carbon source after glucose depletion.

**GFP Expression Kinetics and Galactose Concentration Effect**

In order to have a complete picture of the galactose concentration effect on GFP expression, a wide range of galactose concentrations from 0–20 g/L were used to induce the expression of GFP. For all experiments, the cells were grown in 1-L flasks until glucose was depleted (about 22–24 h) and then aliquots of 44-ml culture were transferred to 250-ml shake flasks. By doing this, the cultures were identical at the time of induction. Concentrated galactose solution (40%, w/v) to induce and distilled water (control) were added so all the cultures had similar volume but different galactose concentrations. In order to minimize the fluorescence background from the culture medium, samples were centrifuged at 13,000 g for 3 min and the cell pellets were resuspended with 1x PBS buffer. The results are shown in Figures 4 and 5.

**Threshold [gal] for GFP Expression**

Figure 4a shows that [gal] of as low as 0.05 g/L was able to induce the GFP expression two times over the control background. However, the expression of GFP was very slow at this low [gal]. Interestingly, when 0.1 g/L of gal was added, GFP expression was much faster and the net increase of fluorescence signal was 4.5 times higher. For all those induced with a [gal] higher than 0.1 g/L, the same trend was observed (Fig. 4b). This result suggests that the two commonly known galactose transport mechanisms were involved in this GAL regulatory system, namely, the high affinity and low affinity transport mechanisms (Ramos et al., 1989). Although the intracellular galactose concentration needs to be measured to study the galactose transport mechanism so that we can confirm this suggestion, our results show an advantage of using GFP to study this system, namely, sensitivity. Use of GFP allows measurement in real time, unlike the traditional enzyme-based reporter gene measurement.

**Kinetics of GFP Expression and Galactose Uptake**

Figures 4 and 5 show the GFP expression profiles, galactose uptake profiles, GFP expression rates, and galactose uptake rates for initial [gal] range from 0.1–20 g/L. Figure 4b shows that with different amounts of galactose added GFP expression leveled off at different times. This was due to the depletion of galactose at different times, as shown in Figure 4c. Figure 4c also shows that for initial [gal] of below 10 g/L, the cells were able to consume all of the galactose added. When 20 g/L of galactose was added, GFP expression leveled off, while there was still about 5 g/L of galactose left in the culture. This was probably due to other nutrient limitation because in other experiments by doubling the medium concentrations (except for glucose) GFP expression level (AU) was about 1.5 times higher and the specific expression level (AU/OD) about 1.2 times higher.

**Figure 4.** a: GFP expression profiles for initial [gal] of 0–0.1 g/L. b: GFP expression profiles for initial [gal] of 0.1–20 g/L. c: Galactose uptake profiles. Galactose was added after glucose was depleted. Samples were centrifuged and resuspended in 1x PBS buffer to remove the autofluorescence background from culture media. Galactose concentrations never reach 0 for all cultures. The background levels are about 0.2 g/L. Data are average of duplicate experiments. Strain DMY11[pGAL110-GFP] was used.
Also, 20 g/L of galactose was completely consumed by the end.

In order to examine kinetics of the galactose concentration effect on GFP expression and galactose uptake, GFP expression rate and galactose uptake rates were calculated based on Figure 4. The rates were the average rates calculated for the periods that appeared to be in a first-order kinetics. Figure 5a shows that starting with initial [gal] of 0.05 g/L, the GFP expression rate increased rapidly with increase of initial [gal], and it reached the maximum rate when initial [gal] was 3 g/L. For galactose uptake (Fig. 5b), the maximum uptake rate (0.35 g/L/h) was reached when initial [gal] was 5 g/L. Note that the gal uptake rate did not increase beyond initial [gal] of 5 g/L. The results suggest that the GFP expression rate depends on the galactose uptake rate (see detailed discussion in the galactose uptake part in the model development section, below).

Specific GFP Yield Vs. Initial Galactose Concentration Added

Since galactose is an expensive inducer and the amount needed for full GFP expression is significant, it is of practical interest to look at the initial [gal] effect on specific GFP yield as shown in Figure 5c. The results show that GFP specific yield increased rapidly with the increase of initial [gal] and reached the maximum when initial [gal] is 0.1 g/L—a concentration point where we believe that the cells began to utilize low affinity galactose transport mechanism. The specific yield then fell and continuously decreased with higher initial [gal] added (see detailed discussion in model section, below). Compared to other initial [gal], 0.1–1 g/L gave relatively high specific yields. However, at initial [gal] of 0.1 g/L, GFP expression rate was just approximately 10% of the maximum. For practical purposes, the balance between a high expression rate and a high specific GFP yield could probably be found at an initial galactose concentration of 0.5–3 g/L.

Potential of Using 96-Well Plates for GFP Expression Studies

One of the great advantages of using GFP is its potential application in high-throughput technology. As a preliminary effort, we compared the galactose concentration effect on GFP expression levels between cultures grown in shake flasks and in 96-well plates (Fig. 6). Although incubated with shaking, mixing was still insufficient in the plates and cells tended to precipitate. As a result, cells grew slower in the plates and thus GFP was expressed at a slower rate. Nevertheless, Figure 6 shows that galactose concentration effect on GFP expression level followed the same trend for both cultures grown in shake flasks and in plates. There was a higher standard deviation observed in the plate cultures as compared to the shake flask studies. This was probably due to poor mixing in the plate cultures. The important factor is the tremendous labor savings brought by the plate studies. The plate experiment allowed essentially 96 independent experiments, about four times more than with shake flasks, and the plate reader finished each measurement in a few minutes, whereas it took more than 1 h to do just one fluorescence intensity measurement manually for each of the flasks.
Analytical Model for GFP Expression in Recombinant Yeast S. cerevisiae Strains

Based on the experimental results obtained from galactose concentration effect on GFP expression, an unstructured mathematical model was constructed and tested.

Model Development

Protein Expression

Equation (1) was used to describe the GFP expression rate per single cell:

\[
\frac{dy}{dt} = kq(P - y)
\]

where \( y \) = GFP concentration in a single cell (AU/OD), \( t \) = time after induction (h), \( k \) = expression rate coefficient (L/g), \( q \) = galactose uptake rate (g/L/h), \( P \) = maximum GFP concentration per single cell produced under specific culture conditions (temperature, media formulation, etc.) (AU/OD).

This model is constructed based on the following assumptions and concepts: 1) GFP expression is nongrowth-associated. 2) GFP expression requires the availability of a sufficient intracellular inducer (galactose) concentration, which requires continuous uptake of galactose due to its metabolism. Galactose transport is likely to be the rate-limiting step in the galactose metabolism pathway, as is glucose transport (Einsle et al., 1978). Therefore, the intracellular inducer concentration depends on the galactose transport/uptake rate, assuming that galactose is consumed at the same rate it is transported into the cell. Thus, we propose that GFP expression rate is proportional to galactose uptake rate, q. 3) As the recombinant protein accumulates in the cell, the system becomes less efficient in producing more protein due to an inhibition effect from the intracellular recombinant protein accumulated, i.e., the “driving force” for the continuous production of GFP decreases. It is well known that overexpression of many recombinant proteins are inhibitory to host cell growth (Lee et al., 1985; Bentley et al., 1990). For the system we studied, cell growth was not adversely affected by GFP expression, as mentioned earlier. Nonetheless, we believe that the inhibition effect applies to protein production itself. Also, the concept of maximum protein concentration in a single cell has been previously used by other researchers (Lee et al., 1985). 4) Since GFP is very stable in yeast, the degradation of GFP is neglected. 5) \( k \) is a constant for a given strain and culture condition.

Galactose Uptake

Addition of galactose induces two processes, a galactokinase (encoded by the \( GAL1 \) gene)-independent, low affinity process and a galactokinase-dependent, high affinity process. Both processes require the presence of the \( GAL2 \) protein (Ramos et al., 1989). The expression of \( GAL1 \) and \( GAL2 \) proteins, on the other hand, require galactose induction. Therefore, if the initial [gal] is not high enough, and so optimum induction is never reached, the gal transport will not operate at its full efficiency. The situation then becomes more complicated because of the interconnection between gal uptake and the \( GAL \) gene induction/expression. From Figure 4c it appears that for initial [gal] of above 3 g/L, after the initial period of induction, galactose uptake was at an almost constant rate until its depletion. We believe that in this system initial [gal] of 3 g/L is necessary for the optimum uptake to occur. For simplicity, we consider initial [gal] above 3 g/L. Thus it is reasonable to describe the galactose uptake rate as:

\[
q = \frac{dS}{dt} = \text{constant}
\]

Where \( q \) is gal uptake rate (g/L/h), \( S \) is gal concentration (g/L). The analytical solution for Eq. (1) is then easily obtained as:

\[
y = P(1 - e^{-kqt})
\]

And from Eq. (2), for initial galactose amount of \( S_0 \) added, the time needed to consume it is \( t_0 = \frac{S_0}{q} \). Thus, from Eq. (3) for each batch fermentation with initial [gal] = \( S_0 \) the final GFP production level will be:

\[
y = P(1 - e^{-kS_0t})
\]

Parameter Estimations

There are three parameters in this model: \( k \), \( q \), and \( P \).

Estimation of \( q \)

The value of \( q \) is estimated from galactose uptake profiles. From Figure 5b the average of the maximum gal uptake rates (initial [gal] of above 5 g/L) is used as the value of \( q \).
Estimation of k and P

k and P are estimated using Eq. (3). Experimental data with initial \([\text{gal}] = 20 \text{ g/L}\) are used. Both strains DMY11[pGAL110-GFP] and CF36[pGAL110-GFP] are tested to give their own k and P values. Non-linear regression method is used and sum of square error (SSE) is defined as:

\[
SSE = \sum_{i=1}^{n} \frac{(y_i - \bar{y}_i)^2}{y_i}
\]

Where \(y\) is experimental result and \(\bar{y}_i\) is the corresponding predicted result, \(i\) is sample point.

Simulation Results

GFP Expression Profiles

The model does not describe the initial period of induction. In the beginning of induction, the cells have to make mRNA, synthesize transport proteins, etc., and thus the uptake of galactose is slow, resulting in slow expression of GFP. From experimental results of shake flask studies, this period lasts about 3 h for both CF36[pGAL110-GFP] and DMY11[pGAL110-GFP] strains. The amount of GFP produced during this initial period is very small compared to the whole process. In this model, we neglect this portion of expression. Meanwhile, there is a 95-min time required for the chromophore to fold correctly before the fluorescence can be detected. Thus, simulations start at 4.5 (3 + 1.5) h after induction. The results are shown in Figure 7a.

Galactose Concentration Effect on GFP Specific Yield

For a batch fermentation, the specific yield is:

\[
Y = \frac{\bar{y}}{S_0} = \frac{P}{S_0} (1 - e^{-kS_0})
\]

With the \(P\) and k value obtained, we can estimate the GFP specific yield for a given amount of initial \([\text{gal}]\). The results are shown in Figure 7b. As both the predicted and experimental results show, GFP specific yield fell continuously with increased initial \([\text{gal}]\). This is caused by the decreased efficiency of the cells to produce GFP due to the inhibitory effect of GFP overproduction, as stated in model assumption 3 (above).

CONCLUSION

Our results demonstrate that the expression of GFP in S. cerevisiae could be monitored quantitatively by measuring the fluorescence intensity of the culture broth. Moreover, with the GFP sensor developed (Randers-Eichhorn et al., 1997), the expression could also be monitored on-line and in real time. These results show great potential for using GFP as a tool for bioprocess monitoring, control, and optimization. Also, the 95-min time lag for GFP’s chromophore to fold correctly would have much less impact in yeast fermentations compared to E. coli fermentations.

GFP was also shown to be very useful for elucidating promoter mechanism studies. For the GAL1 promoter studied, induction of GFP expression could be accurately detected with as little as 0.05 g/L galactose added. Overproduction of the GAL4 protein by the GAL10p-GAL4 integrated cassette clearly enhances the GFP expression level. The specific GFP productivity reaches the maximum when initial galactose concentration is 0.1 g/L. However, to fully activate the induction system and for optimum operation, the initial galactose concentration required is between 1–3 g/L. The maximum galactose uptake rate is approximately 0.35 g/L/h in shake flasks cultures and slightly higher in the batch fermentations (about 0.45 g/L/h). This suggests that in chemostat or fed-batch fermentations, the galactose feed rate required in order to maintain high expression level will probably be about 0.45 g/L/h. It will also be interesting to study what level of low glucose concentration is nonrepress-
ing. A simple model with two parameters was constructed and tested to describe GFP expression kinetics.

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REFERENCES


