Rapid and sensitive detection of β -galactosidase-producing yeasts by using microtiter plate assay

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Yeast β -galactosidase activity was detected by a microtiter plate assay using pNPG or Xgal as substrate in 30 minutes. The detection gave a clear result which is well correlated with the specific β -galactosidase activity present in each strain studied. The microtiter plate assay is an effective method to improve the detection and quantify the β -galactosidase gene in recombinant strains of *Saccharomyces cerevisiae*.

Introduction

Since the first description of an active β -galactosidase chimera (Müller and Kania, 1974), many cloning systems have been described that make use of the fact that the 25 N terminal residues of β -galactosidase can be replaced by peptides of any size and origin without destroying its activity (Koenen *et al.*, 1985). In fact, the β -galactosidase structural gene can be fused to the promoter and controlling elements of other genes as a way to provide an enzymatic marker for gene expression. Currently, heterologous β -galactosidase is employed as a standard marker for genetic analyses of *Saccharomyces cerevisiae*. Therefore, the screening of β -galactosidase producing strains turns out to be very important to current genetic analyses.

Two different substrates are commonly used to detect β -galactosidase activity. Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) is employed for *in vivo* detection of enzyme activity on plates since it readily enters the cells. The Xgal substrate is commonly used since its β -galactosidase hydrolysis product dimerizes and forms a blue precipitate that does not diffuse through the agar. Nevertheless, as it is referred to by O'Kennedy and Patching (1995), significant differences may occur in the degree of colour development on Xgal containing plates and consequently, it may be difficult to distinguish between β gal⁺ and β gal⁻ clones. The β -galactosidase activity can also be detected by the hydrolysis of the chromogenic substrate pNPG (p-nitrophenyl- β -D-galactopyranoside). The pNPG is unable to enter intact cells. Therefore, the cells have to be permeabilized before determination of enzyme activity. For

S. cerevisiae a method described by Miller (Miller, 1972) has been usually employed, with chloroform and SDS (sodium dodecyl sulfate) as permeabilizing agents.

The Xgal substrate has been traditionally employed for determining β -galactosidase activity in bacteria using a solid medium. As there has been an increasing interest on yeast β -galactosidase gene (Sreekrishna and Dickson, 1985; Vieth, 1994; Bang *et al.*, 1995) and in cloning systems employing this yeast gene, there is a demand for a method for rapid detection of yeast β gal⁺ clones.

In this study, the substrates Xgal and pNPG are used to detect yeast strains having β -galactosidase activity in microtiter plate and its performance is compared with the specific β -galactosidase activity determined in crude extracts by following the reaction kinetics. Both microtiter plate assays turn out to be simple, rapid and highly sensitive.

Materials and methods

Yeast strains

The yeast strains used in this study were *Kluyveromyces marxianus* ATCC10022, *Saccharomyces cerevisiae* NCYC869 (Mat α , Flo1) and two *S. cerevisiae* NCYC869-A3 (Mat α , Flo1, ura3) transformants named T1 and T2, harboring a *K. lactis* β -galactosidase gene. These transformants were obtained by transforming *S. cerevisiae* NCYC869-A3 by the lithium acetate method (Ito *et al.*, 1983) with a recombinant vector resulting from the coupling of pKR1B-lac4-1 (ATCC n°40186, U.S. patent n°5, 047, 340 dated Sept 10, 1991; Sreekrishna, K. and Dickson, R.C., 1985) and pYAC4.

Microtiter plate assay with pNPG

The yeast cells were grown overnight in YNB-Lactose medium at 30°C. The YNB-Lactose medium was composed of 0.67% (w/v) yeast nitrogen base and 0.5% (w/v) lactose. A 100 µl cells culture (*c.a.* 10⁶ cells) was plated in a well of the 96 well microplate. Then each well was added with 200 µl of SDE (0.05 M Tris/HCl pH 7.5–8.0; 0.01 M EDTA; 1 M KCl; 0.05 M 2-mercaptoethanol), a drop of 0.1% (w/v) SDS and a drop of chloroform. The β-galactosidase activity was detected by dropping 50 µl of 4 mg/ml pNPG. The cells were exposed to the testing medium in eight replicates and for each case study a blank containing all components except the pNPG reagent was present also in eight replicates. The microtiter plates were then incubated at 30°C for 30 min. Positive strains developed an unequivocal yellow colour and negative strains and blanks revealed colourless. The plates were read at 405 nm every 5 minutes on a scanning multiwell spectrophotometer SLT Spectra. For each sample value the correspondent blank was subtracted as well as the time zero value.

Microtiter plate assay with Xgal

The yeast cells were grown overnight in YNB-Lactose medium at 30°C. A 200 µl cells culture (*c.a.* 10⁶ cells) was plated in a well of a 96 well microplate. Then each well was added with 5 µl Xgal (20 mg/ml) and incubated at 30°C. The direct observation of a clear blue colour revealed the presence of β-galactosidase activity. In order to dissolve the formazan crystals, 100 µl of DMSO were added at the end of the assay (24 hours) and the plates were read at 750 nm on a scanning multiwell spectrophotometer SLT Spectra. Once again, cells were exposed to the testing medium in eight replicates and for each case study a blank containing all components except the Xgal reagent was present, also in eight replicates. For each sample value the correspondent blank was subtracted.

β-Galactosidase specific activity

The β-galactosidase specific activity was assayed in cellular crude extracts and determined in a total volume of 1.0 ml by following the hydrolysis of 2.3 mM pNPG in Z buffer (Miller, 1972), pH 7.0, spectrophotometrically at 420 nm. One unit of enzyme activity hydrolyses 1.0 nmol of pNPG/min at 25°C. The value of the extinction coefficient of pNPG was determined to be 6.6 mM⁻¹ cm⁻¹. Soluble protein concentrations were assayed using Coomassie Brilliant Blue according to Bradford.

Results and discussion

The yeast strains tested for β-galactosidase activity are listed in Table 1. The *Saccharomyces cerevisiae* NCYC869

strain was used as a control and at the end of both tests the wells inoculated with this strain remained colourless. Both *K. marxianus* and the transformed *S. cerevisiae* strains gave positive reaction at the end of the assays. The specific β-galactosidase activity was quantified in crude extracts of these strains with the aim of supporting the results obtained with the microtiter plate assay (see table 1).

Table 1 Yeasts tested for β-galactosidase activity.

Strains	Assay Method		
	Microtiter plate assay with Xgal ^a	Microtiter assay with pNPG ^b	β-Galactosidase specific activity ^c (U/mg protein)
<i>Kluyveromyces marxianus</i>	+	+	2360 ± 301
<i>Saccharomyces cerevisiae</i> NCYC869	–	–	0
<i>Saccharomyces cerevisiae</i> T1	+	+	343 ± 200
<i>Saccharomyces cerevisiae</i> T2	+	+	138 ± 12

a. +, blue colour; –, colourless.

b. +, yellow colour; –, colourless.

c. mean of at least three independent assays ± standard deviation.

Both microtiter plate assays are suitable for qualitative screening since positive strains can be easily selected from negative strains by the presence of colour being possible to distinguish between different colour developments according to different enzyme production levels.

As presented in figure 1, there is a linear time dependence in the pNPG assay and the β-galactosidase activity can be detected within 30 minutes after the pNPG addition. The validity of the method was also confirmed by obtaining a linear relationship between the β-galactosidase activity and biomass concentration (figure 2) in the biomass concentration range studied (0.06–0.25 g/l).

In contrast, the Xgal assay is better used as an end point method. The blue precipitate that is formed has to be dissolved in order to obtain reproducible results. This can be achieved with DMSO that destroys the cells and hence stops the enzyme reaction. As a consequence, once DMSO is added there is no further colour development. Taking this into account, DMSO reagent must be added when the reaction is complete. The time chosen to take the end point values shown in figure 3 was 24 hours

Figure 1 pNPG assay dependence with time for a biomass concentration of 0.25 g/l (dry weight). (Km) *K. marxianus*; (T1) and (T2) *S. cerevisiae* transformants; (Sc) *S. cerevisiae*.

Figure 2 pNPG assay dependence with biomass concentration for *Kluyveromyces marxianus* strain and at 30 minutes.

even though the blue colour can be visually detected much sooner than that (for *Kluyveromyces marxianus* at 30 minutes a light blue colour is already present). A linear biomass dependence for the Xgal assay (figure 4) was also found in the biomass concentration range studied (0.4–2 g/l).

As shown in figures 5 and 6, both microplate assays were very well correlated with the β -galactosidase specific activity determined by following the enzyme kinetics in crude extracts.

Figure 3 Xgal assay values at 24 hours for a biomass concentration of 2 g/l (dry weight). (Km) *K. marxianus*; (T1) and (T2) *S. cerevisiae* transformants; (Sc) *S. cerevisiae*.

Figure 4 Xgal assay dependence with biomass concentration for *Kluyveromyces marxianus* strain at 24 hours.

Screening for β -galactosidase producing strains and quantitative determination of enzymatic activity using the microtiter plate assay is time-saving since it requires only about 30 minutes for the pNPG assay. However, for complete colour development 24 hours are needed for the Xgal assay, if quantitative determination is wanted. If a qualitative screening is desired 30 minutes are enough to detect β -galactosidase activity since a blue colour is already noticed. The microtiter plate assay gave clear determination for both assays. The pNPG microtiter plate assay is more rapid but more laborious since the cells have to be permeabilized. The Xgal microtiter plate assay only needs to inoculate the yeasts

Figure 5 Correlation between the values obtained by the pNPG assay at 30 minutes for the four strains tested and the β -galactosidase specific activity in each strain.

Figure 6 Correlation between the values obtained by the Xgal assay at 24 hours for the four strains tested and the β -galactosidase specific activity in each strain.

in the microtiter wells, add Xgal, wait for colour development and finally at the end of the assay to dissolve with DMSO.

In conclusion, both microtiter plate assays are simple, rapid, sensitive, well correlated with the standard method and suitable for screening large number of recombinant colonies containing β -galactosidase activity.

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References

- Bang, J.H., Nam, D.H., Kang, D.O., Ahn, J.S. and Ryu, D.D.Y. (1995). *J. Microbiol. Biotechnol.*, 5, 6–13.
- Ito, H., Fukada, Y., Murata, K. and Kimura, A. (1983). *J. Bacteriol.* 153, 163.
- Koenen, M., Griesser, H. and Müller-Hill, B. (1985) Immunological Detection of Chimeric β -galactosidase expressed by plasmid vectors. In: *DNA Cloning*, D.M. Glover, ed. vol. 1. UK: IRL Press.
- Miller, G.L. (1972) Experiments in Molecular Genetics, pp. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Müller-Hill, B. and Kania, J. (1974). *Nature*, 249, 561.
- O'Kennedy, R. and Patching, J.W. (1995). *Biotech. Techniques*, 9, 921–924.
- Sreekrishna, K. and Dickson, R.C. (1985). *Proc. Natl. Acad. Sci. U.S.A.*, 82, 7909–7913.
- Vieth, W.R. (1994) *Bioprocess Engineering. Kinetics. Mass Transport. Reactors and Gene Expression*. New Jersey: John Wiley & Sons, Inc.

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