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Effect of Galactose and Dextrose on Human Lipocortin I Expression in Recombinant *Saccharomyces cerevisiae* Carrying Galactose-Regulated Expression System

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The expression kinetics of human lipocortin I (LCI), a potential anti-inflammatory agent, was studied in the shake-flask and fermenter cultures of *Saccharomyces cerevisiae* carrying a galactose-inducible expression system. The cell growth, expression level of LCI, and the plasmid stability were investigted under various galactose induction conditions. The expression of LCI was repressed by the presence of a very small amount of dextrose in the culture medium, but it was induced by galactose after dextrose became completely depleted. The optimal ratio of dextrose to galactose for lipocortin I production was found to be 1.0 (10 g/*l* dextrose and 10 g/*l* galactose). With optimal D/G ratio of 1.0 and the addition of galactose prior to dextrose depletion, LCI of about 100~130 mg/*l* was produced. LCI at a concentration of 174 mg/*l* was porduced in the fed-batch culture, which was nearly a twice as much of that produced in the batch culture. The plasmid stability was very high in all culture cases, and thus was considered to be not an important parameter in the expression of LCI.

The yeast *Saccharomyces cerevisiae* has been widely used as a host for the production of heterologus proteins due to its many advantages over other microorganisms. It is non-pathogenic, produces no endotoxins, and has been cultivated on an industrial scale for centuries (10). In order to ensure both stable and continuous expression of the heterologous proteins in recombinant yeast, the vector and promoter should be of great relevance to the plasmid copy number, promoter strength and plasmid stability. Based on the use of either constitutive or inducible yeast promoters, a variety of expression vector systems have been developed. Constitutive expression of heterologous proteins in yeast might be toxic for the cells (5, 18), and could consequently result in the decrease in cell density and plasmid stability. Furthermore, it might lead to rapid degradation of the recombinant protein during the cell growth (20). These problems can be alleviated by the use of inducible and strong promoters. These promoters which are suitable for yeast have been derived from the galactose-regulated genes (9, 13). When cloned in plasmids of high copy number, the galactose inducible promoters such as *GAL1*, *GAL7*, *GAL10*, and their hybrids with *CYC1* or UAS_G are capable of increasing the gene expression level (1, 4, 6, 7, 12, 15, 17, 22). The galactose regulatory circuit is subject to catabolite repression, and the mutant cells capable of inhibiting this repression can be utilized as hosts for galactose induction in a glucose-containing medium (3, 9).

In an induction system which employs the galactose inducible promoter, the cells were first cultivated in a complex medium containing glucose as a carbon source to repress the expression of cloned-gene, and then the synthesis of cloned-gene product was induced by the addition of galactose (1, 3, 15). Da Silva and Bailey (4) reported that the ratio of galactose to glucose and the galactose concentration were very important parameters in determining the level of gene expression. However, there is still very limited information on the effects of galactose and glucose.

Recently, we constructed a yeast expression and secretion vector, YEGα-LC, by using the prepro leader sequence (ppL) of mating factor α and *GAL10* promoter for the production of human lipocortin I (LCI) (19). In

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the present paper, we have described how the initial ratio of glucose to galactose and the addition timing of galactose affect the cell growth and the LCI expression in recombinant *S. cerevisiae*. In order to enhance the level of LCI expression, a fed-batch culture was conducted with intermittent feedings of galactose, and the results were compared with those of the batch culture.

MATERIALS AND METHODS

Yeast Strain and Plasmid

Saccharomyces cerevisiae 2805 (*MATa pep4::HIS3 pro1-δ can1 GAL2 his3δ ura3-52*) was used as the host cell in this study. Plasmid YEGα-LC is a shuttle vector containing *URA3* gene, the yeast 2μ origin, and the yeast *GAL10* promoter which regulates the expression of human LCI. For the secretion of LCI into an extracellular culture medium, the prepro leader sequence (about 300 bp) of mating factor α was connected between *GAL10* promoter and LCI gene (19). The resulting plasmid YEGα-LC (6.9 kb) was transformed into *S. cerevisiae* 2805 strain by the lithium-acetate method (8).

Media and Culture Conditions

Minimal YCAD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid and 2% dextrose) was used for the selection of transformant cells and the seed culture. A single yeast transformant on the YCAD agar plate was inoculated into 10 *ml* YCAD medium and incubated for overnight at 30℃. For further activation, the seed was transferred into 100 *ml* YCAD medium and incubated for overnight. 5 *ml* of this seed was finally inoculated into a 500 *ml* Erlenmeyer flask containing 100 *ml* of production medium. For the production of LCI, YEP medium (1% yeast extract and 2% peptone) was used as a basal medium, and dextrose and galactose of varying concentrations were used as the carbon sources. The fermentations were performed in a 2 *l* jar fermentor (KFC Co., Korea) containing 1 *l* liquid medium. The culture temperature and pH were maintained at 30℃ and 5.5, respectively. The aeration rate was 1 vvm, and the agitation speed was kept between 400~700 rpm to maintain the concentration of the dissolved oxygen above 10% air saturation. Dextrose and galactose, each at concentrations of 10 g/*l* (D/G ratio=1), were used as carbon sources in the batch fermentor culture. In the case of the fed-batch culture, the initial dextrose concentration was at 5 g/*l*, and dextrose was continuously fed to the culture for 24 hours. After 24 hr of culture, galactose was intermittently fed into the fermentor for the LCI expression.

SDS-PAGE and LCI Measurement

Extracellular proteins in the culture supernatant were precipitated by adding trichloroacetic acid (TCA) and sodium deoxycholate, and collected after centrifugation at 15,000 rpm for 5 min. The concentrated protein solution was subjected to a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein bands on the gel were scanned by a densitometer (Biomed Instrument SCR 2D/1D, USA), and then the LCI concentration was calculated by multiplying the scanned peak area of LCI band (40 kd) by the total concentration of protein loaded on the gel.

Analytical Methods

The growth of yeast cell was monitored by measuring the optical density (OD) at 600 nm (UVICON 930, Switzerland). The concentration of glucose in the culture broth was measured by using a glucose analyzer (Yellow Springs Instrument 2700-D, USA), and the concentration of galactose was measured by the enzymatic method (Boehringer Mannheim kit #176303, FRG). The ethanol concentration was measured using a gas chromatograph equipped with a flame ionization detector (Hewlett Packard 5890, USA). Plasmid stability was determined by spreading the diluted samples onto YEPD agar plates and incubating them at 30℃ for 2 days. Colonies were then replicated onto plates of defined YCAD media both with and without uracil supplement. The ratio of the number of CFUs (colony forming units) on the selective (ura^-) agar plates to those on the nonselective (ura^+) plates was used to calculate the percentage of cells containing the plasmid. The concentration of the extracellular protein was measured by the modified Lowry method (Sigma protein kit #5656, USA).

RESULTS AND DISCUSSION

Effect of Dextrose/Galactose Ratio on LCI Expression

To optimize the induction conditions, the effect of dextrose/galactose (D/G) ratio on the LCI expression was examined by using a shake-flask culture. As shown in Fig. 1, the recombinant yeast grew rapidly when the D/G ratio was increased, and after 48 hr of culture the final cell densities reached about 40 OD in all cases. When the dextrose was exhausted at the 12th hr, the cells began to utilize ethanol and galactose as carbon sources and to produce LCI. The effects of D/G ratio on the plasmid stbility and the LCI expression are summarized in Table 1. The expression of LCI was strictly repressed by the presence of dextrose, and was induced by galactose after the dextrose became completely depleted. The highest level of LCI expression was obtained at a D/G ratio of 1.0. These results demonstrate that it is better to use both dextrose and galactose from the beginning of shake-flask culture than to use galactose as a sole carbon source. At the D/G ratio of 1.0, the

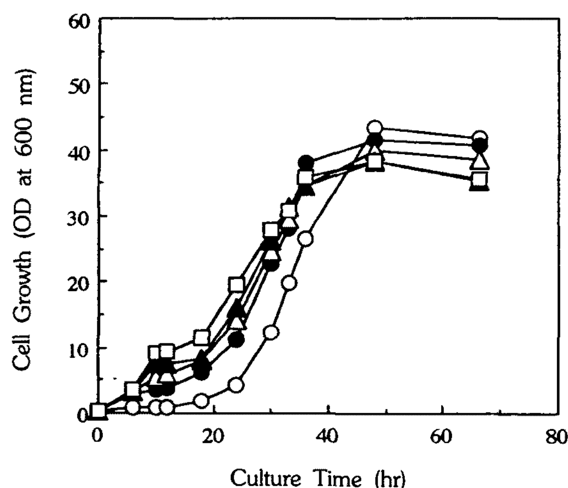

Fig. 1. Effect of dextrose/galactose (D/G) ratio on the cell growth of *S. cerevisiae* 2805/YEGα-LC in the shake flask culture.

Dextrose 0 g/*l*+galactose 20 g/*l*, ○; dextrose 5 g/*l*+galactose 15 g/*l*, ●; dextrose 10 g/*l*+galactose 10 g/*l*, △; dextrose 15 g/*l*+galactose 5 g/*l*, ▲; dextrose 20 g/*l*+galactose 0 g/*l*, □.

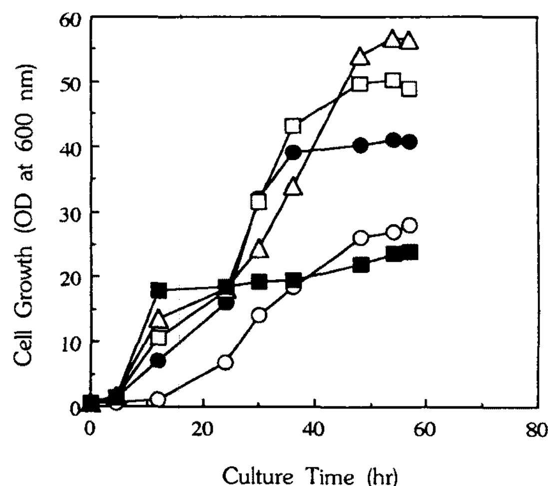

Fig. 2. Effect of initial dextrose concentration on the cell growth of *S. cerevisiae* 2805/YEGα-LC in the shake flask culture.

Dextrose at varying concentrations was initially added to the culture medium containing 10 g/*l* of galactose. Initial dextrose concentrations were 0 g/*l* (○), 10 g/*l* (●), 20 g/*l* (□), 30 g/*l* (△), and 50 g/*l* (■).

Table 1. Effect of dextrose/galactose ratio on plasmid stability and lipocortin I expression

Dextrose: Galactose (g/*l*)	Plasmid stability (%)	Extracelluar protein conc. (mg/*l*)	Lipocortin I conc. (mg/*l*)
0:20	32	267	74
5:15	40	281	101
10:10	52	305	131
15: 5	68	301	113
20: 0	85	205	ND*

ND*; not detectable

transition period from dextrose- to galactose-utilization was very short, and this appeared to result in an increase in the expression rate of LCI.

When 2% (w/v) galactose was used as the sole carbon source, the cell growth was retarded, and the plasmid stability decreased to a considerable degree. It has previously been observed that the stability of recombinant plasmid carrying 2μ-origin decreased as the growth rate declined (2, 11, 14). These results were consistent with our observation that a decrease in the growth rate at 2% galactose yielded the lowest plasmidd stability of 32%.

Effect of Initial Dextrose Concentration on LCI Expression

To further increase the level of LCI expression, the effect of dextrose concentration on LCI expression was investigated in a shake-flask culture. In these experiments, a variety of dextrose concentrations ranging from 0 to 50 g/*l* were initially added to the culture media containing 10 g/*l* galactose. As shown in Fig. 2, the cell growth rate and the final cell concentration increased when the initial dextrose concentration was increased. However, the initial dextrose concentration was at 50 g/*l*, the cells were not capable of consuming dextrose, ethanol and galactose due to accumulation of ethanol above the concentration of 18 g/*l* in the culture broth (Fig. 3). While the final cell concentration increased when the initial dextrose concentration was increased, the hi ghest level of LCI expression was achieved at the initial dextrose concentration of 10 g/*l* (Table 2). As expected, the lowest plasmid stability was observed when only galactose was used as the carbon source. Although the plasmid stability was also very high at 2% and 3% initial dextrose concentrations, the final levels of LC1 expression were lower than that at 1% initial dextrose concentration. Thus, it appeared that the final level of LC1 expression was not so greatly affected by the plasmid stability in the recombinant yeast system used in this study.

The initial dextrose concentration of 10 g/*l* yielded the highest level of human LCI expression in recombinant *S. cerevisiae* due to the low accumulation of ethanol and the high specific expression rate. This was also observed from the two-step culture experiment; cells exponetially grown in YPD medium were harvested and then transferred to YCADG medium composed of 0.67 % yeast nitrogen base without amino acids, 0.5% casa-

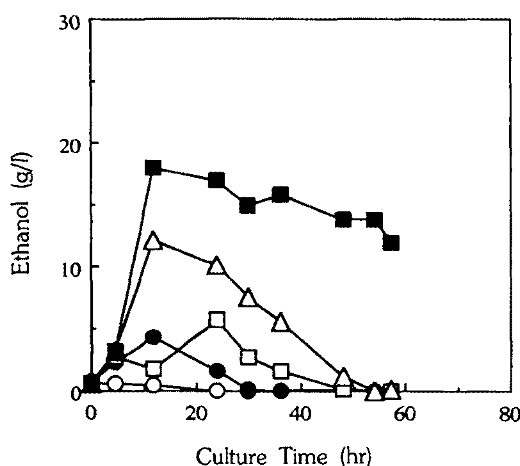

Fig. 3. Ethanol accumulation at varying initial dextrose concentrations in the shake flask culture.
The symbols are the same as in Fig. 2.

Table 2. Effect of initial dextrose concentration on plasmid stability and lipocortin I expression

Dextrose conc. (g/l)	Plasmid stability (%)	Extracellular protein conc. (mg/l)	Lipocortin I conc. (mg/l)
0	20	268	60
10	56	289	129
20	55	280	80
30	50	274	74
50	77	272	22

mino acid, 1% galactose and various concentrations of dextrose. The highest level of LCI expression was also obtained at the dextrose concentration of 10 g/*l* (data not shown).

During the batch culture of yeast, the accumulation of ethanol resulting from fermentation affects, in an adverse manner, the states and metabolic activities of the yeast cell, such as specific growth rate, viability and transport rate of sugars and amino acids (21). The sugar-utilizing activity and viability of the recombinant yeast used in this study were reduced significantly by the accumulation of ethanol above the concentration of 15 g/*l*, which consequently prevented the cells from growing even in the presence of dextrose.

Effect of Galactose Addition Time on LCI Expression

The final expression level of a heterologous gene under *GAL* promoter regulation is known to be strongly dependent on the time at which galactose was added (3, 5, 16). To investigate the effect of galactose addition time on LCI expression, galactose at a concentration of 10 g/*l* was added to the cells growing in YEPD medium (YEP plus 1% dextrose) at different cell growth phases. As shown in Fig. 4, upon the addition of galactose within 12 hr of culture, no significant differences in the cell growth and the final cell concentration were observed. When galactose was added at the 24th or the 30th hr, the cell was not capable of fully restoring its original galactose-metabolizing activity (Fig. 5), and this resulted in a decrease in the LCI expression (Table 3). The plasmid stability was maintained above 50% even in the cases of galactose addition at the 24th hr and the 30th hr. In general, the plasmid instability in recom-

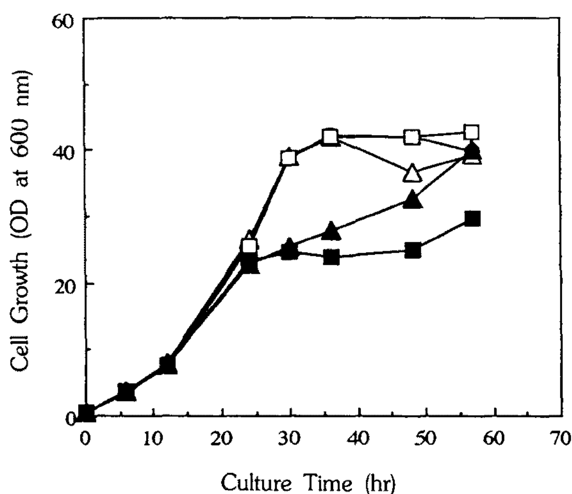

Fig. 4. Effect of galactose addition time on the cell growth of *S. cerevisiae* 2805/YEG-α-LC in the shake flask culture.
Galactose at a concentration of 10 g/*l* was added to the culture medium containing 10 g/*l* of dextrose at 0 hr (○), 6 hr (△), 12 hr (□), 24 hr (▲) and 30 hr (■) after cell inoculation.

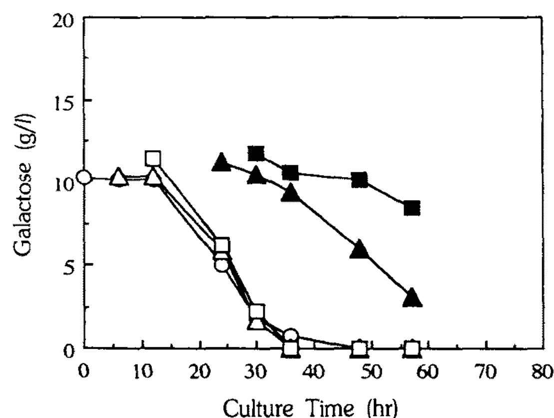

Fig. 5. Effect of galactose addition time on galactose consumption in the shake flask culture.
The symbols are the same as in Fig. 4.

Table 3. Effect of galactose addition time on plasmid stability and lipocortin I expression

Addition time (hr)	Plasmid stability (%)	Extracellular protein conc. (mg/*l*)	Lipocortin I conc. (mg/*l*)
0	72	278	109
6	70	247	96
12	64	252	96
24	64	285	40
30	79	246	2

binant yeast fermentation is one of the major factors which affect the expression level of heterologous gene (3, 4). However, as mentioned already in the varying D/G ratio experiment, the plasmid stability in this recombinant yeast system was not a major parameter of the production of LCI.

Batch and Fed-batch Culture in a Jar Fermentor

The batch production of LCI was carried out at an initial D/G ratio of 1.0 (1% dextrose+1% galactose), and the results were compared with those of the fed-batch culture. In the fed-batch culture, galactose was intermittently fed at the culture times indicated by arrows in Fig. 6. Until the 24th hr, the dextrose feeding was manually controlled to avoid carbon-limited condition. As illustrated in Fig. 6, the cell growth in the batch culture was very similar to that of the shake-flask culture, and the final cell concentration reached 35 OD. In the fed-batch culture, the cells grew rapidly while dextrose was being fed, and then the cell growth rate decreased significantly during galactose consumption. The final cell concentration in the fed-batch culture was about 77 OD, which was a two-fold increase over that of the batch culture. Upon the depletion of dextrose, the expression of LCI began to be induced by galactose (Fig. 7). The expression rate increased dramatically between the 3rd hr and the 5th hr after dextrose depletion, and then decreased gradually. The results of the comparison of various cultures are summarized in Table 4. In the fed-batch culture, ethanol at a concentration of 15 g/*l* was produced during the initial dextrose-feeding phase, and the plasmid stability was stably maintained above 80% in the prolonged culture time of 72 hr. It is unclear yet why the plasmid stability was so high in the fed-batch culture. Further studies will be carried out to elucidate this phenomena.

The yield values were evaluated and also compared for the three culture cases. In the batch cultures performed in the shake-flask and the fermentor with the D/G ratio of 1.0, the values of $Y_{X/S}$ was in the range of 1.7~2.0 OD/g sugar and $Y_{P/X}$ was 3.0 mg LCI/OD cell. However, these yield values significanly decreased to 50~70% in the fed-batch culture (Table 4). It appeared that the accumulation of ethanol above the concentration of 15 g/*l* decreased the galactose-metabolizing activity of the

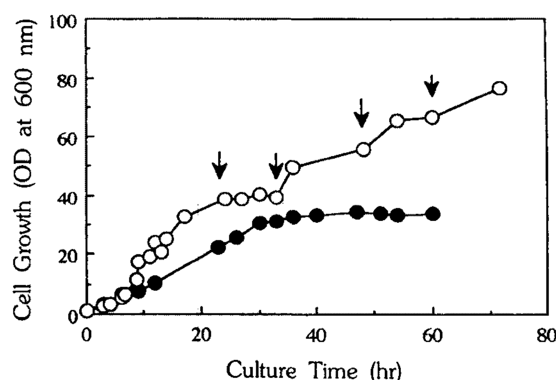

Fig. 6. Cell growth during the batch (●) and fed-batch culture (○) in a jar fermentor.
The arrows indicate the galactose addition times in the fed-batch culture.

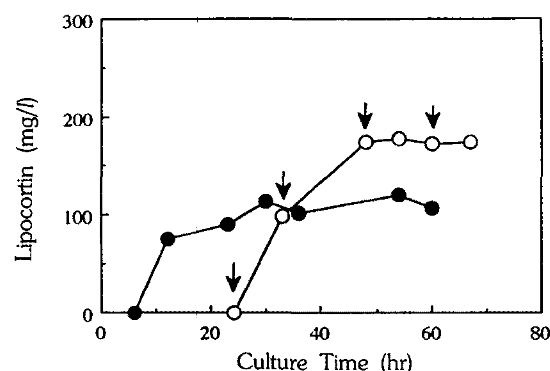

Fig. 7. Lipocortin I expression during the batch (●) and fed-batch culture (○) in a jar fermentor.
The arrows indicate the galactose addition times in the fed-batch culture.

Table 4. End-of-fermentation data and calculated yields for the batch and fed-batch cultures

	Flask culture	Fermentor culture	
		Batch	Fed-batch
Cell conc. (OD_{600})	40.7	34.6	76.62
Ethanol conc. (g/*l*)*	6.2	5.2	16.0
Plasmid stability (%)	60	55	86
Lipocortin I conc. (mg/*l*)	123	107	174
Total sugar added (g)	20	20	95
$Y_{X/S}$ (OD_{600}/g sugar)	2.03	1.73	0.81
$Y_{P/x}$ (mg LC I/OD_{600})	3.0	3.1	2.2

*Ethanol concentration was measured at the 12th hr for the batch culture and the 24th hr for the fed-batch culture.

cell, which resulted in the reduced expression level of LCI. This indicates that a high cell-density culture without deterioration in galactose-utilizing activity by ethanol is required to enhance the production of LCI.

In conclusion, the *GAL* promoter system used in this study was rapidly induced by adding galactose prior to the complete depletion of dextrose. During the initial growth phase, the dextrose which was used as the main carbon and energy source repressed strongly the cloned-gene expression. When dextrose was exhausted, the cells began to utilize ethanol and galactose simultaneously as carbon sources. As soon as galactose was metabolized by the cells, the expression of the cloned-gene began to be induced. The plasmid stability of the recombinant yeast used in this study was very high even in the galactose-metabolizing induction phase. In addition, the plasmid stability in the fed-batch culture was higher than that in the batch culture in spite of the prolonged culture time. This indicates that a potential for the large-scale production of recombinant human LCI could be possible by empolying a fed-batch culture mode.

REFERENCES

1. **Broker, M., D. Baumal, A. Gottig, J. Ochs, M. Bodenbenner and E. Amann.** 1991. Expression of human blood coagulation protein Factor XIII in *Saccharomyces cerevisiae*: Dependence of the expression levels from host-vector systems and medium composition. *Appl. Microbiol. Biotechnol.* **34**: 756-764.
2. **Caunt, P., A. Inopoolsup and P.F. Greenfield.** 1988. Stability of recombinant plasmids in yeast. *J. Biotechnol.* **8**: 173-192.
3. **Da Silva, N.A. and J.E. Bailey.** 1989. Effect of inducer concentration on *GAL* regulated clone gene expression in recombiant *Saccharomyces cerevisiae*. *J. Biotechnol.* **10**: 253-266.
4. **Da Silva, N.A. and J.E. Bailey.** 1991. Influencel of plasmid origin and promoter strength in fermentations of recombinant yeast. *Biotechnol. Bioeng.* **37**: 318-324.
5. **Fieschko, J.C., K.M. Egan, T. Ritch, R.A. Koski, M. Jones and G.A. Bitter.** 1987. Controlled expresion and purification of human interferon from high cell density fermentations of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **29**: 1113-1121.
6. **Guarente, L., P.R. Yocum and P.A. Gifford.**1982. *GAL 10-CYC1* hybrid yeast promoter identifies the *GAL4* regulatory region as an upstream site. *Proc. Natl. Acad. Sci. USA.* **79**: 7410-7414.
7. **Hjortso, M.A. and J.E. Bailey.** 1984. Plasmid stability in budding yeast populations: steady state growth with selective pressure. *Biotechnol. Bioeng.* **26**: 528-536.
8. **Ito, H., Y. Fukuda, K. Murata and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**: 163-168.
9. **Johnston, M.** 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. *Microbiol. Rev.* **51**: 458-476.
10. **Kingsman, S.M., A.J. Kingsman and J. Mellor.** 1987. The production of mammalian proteins in *Saccharomyces cerevisiae*. *Trends Biotechnol.* **5**: 53-57.
11. **Kleinman, M.J., E.B. Gingold and P.F. Stanbury.** 1986. The stability of the yeast plasmid pJDM 248 depends on growth rate of the culture. *Biotechnol. Lett.* **8**: 225-230.
12. **Lotti, M., D. Porro, E. Martegani and L. Alberghina.** 1988. Physiological and genetic modulation of inducible expression of *Escherichia coli* β-galactosidase in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **28**: 160-165.
13. **Mylin, L.M., K.J. Hofmann, L.D. Schultz and J.E. Hopper.** 1990. Regulated *GAL4* expression cassette providing controllable and high-level output from high-copy galactose promoter in yeast. p. 297-308. *In* D.V. Goeddel (ed.), *Methods in Enzymol.*, Vol. **185**, Academic Press, New York, London.
14. **Parker, C. and D. DiBiasio.** 1987. Effect of growth rate and expression level on plasmid stability in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **29**: 215-221.
15. **Porro, D., E. Martegani, B.M. Ranzi and L. Alberghina.** 1991. Heterologous gene expression in continuous culture of budding yeast. *Appl. Microbiol. Biotechnol.* **34**: 632-636.
16. **Porro, D., M. Lotti, E. Martegani, B.M. Ranzi and L. Alberghina.** 1992. Enhanced expression of heterologous proteins by the use of a superinducible vector in budding yeast. *Appl. Microbiol. Biotechnol.* **36**: 655-658.
17. **Schultz, L.P., K.J. Hofmann, L.M. Mylin, D.L. Montgomery, R.W. Ellis and J.E. Hopper.** 1987. Regulated overproduction of the *GAL4* gene product greatly increases expression from galactose inducible promoters on multicopy expression vectors in yeast. *Gene* **61**: 123-133.
18. **Schultz, L.D., J. Tanner, K.J. Hofmann, E.A. Emini, H.J. Condra, R.E. Jones, E. Kieff and R.E. Ellis.** 1987. Expression and secretion in yeast of a 400-kDa envelope glycoprotein derived from Epstein-Barr virus. *Gene* **54**: 113-123.
19. **Sohn, J.H., D.S. Na and S.K. Rhee.** 1991. Gene expression and secretion of human lipocortin-1 in *Saccharomyces cerevisiae*. *Korean Biochem. J.* **24**: 453-460.
20. **Stepien, P.P., R. Brousseau, R. Wu, S. Narang and D.Y. Thomas.** 1983. Synthesis of a human insulin gene. VI. Expression of the synthetic proinsulin in yeast. *Gene* **24**: 289-297.
21. **Thomas, D.S., J.A. Hossack and A.H. Rose.** 1978. Sterol and phospholipid composition influence ethanol tolerance in *Saccharomyces cerevisiae*. *Arch. Microbiol.* **117**: 239-245.
22. **Velati-Bellini, A., P. Pedroni, E. Martegani and L. Alberghina.** 1986. High levels of inducible expression of cloned β-galactosidase of *K. latis* in *S. cerevisiae*. *Appl. Microbiol. Biotechnol.* **25**: 124-131.

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