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Effect of Galactose Feeding Strategy on Heterologous Human Lipocortin-I Production in the Fed-Batch Culture of *Saccharomyces cerevisiae* Controlled by the *GAL10* Promoter

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Fed-batch fermentations were conducted to produce human lipocortin-I (LC1), a potential anti-inflammatory agent, from recombinant *Sacchromyces cerevisiae* carrying a galactose-inducible expression system. The cell growth, expression level of LC1, and the plasmid stability were investigated under various LC1 induction modes performed by three different galactose feeding strategies. Galactoe was fed to induce the expression of LC1 from the beginning (initial induction) of culture or when the cell concentration reached 120 OD (mid-phase induction) or 300 OD (late induction). Among the three galactose-induction modes tested, the initial induction mode yielded the best result with respect to a final expression level of LC1. Fed-batch fermentation with initial induction mode produced LC1 at a conentration of 220 mg/l, which corresponded to 1.38- and 1.53-fold increases over those produced by mid-phase and late induction modes.

A number of heterologous genes from human, animal, plant and viral origins have been expressed in the yeast *Saccharomyces cerevisiae* 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 (9). In addition, a simple purification process of correctly processed protein can be easily achieved by secreting the heterologous proteins of interest extracellularly and harvesting them directly from the culture broth. For the efficient expression of heterologous proteins in *S. cerevisiae*, a variety of vector systems have been designed and constructed so as to contain appropriate constitutive or inducible promoters. Among the inducible and strong promoters used in yeast, the galactose-inducible promoters such as *GAL1, GAL7, GAL10* and their hybrids with *CYC1* or UAS_G have been more frequently used to produce heterologous proteins from *S. cerevisiae* (1-5, 8, 13-15). In the galactose-inducible system, the yeast cells were first grown in a complex medium containing dextrose as a carbon source to repress the expression of the cloned-gene, and then galactose was added to the culture to induce the sysnthesis of the cloned-gene product. Therefore, the feeding strategy of galactose as well as dextrose is an important parameter to determine the final expression level of the cloned-gene product.

Lipocortin-1 (LC1) has been implicated as a glucocorticoid-induced, Ca^{++}-dependent and membrane-binding protein which mediates the anti-inflammatory function through phospholipase A_2 (PLA_2) inhibition (17). A cDNA coding for human LC1 has been cloned and expressed in *Escherichia coli* (6, 16) and *S. cerevisiae* (17). More recently, we have investigated the effects of carbon sources (dextrose and galactose) and transcriptional terminators on human LC1 expression in recombinant *S. cerevisiae* carrying the galactose-regulated expression system (12, 7).

In this study, the cell growth and product induction phases were separated in a manner so that the controlled-feeding of dextrose was first performed for cell growth, and human LC1 expression was subsequently induced by the continuous feeding of galactose. We have investigated the detailed effect of a galactose feeding strategy on the fed-batch production of human LC1 by recombinant *S. cerevisiae* carrying the galacoste-regulated expression system.

MATERIALS AND METHODS

Strain and Plasmid

Saccharomyces cerevisiae 2805 (*MATα pep4::HIS3 pro1-δ can1 GAL2 his3δ ura3-52*) was used as the

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host cell in this study. Plasmid YEGα-LC is a shuttle vector containing *the URA3* gene, the yeast 2μ origin, and the yeast *GAL10* promoter which regulates the expression of human LC1. For the secretion of LC1 into an extracellular medium, the prepro leader sequence (about 300 bp) of mating factor α was connected between *GAL10* promoter and LC1 gene (12). The resulting plasmid YEGα-LC (6.9 kb) was transformed into *S. cerevisiae* 2805 strain by the lithium-acetate method (7).

Media and Seed Culture Conditions

Minimal YNBCAD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid and 2% dextrose) was used for the selection of transformed cells and the seed culture. A single yeast transformant on the YNBCAD agar plate was inoculated into 10 ml YNBCAD medium and incubated overnight at 30°C. For further activation, the seed was transferred into a 500 ml Erlenmeyer flask containing 100 ml of the same medium, and incubated as above. This culture was used as an inoculum for the fed-batch fermentation.

Fed-Batch Fermentation

Table 1 shows the media compositions used for the fed-batch fermentation. After 100 ml of seed culture was transferred to a 2.5 l jar fermentor (KFC Co., Korea) containing 900 ml of initial medium, the cells were grown for activation and adaptation. As soon as dextrose was exhausted and dissolved oxygen concentration began to increase rapidly, the growth medium was continuously fed to the fermentor. The growth medium was switched to the induction medium for gene expression at appropriate culture times. The feeding rate was manually and intermittently controlled to keep the concentrations of dextrose and galactose zero. The culture temperature and pH were maintained at 30°C and 5.5, respectively. The aeration rate was 1 vvm, and the agitation speed was kept between 400-1,000 rpm to maintain dissolved oxygen concentration above 10% air saturation.

Table 1. Media compositions for fed-batch fermentations.

components	media (g/l)		
	initial	growth	induction
dextrose	5	400	
galactose	-	-	400
yeast extract	3	30	30
casamino acid	-	5	5
$(NH_4)_2SO_4$	3	40	40
sodium citrate	0.2	1.8	1.8
K_2HPO_4	1.5	14.9	14.9
$MgSO_47H_2$	0.5	5.7	5.7
*trace solution	5 ml	10 ml	10 ml

*trace solution: $(NH_4)_6MoO_{24}$, 3.0 μM; H_3BO_3, 400 μM; $CuSO_4$, 10 μM; $MnCl_2$, 80 μM; $ZnSO_4$, 10 μM.

SDS-PAGE and LC1 Assay

Extracellular proteins in the culture supernatant were precipitated by adding 10% (v/v) trichloroacetic acid (TCA) and 10% (v/v) 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 (SDS-PAGE). The protein bands on the gel were scanned by a densitometer (Biomed Instrument SCR 2D/1D, USA), and then the LC1 concentration was calculated by multiplying the scanned peak area of LC1 band (about 40 KDa) by the total concentration of protein loaded on the gel.

Analytical Methods

The growth of yeast cells was monitored by measuring the optical density (OD) at 600 nm (UVICON 930, Switzerland). The concentration of dextrose in the culture broth was measured by using a dextrose 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 gas chromatography equipped with a flame ionization detector (Hewlett Packard 5890, USA). Plasmid stability was determined by spreading the diluted samples onto YEPD (1% yeast extract, 2% bacto-peptone and 2% dextrose) agar plates and incubating them at 30°C for 2 days. Colonies were then replicated onto plates of defined YNBCAD 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

Fundamental Fermentation Parameters

The batch culture of recombinant *S. cerevisiae* was performed to obtain the fundamental fermentation parameters based on dextrose and galactose consumption. The cells were cultivated in a jar fermentor containing YEPDG medium composed of 1% yeast extract, 2% bacto-peptone, 1% dextrose and 1% galactose. The cells consumed only dextrose for growth for 12 h after inoculation, and then began to induce the expression of LC1 by using galactose. The fermentation parameters were estimated from these

Table 2. Fundamental fermentation parameters based on dextrose and galactose consumption.

	dextrose	galactose
specific growth rate (μ; h^{-1})	0.123	0.044
celluar growth yield ($Y_{x/s}$; g DCW/g carbon)	0.436	0.722
specific consumption rate of carbon source (q_s: g carbon/g DCW h)	0.282	0.061

batch culture data (Table 2) and used for the contol of medium feeding rate in fed-batch cultures. As expected, the specific growth rate and the specific substrate consumption rate based on dextrose consumption were bigger than those on galactose, while the cellular growth yield was less.

Dextrose and Galactose Feeding Strategies

Three different fed-batch fermentations were performed to investigate the effect of galctose feeding strategy on LC1 expression. In the initial induction mode, galactose was continuously fed to the culture as the sole carbon source during an overall period of culture, which resulted in simultaneous cell growth and LC1 expression. In the mid-phase induction mode, the growth medium was first fed to the culture for cell growth until the cell concentration reached about 120 OD, and then the induction medium started to be fed for LC1 gene expression. Finally, in the late induction mode, the cells were cultivated by supplying the growth medium until the cell concentration reached 300 OD, and then the induction medium was fed for LC1 gene expression.

In these fed-batch fermentations, the feeding rate was estimated by the following well-known equation in a singular region (10).

$$F = \frac{\mu XV}{(S_f - S)Y_{X/S}}$$

where F, feeding rate (l/h); μ, specific cell growth rate (1/h); X, cell concentration (g/l); S, carbon source concentration (g/l); S_f, carbon source concentration in the feed (g/l); $Y_{X/S}$, cellular yield coefficient based on carbon source consumption (g cell/g carbon source). The feeding rate was computed by using the parameters in Table 2.

Fed-Batch Fermentations with Various Galactose Feeding Strategies

Fig. 1 shows the results of fed-batch fermentations performed by initial galactose induction mode. Galactose concentration was increased by the continuous feeding of galactose and reached 30 g/l after 20 h of culture. After then, galactose levels declined rapidly

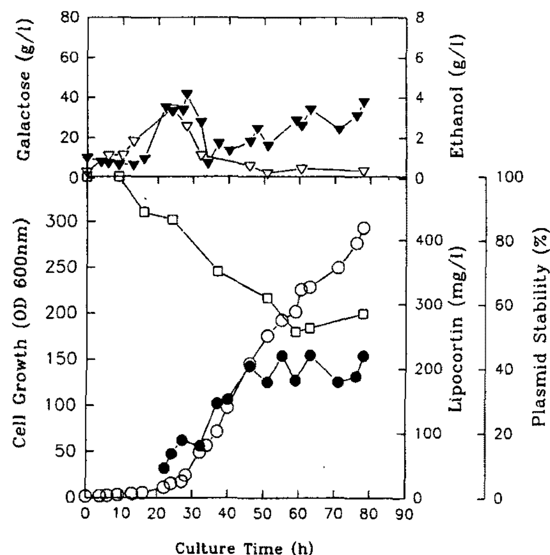

Fig. 1. *Time profiles of fed-batch fermentation with initial induction mode.*

○, cell concentration; ●, LC1 concentration; □, plasmid stability; △, galactose concentration; ▲, ethanol concentration.

and leveled off at around 5 g/l. Cell concentration began to increase at the 15 h after inoculum, and reached 293 OD (94 g DCW/l) after 78 h of culture. The secreted level of LC1 increased with increased cell concentration for 45 h of culture, and levelled off at around 220 mg/l after 45 h. The plasmid stability was rapidly decreased until 60 h of culture, and then maintained around 57%. The lag period observed by 15 h after inoculum appears to be caused by the induction of galactose-utilizing enzymes such as β-galactosidase.

To alleviate the metabolic stress resulting from the prolonged induction time, another mode of fed-batch fermentation were performed in such a manner that the cell growth and product induction phases were separated. In mid-phase induction, the growth medium containing dextrose as the sole carbon source was fed to the culture broth for 33 h after inoculation, and then the induction medium containing galactose as the sole carbon source was switched and continuously fed for another 43 h. The cell concentration just before the medium-switching was 120 OD. As shown in Fig. 2, the LC1 gene began to be expressed upon the addition of the induction medium. Decreased cell growth rate was observed during the induction period. Plasmid stability in mid-phase induction mode was higher than that in the initial induction mode. This, of course, which appeared to result from decreased induction time. LC1 at a concentration of about 160 mg/l was finally produced

with a final cell concentration of 315 OD.

In late induction mode, the cells were grown for initial 47 h by feeding the growth medium, and then the induction medium was switched. During the cell growth period, cells grew rapidly and reached about 300 OD after 47 h of culture. After LC1 gene induction by the addition of galactose-containing induction medium, a significant decrease in cell growth was not observed, resulting in a final cell concentation of 361 OD. At the end of fermentation, the plasmid stability and LC1 concentration were about 90% and 144 mg/l, respectively. During an overall period of culture, ethanol concentration was controlled not to exceed 6 g/l. Although the feeding rate of galactose-containing induction medium was also controlled as previously, the accumulation of galactose was observed especially in the initial period after induction. This also resulted from the time required for the induction of galactose-utilizing enzymes as observed in the mid-phase induction mode. Therefore, the actual feed rate of galactose needs to be lower than the theoretical one after switching to the galactose-containing induction medium.

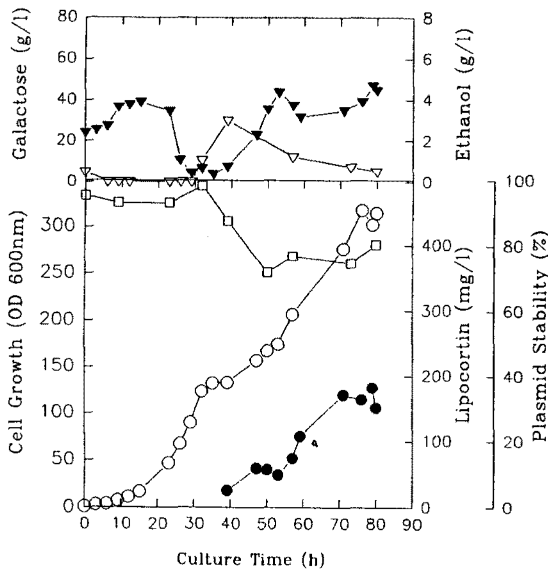

Fig. 2. Time profiles of fed-batch fermentation with mid-phase induction mode.
○, cell concentration; ●, LC1 concentration; □, plasmid stability; △, galactose concentration; ▲, ethanol concentration.

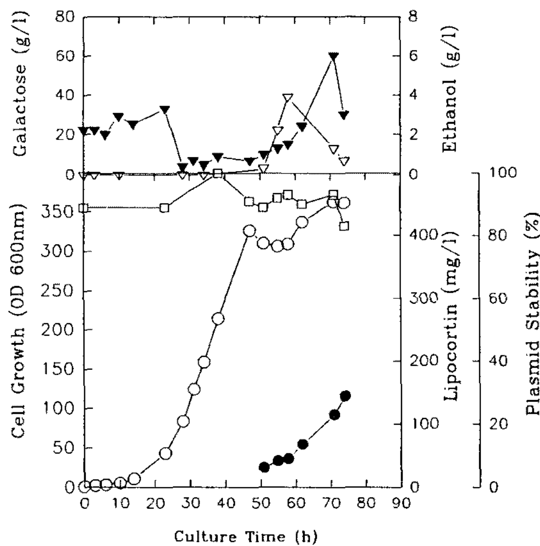

Fig. 3. Time profiles of fed-batch fermentation with late phase induction mode.
○, cell concentration; ●, LC1 concentration; □, plasmid stability; △, galactose concentration; ▲, ethanol concentration.

In fed-batch fermentations with mid-phase and late induction modes, the plasmid stabilities and final cell concentrations were increased, which appeared to result from less metabolic stress by shortened gene expression times. However, the amounts of secreted LC1 in these cases were lower than that produced by the initial induction mode. Among the induction modes tested, the highest expression level of LC1 was obtained by the initial induction mode.

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 (12). In all fed-batch fermentations performed, ethanol concentrations were well controlled not to exceed 6 g/l. Therefore, the effects of ethanol on LC1 expression could be minimized in this study.

In conclusion, the comparative results of all fed-batch fermentations are summarized in Table 3. It is of interest that the initial induction mode resulted in the best result with respect to a final expression level of LC1. Further effort is currently being made to furth-

Table 3. End-of-fermentation data for the fed-batch fermentations performed by various galactose feeding strategies.

	galactose induction modes		
	initial	mid-phase	late
culture time	78	79	74
dextrose fed (g)	0	63	250
galactose fed (g)	200	180	210
final cell conc. (g/l)	93.3	100.8	115.5
plasmid stability (%)	57	78	83
LC1 conc. (mg/l)	220	160	144
*specific LC1 expression rate (mg/g DCW h)	0.046	0.04	0.048

*based on the plasmid-containing cell.

er enhance the expression level of LC1, and the results will be reported soon.

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