BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY 14, 82–92 (1991)

Efficient Production of Recombinant DNA Proteins in *Saccharomyces cerevisiae* by Controlled High-Cell-Density Fermentation

LILIA ALBERGHINA, DANILO PORRO, ENZO MARTEGANI, AND BIANCA MARIA RANZI

Dipartimento di Fisiologia e Biochimica Generali, sez. di Biochimica Comparata, via Celoria 26, Università di Milano, 20133 Milano, Italy

ALBERGHINA, L., PORRO, D., MARTEGANI, E., AND RANZI, B. M. Efficient Production of Recombinant DNA Proteins in *Saccharomyces cerevisiae* by Controlled High-Cell-Density Fermentation. *Biotechnol. Appl. Biochem.* **14,** 82–92 (1991).

High levels of expression of heterologous proteins (from 5 to 15% of total cell proteins) in the budding yeast *Saccharomyces cerevisiae* have been obtained previously by the use of the inducible strong hybrid promoter $UAS_{GAL}/CYC1$, in batch as well in continuous cultures. However, in order to maximize the yield of heterologous proteins, a computer controlled fed-batch fermentation is essential. For this reason we have developed a fed-batch system based on a semiconductor gas detector that measures ethanol in the outflow gases. The optimal conditions are described for very high production (up to 1550 mg/liter), with both high productivity (up to 100–120 mg/liter/h) and high yield (up to 15 mg of protein/g of dry biomass), of heterologous protein driven by the $UAS_{GAL}/CYC1$ promoter in a completely computer controlled fed-batch fermentation of budding yeast. However, high production was dependent upon the addition of a large amount of galactose. The process was improved by developing a new, more easily inducible, vector system obtained by subcloning the GAL4 gene.

Recombinant DNA technology applied to cellular systems which are able to grow in large-scale fermenters has given the opportunity to obtain large quantities of proteins that would otherwise be difficult or impossible to obtain. For this purpose the use of the yeast *Saccharomyces cerevisiae,* as a host microorganism for the expression of heterologous proteins, has been particularly attractive. It is generally recognized as a safe organism (GRAS), lacking endotoxins and lytic viruses and is able to perform many post-translational modifications, including glycosylation, acylation and correct folding (1, 2). It has a secretory system very similar to that of higher eukaryotes (3), facilitating the recovery of the secreted product. The molecular biology of *S.cerevisiae* is very well developed and at present it is the only eukaryotic cell which is very near to the goal of having an entire chromosome (chromosome III) fully sequenced (4). It is possible to optimize the productivity of the *S.cerevisiae* expression system by attention to the genetic determinants of the promoter sequence, the growth conditions of the culture, and its volumetric productivity.

In previous reports from our laboratory it has been demonstrated that fairly high yields of heterologous proteins (from 5 to 15% of total cell proteins) are produced using a strong inducible $UAS_{GAL}/CYC1$ promoter in batch cultures (5–10). Several genes have been expressed in this system, *Escherichia coli* and *Kluyveromyces lactis* β-galactosidase, maize zein, *Saccharomyces diastaticus* glucoamylase, human tissue

0885-4513/91 $3.00

Culture Conditions and Methods

Fermentation experiments were performed in a 1.5-liter bioreactor equipped with temperature and pH controllers (19). Fed-batch processes were performed at 30°C and pH 4.95. Dissolved oxygen was measured with a galvanic (Pt/Pb) electrode. During fermentation the bioreactor was aerated with an air flow of 2.5 liter/min and dissolved oxygen tension was controlled by agitation (between 360 and 1000 rpm). At the beginning of each experiment, the dissolved oxygen tension was about 100% of saturation. When the biomass increased at the end of the experiment it was still above 20% of saturation. The pH was maintained at 4.95 by addition of 4 M ammonia (which also constituted the nitrogen source). Foam, when significant during the culture, was suppressed by the addition of drops of dilute and sterilized antifoamer (polypropylene glycol 2000).

Ethanol concentration in the culture liquid was measured by the FMC system (Arge Biotechnologie, Austria). The system consists of a porous silicone tubing sensor, a fermentation microcomputer and a dialogue station with a keyboard and a monitor (19). A further description of the continuous ethanol determination appears under Results. Fermentation parameters were acquired on line with a MINC 11/23 computer (Digital Equipment Corp., Maynard, MA).

For dry mass weight measurements, glass microfiber filters (Whatman, GF/A) were used; after removal of the medium by suction, the filter with the pellet was washed with deionized water and then dried to constant weight at 100°C. The weight of cells per liter was calculated.

Cell viability and plasmid stability were established by spreading culture samples on X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside, 50 μg/ml)–YNB–galactose (selective) and on YEPD (yeast–extract–peptone–glucose, nonselective) plates. Blue colonies on the selective plates represented the plasmid containing cells, while white colonies on the nonselective plates indicated total viable cells.

Determination of Glucose and Galactose

Glucose was determined by standard enzymatic methods as previously described (20). Galactose was determined by a standard enzymatic method using a Lambda-3 spectrophotometer (Perkin–Elmer) according to Kurz and Wallenfels (21).

β-Galactosidase Assay

Small samples of the culture were washed with distilled water and specific β-galactosidase activity (mg/liter) was detected as previously described (6); units were calculated according to Miller (22).

Electrophoresis and Immunoblotting

Total yeast proteins were prepared, fractionated by 8% SDS–polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose filters and immunodetected with polyclonal anti-β-galactosidase antibodies as previously described (23).

RESULTS

A Fed-Batch Controlled by Ethanol Production

In order to achieve a high yield of glucose conversion in biomass and an elevated volumetric productivity, the supply rate of glucose to the culture has to be regulated in such a way as to prevent ethanol production due to the Crabtree effect (24).

We have developed a fed-batch control system based on the determination of the ethanol in the bioreactor, using a gas sensor probe with a silicone membrane. The control system regulated the feed rate of the growth medium in order to maintain the ethanol concentration at a low and constant preset level (19). With this control system, we obtained high cell densities (up to 100 g dry weight per liter) with an overall volumetric productivity of 1.55 g dry mass/liter/h, starting from 19.36 g dry mass per liter, with a yield of 0.46.

However, the efficiency of this control system was largely reduced by the slow response of the submerged ethanol probe, due to the relatively long period of time required by the silicone tubing probe to detect small changes of ethanol concentration. This fact caused a large accumulation of ethanol in the medium which in turn required a long time to be oxidized (data not shown). The process was greatly improved by measuring ethanol directly in the outflow gases. This was performed by constructing a stainless steel chamber that surrounds the semiconductor gas sensor, and forcing the outflow gases to circulate in this chamber. With this modified control system, the same cell density, but with a volumetric productivity of 3.58 g dry mass/liter/h (starting from 14.82 g/liter of dry mass) was obtained.

Production of Heterologous Proteins in Controlled Fed-Batch Fermentation

The *E.coli* β-galactosidase gene borne by the pLA41 (12) plasmid under the strong inducible promoter $UAS_{GAL1-10}/CYC1$ (Fig. 1) was initially used as a model gene for the expression system.

The yeast strain GRF18[pLA41] was grown in fed-batch using glucose as the sole carbon source, and the synthesis of heterologous β-galactosidase was then induced by adding galactose.

Different protocols were used to obtain full induction. Initially we simply added galactose (10 g/liter final concentration) to the fermenter. Under these conditions (Fig. 2A) a rather low level of heterologous protein was obtained; a maximal level of about 100 mg/liter was measured after a few hours, and this value corresponded to only 0.5% of total yeast proteins, much less than the levels of expression previously obtained in batch and continuous cultures (6, 12). This poor expression efficiency could be understood when the inducer concentration in the medium was measured. In fact, since the glucose concentration was very low (between 5 and 50 mg/liter) the yeast cells were derepressed for catabolite repression (14) and immediately metabolized the added galactose, so that it rapidly became undetectable in the culture medium (data not shown).

To overcome this problem we induced expression by using a mixed substrate in the feed, i.e., glucose plus increasing amounts of galactose, starting from a 10:1 ratio (45.5 g of glucose plus 4.5 g of galactose in 100 ml of feed medium) to a 2:1 ratio (33 g of glucose and 16.5 g of galactose). The results of the different procedures used are shown in Figs. 2B and 2C.

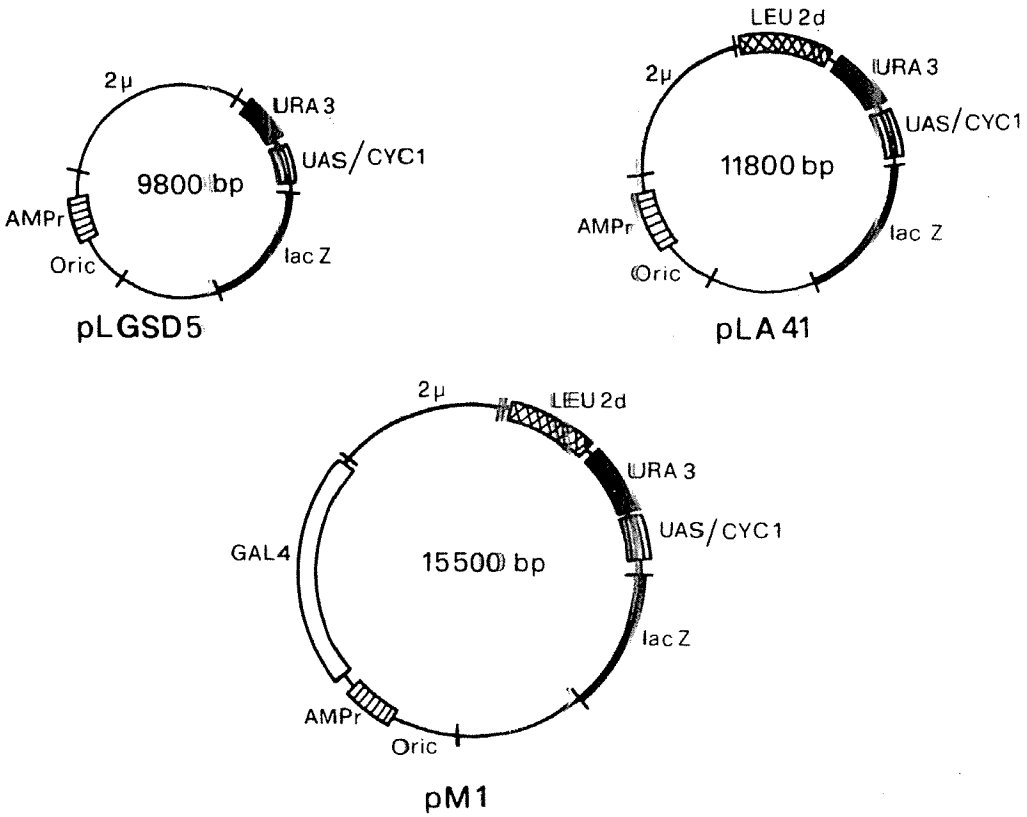

FIG. 1. Plasmids used in this work. pLGSD5 (15) contains the hybrid $UAS_{GAL1\text{-}10}/CYC1$ yeast promoter fused with the *E.coli* β-galactosidase gene. Expression of the heterologous gene is induced by galactose and repressed by glucose. Plasmid pLA41 (12) was constructed by replacing a 700-basepair 2-μm fragment of pLGSD5 with a 2000-basepair fragment spanning the *LEU2d* gene and part of the 2 μm sequences. Plasmid pM1 (13) was obtained by subcloning the *GAL4* gene in pLA41.

A low galactose concentration (10:1) immediately induced a low level of expression; however, if galactose was present in a 2:1 ratio a stronger and faster induction was observed to a level of 1400 mg/liter of β-galactosidase at a cell density of 100 g of dry weight/liter. This level of expression corresponded to approximately 7% of total yeast proteins, a value comparable with the maximal levels obtained in batch cultures (6).

In all these experiments glucose remained the predominant carbon source, and this allowed the computer to control the process, since the cells continued to grow during the production phase.

As shown in Fig. 2C no marked increase could be obtained by using only galactose (50% (w/v)) in the feed medium. The arrow F in Fig. 2C indicates the time at which the growth medium was changed to a continuous galactose feeding (100 ml/h, 50% (w/v)), so that galactose was the only carbon source. After a slight increase in enzyme productivity (from 1400 to 1550 mg/liter), both heterologous expression and biomass concentration approached an apparent steady state. Under these conditions, a very high heterologous productivity was obtained for several hours (100–120 mg of protein produced per liter in 1 h of fermentation), with high heterologous protein yield (15.5 mg of heterologous protein per gram of dry mass, corresponding to 8% of total yeast proteins).

All the levels of expressed enzyme were calculated on the basis of β-galactosidase activity, measured either in cell free extracts (6) or in permeabilized cells (25). How-

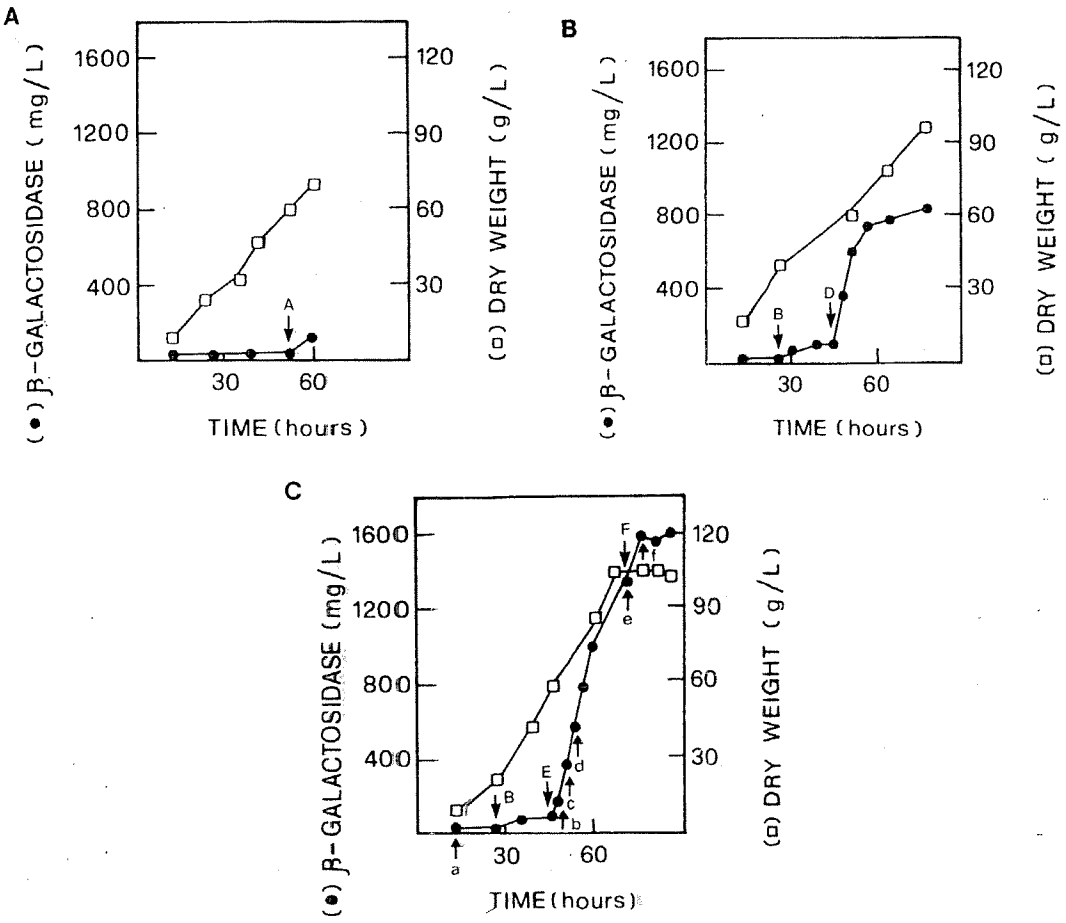

FIG. 2. Yeast biomass and β-galactosidase production in fed-batch cultures of transformant GRF-18[pLA41]. Fed-batch fermentations were performed in a 1.5-liter bioreactor equipped with pH and temperature controllers. Experiments were conducted at 30°C and pH 4.950. 50% (w/v) is the concentration of the total carbon source in the semisynthetic growth medium employed. Our control system was based on the ethanol concentration determined in the outflow gases from the bioreactor. Different methods of induction were used: after the fed-batch growth of the recombinant biomass on glucose, we induced heterologous synthesis by addition of galactose (A). Arrow A indicates the time at which galactose was pulsed to the bioreactor (10 g/liter the final concentration). The optimal conditions of heterologous production were found to be step replacements of the growth medium with mixed glucose–galactose medium at a higher concentration of galactose (B and C). (B) Arrows indicate the time at which the growth medium was changed as follows: B, glucose feed replaced by a glucose/galactose (ratio 10:1) feed; D, glucose–galactose (ratio 10:1) feed replaced by a glucose–galactose (ratio 3:1) feed. (C) Arrows indicate: B, glucose feed replaced by a glucose/galactose (ratio 10:1) feed; E, glucose–galactose (ratio 10:1) feed replaced by a glucose–galactose (ratio 2:1) feed; F, glucose–galactose (ratio 2:1) feed replaced by a galactose feed. The small arrows (from a to f) indicate the time points at which small samples of culture were rapidly collected; the relative total cell proteins were analyzed as shown in Fig. 3. (●) β-Galactosidase production, mg/liter; (□) biomass production, g dry weight/liter.

ever, a Western blot analysis performed with anti-β-galactosidase antibodies demonstrated that the major product was a 116-kDa polypeptide (Fig. 3).

Development of a Derepressed Superinducible Expression System

Under the conditions indicated above enhanced productivity was dependent upon the addition of high amounts of galactose; in fact, for the experiment shown in Fig.

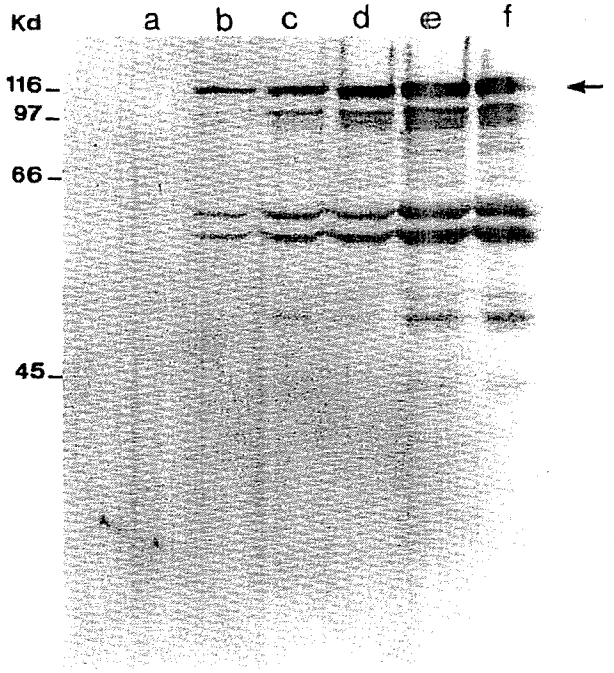

FIG. 3. Immunoblot of total cell protein extracted at selected times (indicated by the small arrows a to f) from the fed-batch fermentation shown in Fig. 2C. Proteins were fractionated by 8% SDS–PAGE, transferred to nitrocellulose and probed with anti-β-galactosidase polyclonal antibodies. 10 μg of total yeast proteins were loaded in each lane. The major product accumulated was identified as a 116-kDa protein. Other immunoreactive species of low molecular weight were detected and are likely to represent degradation products of the mature heterologous enzyme, since their levels are proportional to enzyme activity.

2C, about 400–450 g of galactose were used. Since the goal of this project was to achieve a convenient and efficient process, we looked for possible improvements.

The expression of galactose-induced genes in *S.cerevisiae* is principally regulated by the *GAL4* and *GAL80* gene products. GAL4 protein is a transcriptional activator that binds to UAS_{GAL} sequences (14, 16). The activity of the GAL4 protein is inhibited by the binding of the GAL80 protein to its carboxy terminal region. The system is repressed by glucose, which probably inhibits the binding of GAL4 protein to the UAS_{GAL}, and is induced by galactose, which causes the dissociation of the GAL80 protein from the GAL4 protein. The overall ratio of regulation, between a repressed system and a fully inducted one, exceeds 1000-fold (14). The amplification of GAL4 protein causes a constitutive synthesis of the galactose regulated genes (13, 14, 16). Thus, aiming to improve our process, we used yeast cells transformed with the plasmid pM1 (Fig. 1). This vector was previously constructed by cloning the *GAL4* gene with its promoter into the pLA41 plasmid (13). Yeast transformants carrying the *GAL4* gene on a multicopy plasmid showed a relevant increase in β-galactosidase expression during batch growth on glucose (GRF18[pLA41], 0.0002%; GRF18 [pM1], 1.66% of total cell proteins), but no additional expression was obtained during growth on galactose (GRF18[pLA41], 7.8%; GRF18[pM1], 7.4% of total cell proteins). These results indicate that the overexpression of the *GAL4* gene has a positive, but limited effect on the overall production of the cloned gene suggesting that the heterologous gene expression might be controlled by other regulatory elements beside the GAL4 and GAL80 proteins. However, pM1 represents a constitutive expression system. During a controlled fed-batch fermentation with transformed

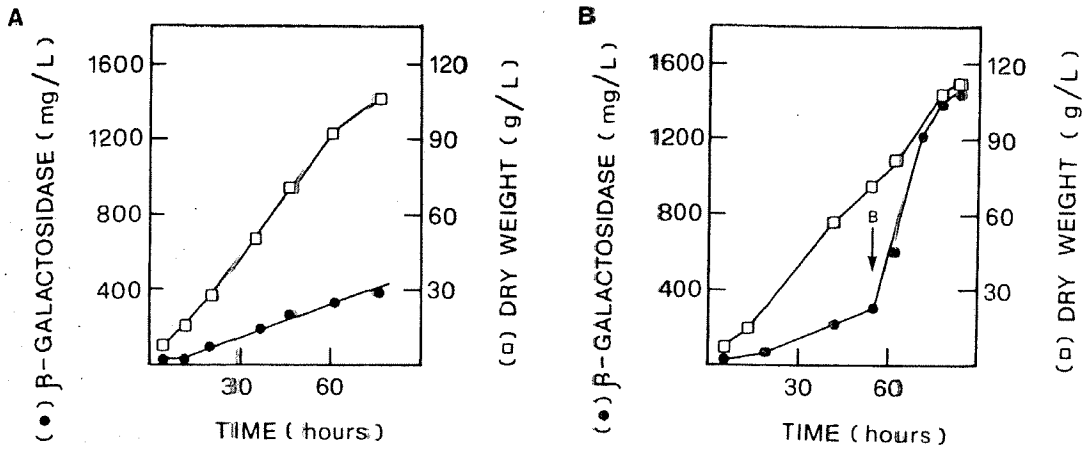

FIG. 4. β-Galactosidase production in fed-batch fermentations of transformant GRF18[pM1]] during growth on either glucose (A) or mixed substrate glucose–galactose (B). Arrow B (in B) indicates the time point at which the substrate was changed from glucose to glucose–galactose (ratio 10:1). (●) β-Galactosidase production, mg/liter; (□) recombinant biomass production, g dry weight/liter.

GRF18[pM1] cells, heterologous β-galactosidase was expressed constitutively at relatively high levels during growth on glucose (up to 400 mg/liter) (Fig. 4A). However, we found that the system was still subject to galactose induction. A very high productivity was reached, again, by using a mixed substrate in the feed medium with a low concentration of galactose (glucose/galactose, ratio 10:1) (Fig. 4B). Also in this case a high yield of heterologous protein was obtained (up to 15 mg of β-galactosidase per gram of dry mass, corresponding to 7–8% of total cell proteins).

DISCUSSION

Efforts have been made in the last few years to develop suitable microbial systems for the expression of recombinant DNA proteins in high quantity, in active form, and under conditions that allow easy purification of a safe product. In this paper we describe fermentation processes that yield high product concentration (about 1500 mg of active rDNA protein per liter of culture), good productivity, and high yield, in a reproducible manner in a GRAS organism such as *S.cerevisiae.*

Several laboratories have developed fed-batch fermentation processes for recombinant yeasts with both constitutive and inducible expression systems (26–29). Generally, the use of constitutive promoters (i.e., α factor, PGK, GDP) does not ensure high heterologous production (27–29) (from 2 to 20 mg/liter). In fact, in some cases, the recombinant cells failed to grow to high cell densities and/or exhibited low plasmid stability. It was demonstrated that these problems are due to the high constitutive expression of the cloned gene (26). For these reasons, inducible promoters are preferred. However, the inducer used might have a relevant effect on the process. This is especially true when the inducer is not gratuitous. For example, galactose which is used for UAS_{GAL} promoters, is very expensive and is needed in large amounts to maintain the induction. In addition, in some of the described processes the fed-batch is controlled in an empirical manner (29) and this obviously makes the process more difficult to control and to reproduce. The process described here is completely computer controlled, and the only manual step required is the inoculum and the withdrawal of culture samples.

TABLE I

Yields of Heterologous Protein in Fed-Batch Cultures of Transformed Yeast

Plasmid	*Carbon source in fed media*	*β-Gal yield (mg/g)*
pLA41	Glucose	0.02
pLA41	Glucose/galactose (ratio 10:1)	1.72
pLA41	Glucose/galactose (ratio 3:1)	8.22
pLA41	Glucose/galactose (ratio 2:1)	14.01
pM1	Glucose	3.80
pM1	Glucose/galactose (ratio 10:1)	14.40

Note. Saccharomyces cerevisiae GRF18 harboring plasmid pLA41 or pM1 was cultured in fed-batch cultures as described under Experimental Procedures. In all the experiments, the concentration of the total carbon source was 50% (w/v); the levels of expressed enzyme were calculated on the basis of β-galactosidase activity (300,000 units/mg). β-Galactosidase yield represents mg β-Gal produced/g dry biomass.

The procedure of induction is also relevant for maximal productivity and we have found that best results are obtained by using a mixed substrate in the feed during the induction period, starting from a biomass at densities of about 30–50 g of dry weight/liter. This procedure has two advantages, (i) less galactose is necessary, since glucose continues to be the main carbon source and (ii) the computer control continues to be operative during the productivity phase. However, it should be noted that during the expression phase only one duplication of biomass occurs (from 50 to 100 g of dry weight/liter) and this fact decreases problems related to plasmid instability; in fact we never observed loss of plasmid under our fed-batch conditions.

We also have exploited the possibility of modifying the regulatory circuit of the *GAL* system through an increase of *GAL4* gene dosage. We have previously shown, using a cotransformation procedure (6), that in most conditions the amount of GAL4 protein appears not to be limiting for the maximal expression of *GAL* regulated genes. However an overexpression of *GAL4* has interesting consequences for our system. The production of heterologous proteins becomes constitutive and a substantial amount of enzyme is produced during growth in glucose. However the system is still induced by galactose, with the advantage that the same level of expression can now be obtained with a much lower inducer concentration. Therefore, for possible industrial applications, the reduction of the level of galactose required for inducing the superinducible plasmid pM1 (Fig. 4B) is quite convenient. However for this process it is important to start with high cell concentrations (not lower than 8–10 g/dry weight/liter) to avoid loss of plasmid during fed-batch growth. The different yields of heterologous protein obtained with the above described fed-batch cultures were compared in Table I.

Finally, the use of a secretion vector constructed in our laboratory using the signal sequence of the glucoamylase II gene of *Saccharomyces diastaticus* (23), under the high-density fed-batch culture conditions described in this paper, might constitute a very efficient system for the production of heterologous secreted proteins. In addition, we have previously shown that analysis of the cell size distribution, like protein distribution (obtained by flow cytometry) and volume distribution (obtained by Coulter counter), provides relevant informations about the growth of the cell popula-

tion and can be used to control the growth dynamics of a yeast population (30). Very important progress in the production of heterologous proteins in fed-batch cultures could result from the control of heterologous productivity by the analysis of biparametric (cell size vs heterologous production) size distributions. Preliminary studies are under way.

ACKNOWLEDGMENT

This work was supported by CNR, special grant P.F. Biotecnologie e Biostrumentazioni, Subproject 3, to B.M.R.

RECEIVED: December 17, 1990; ACCEPTED March 5, 1991

REFERENCES

1. SCHEKMAN, R. (1985) *Ann. Rev. Cell. Biol.* **1,** 115–143.
2. VAI, M., POPOLO, L., GRANDORI, R., LACANÀ, M., AND ALBERGHINA, L. (1990) *Biochim. Biophys. Acta* **1038,** 277–285.
3. TANNER, W., AND LEHLE, L. (1987) *Biochim. Biophys. Acta* **906,** 81–99.
4. Proc. BAP Meeting on Sequencing of the Yeast Chromosome III (1989) Tutzing (D), 31 October.
5. COMPAGNO, C., CORAGGIO, I., RANZI, B. M., ALBERGHINA, L., VIOTTI, A., AND MARTEGANI, E. (1987) *Biochem. Biophys. Res. Commun.* **146,** 459–465.
6. LOTTI, M., PORRO, D., MARTEGANI, E., AND ALBERGHINA, L. (1988) *Appl. Microbiol. Biotechnol.* **28,** 160–165.
7. VELATI-BELLINI, A., PEDRONI, P., MARTEGANI, E., AND ALBERGHINA, L. (1986) *Appl. Microbiol. Biotechnol.* **25,** 124–131.
8. VANONI, M., LOTTI, M., AND ALBERGHINA, L. (1989) *Biochim. Biophys. Acta* **1008,** 168–176.
9. CORAGGIO, I., COMPAGNO, C., MARTEGANI, E., RANZI, B. M., SALA, E., ALBERGHINA, L., AND VIOTTI, A. (1986) *EMBO J.* **5,** 459–465.
10. MARTEGANI, E., VANONI, PORRO, D., RANZI, B. M., FORLANI, N., SCHLEUNING, W. D., AND ALBERGHINA, L. (1989) Symposium on biochemistry for biotechnology, Post *FEBS, S.I.B., Como,* L25.
11. VANONI, M., PORRO, D., LOTTI, M., ALBERGHINA, L., AND MARTEGANI, E. (1988) *Life Sci. Adv. Mol. Genet.* 7, 89–94.
12. PORRO, D., MARTEGANI, E., RANZI, B. M., AND ALBERGHINA, L. (1991) *Appl. Microbiol. Biotechnol.* **34,** 632–636.
13. MARTEGANI, E., PORRO, D., LOTTI, M., AND ALBERGHINA, L. (1990) 115th International Conference on Yeast Genetics and Molecular Biology, The Hague, Netherlands, p. 290.
14. JOHNSTON, M. (1987) *Microbiol. Rev.* **51,** 458–476.
15. GUARENTE, L., YOCUM, R., AND GILFORD, F. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7410–7414.
16. JOHNSTON, S. A., AND HOPPER, J. E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6971–6975.
17. MANIATIS, T., FRITSCH, E. F., AND SAMBROOK, J. (1982) "Molecular Cloning. A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
18. ITO, H., FUKUDA, K., MURATA, K., AND KIMURA, A. (1983) *J. Bacteriol.* **153,** 163–168.
19. PORRO, D., MARTEGANI, E., TURA, A., AND RANZI, B. M. (1991) *Res. Microbiol.* **141,** in press.
20. PORRO, D., MARTEGANI, E., RANZI, B. M., AND ALBERGHINA, L. (1987) *Biotechnol. Bioeng.* **32,** 411–417.
21. KURZ, G., AND WALLENFELS, K. (1983) *in* Methods of Enzymatic Analysis (Bergmeyer, H. V., Ed.), Vol. 3, p. 1279, Academic Press, San Diego.
22. MILLER, J. H. (1972) Experiments in Molecular Genetics, p. 352, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

23. VANONI, M., PORRO, D., MARTEGANI, E., AND ALBERGHINA, L. (1989) *Biochem. Biophys. Res. Commun.* **164,** 1331–1338.
24. DAIRAKU, K., YAMASAKI, Y., KUKU, K., SHIOYA, S., AND TAKAMATSU, T. (1981) *Biotechnol. Bioeng.* **23,** 2069–2081.
25. RANZI, B. M., PORRO, D., COMPAGNO, C., AND MARTEGANI, E. (1987) *J. Biotechnol.* **5,** 227–231.
26. FIESCHKO, J. C., EGAN, K. M., RITCH, T., KOSKI, R. A., JONES, M., AND BITTER, G. (1987) *Biotechnol. Bioeng.* **29,** 1113–1121.
27. COPPELLA, S. J., AND DHURJATI, P. (1989) *Biotechnol. Bioeng.* **33,** 976–983.
28. GIU, M. B., JUNG, K. H., PARK, M. H., SHIN, K. S., AND KIM, K. H. (1989) *Biotechnol. Lett.* **11,** 1–4.
29. HSIEH, J. H., SHIH, K. Y., KUNG, H. F., SHIANG, M., LEE, L. Y., MENG, M. H., CHANG, C. C., LIN, H. M., SHIH, S. C., LEE, S. Y., CHOW, T. Y., FENG, T. Y., KUO, T. T., AND CHOO, K. B. (1988) *Biotechnol. Bioeng.* **32,** 334–340.
30. ALBERGHINA, L., RANZI, B. M., PORRO, D., AND MARTEGANI, E. (1990) Annual Meeting of the AIChE, Chicago, IL, 7A.