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Flow Cytometric Analysis of Human Lysozyme Production in Recombinant *Saccharomyces cerevisiae*

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Abstract Flow cytometric techniques were used to investigate cell size, protein content and cell cycle behavior of recombinant *Saccharomyces cerevisiae* strains producing human lysozyme (HLZ). Two different signal sequences, the native yeast *MFα1* signal sequence and the rat α-amylase signal sequence, were used for secretion of HLZ. The strain containing the rat α-amylase signal sequence showed a higher level of internal lysozyme and lower specific growth rates. Flow cytometric analysis of the total protein content and cell size showed the strain harboring the native yeast signal sequence had a higher total protein content than the strain containing the rat α-amylase signal sequence. Cell cycle analysis indicated that the two lysozyme producing recombinant strains had an increased number of cells in the G_2+M phase of the yeast cell cycle compared with the host strain SEY2102.

Keywords: flow cytometry, *Saccharomyces cerevisiae*, human lysozyme, cell cycle, secretion.

INTRODUCTION

Recombinant expression systems such as *Saccharomyces cerevisiae* have generated a wide range of new applications and products [1-8]. The development of these recombinant DNA systems created the need to understand and to predict their behavior in response to changes in growth conditions. Maximizing the productivity of these recombinant expression systems often requires special attention to the 'trade-offs' involved between cloned-gene expression and the biosynthetic activity of the host cell [9]. Failure to control cloned-gene expression may lead to undesirable overproduction of the cloned-gene protein which is deleterious and may even prove to be fatal to the growth of host cells.

Flow cytometry offers rapid measurements of physical and biochemical characteristics of individual cells, allowing statistical distributions of these properties in the whole population to be obtained in a very short period of time [10]. This analytical technique has been applied to growing yeast cells for variety of purposes including characterization of single-cell growth dynamics, kinetic analysis of heterologous protein production, selection of yeast cells with specific properties, and studies on the metabolic state of cell populations [11-22].

Lysozyme is a hydrolytic enzyme that cleaves the α, 1→4 glycosidic linkages in the murein cell wall of various bacteria [23]. The human lysozyme gene (HLZ) containing 130 amino acids acquires a mass of approximately 14.5 kD and is cross-linked by four disulfide bonds [24].

Based on the previously published work [5,7], we used flow cytometric techniques to investigate the physiological changes and secretion behavior of recombinant *S. cerevisiae* producing cloned human lysozyme.

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MATERIALS AND METHODS

Strain and Plasmids

The *S. cerevisae* strain SEY2102 [MATα *ura* 3-52 *leu* 2-3-112, *his* 4-519] was used for transformation of plasmids pMC614 and pMC632 [5]. These 2 μm based plasmids both contain the *ADH1* promoter, the HLZ structural gene, and the *LEU2* gene as a selection marker. Plasmid pMC614 contains the native yeast *MFα1* signal sequence for the secretion of human lysozyme, while plasmid pMC632 employs the rat α-amylase signal sequence.

Media and Cultivation

Recombinant *S. cerevisiae* strains producing HLZ were grown in leucine-free medium supplemented with 80 g/L glucose. Approximately 1 mL of frozen yeast culture was used to inoculate 5 mL of selective medium. The culture was allowed to grow for 24 h at 30°C in a shaking incubator. From the overnight culture, 2.5 mL was

used to inoculate 50 mL of fresh medium in a 250-mL Erlenmeyer flask. This culture was again placed in the incubator and grown to the late exponential growth phase and then, 30-40 mL of the culture was used to inoculate the fermentor (Queue Systems, Parkersburg, WV. USA). During the fermentation, the temperature was maintained at 30°C and the pH was kept at 5.5 through the addition of sterile 1.0 M phosphoric acid and 1.0 M ammonium hydroxide.

Analysis

Dry cell mass concentration was measured with a spectrophotometer (Bausch and Lomb, Berlin, Germany) at 600 nm. Glucose concentrations were determined either by an enzymatic kit (Sigma Chemical Co., St. Louis, MO, USA) or a glucose analyzer (YSI Life Sciences, Yellow Springs, OH, USA). A Varian 3,700 gas chromatograph (Palo Alto, CA, USA) was used to measure ethanol concentrations. Helium was used as a carrier gas at a flow rate of 30 mL/min. The injector and detector were maintained at 160°C while the column was kept at 130°C. To determine the amount of human lysozyme activity in either the culture broth or inside the cells, a method developed by Morsky [25] was adapted for use with recombinant yeasts. In the assay, crude lysozyme solution was added to 55 mM phosphate buffer (pH 6.2), which contained human serum albumin and *Micrococcus lysodeikticus* (Sigma Chemical Co., St Louis, MO, USA) as a substrate. The mixture was placed in a prewarmed cuvette (37°C) and the decrease in absorbance was followed for three minutes. One unit of lysozyme activity was defined as decrease in absorbance (DA) per minute at 700 nm (1 unit = 0.001 DA/min).

Flow Cytometric Analysis

Cells harvested during the culture were fixed by adding 2.5 mL of a sample to 18 mL of ice-cold 70% ethanol. The mixture was then vortexed and stored at –20°C until a flow cytometric run could be performed. Quantization of the cell size and total protein content was accomplished by running beads of known size and fluorescence through the flow cytometer. For cell size measurement, two different sized beads, 2.07 μm and 6.9 μm were used. Fluorescein isothiocyanate (FITC) was used to stain cellular proteins. Fixed cells were sonicated with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Plainview, NY, USA) for one minute and washed with 0.1 M phosphate buffer (pH 7.4). The cells were then stained with FITC solution (0.05 mg/mL 0.5 M $NaHCO_3$) for 40 min on ice. The stained cells were finally resuspended in 0.85% saline solution. Flow cytometric analyses were carried out on a Coulter Epics flow cytometer (Coulter Corp., Hialeach, FL, USA) utilizing an argon laser at a wavelength of 488 nm and a light output of 0.11 W. A 525 nm band-pass filter (Omega Optical, Brattleboro, VT, USA) was used to detect linear green fluorescence. Total protein content was calibrated in terms of percent brightness [10]. A 12.5% set of beads was run on the flow cytometer and the mean of this distribution was used to find the percent brightness of the cells. For cell cycle analysis, acriflavine was used to stain DNA. RNA was removed with 4 N HCl and cells were stained with acriflavine solution for 20 min. Excess acriflavine was then removed by washing with an acid-alcohol solution (1% HCl in 70% ethanol). After washing three times with acid-alcohol solution, the cells were resuspended in distilled water and stored for the flow cytometric analysis.

RESULTS AND DISCUSSION

Aerobic batch fermentations were performed for the lysozyme producing recombinant yeast strains with leucine-free medium supplemented with 80 g/L glucose. High glucose concentration was used to derepress the *ADH1* promoter, leading to the expression of human lysozyme. The strain containing pMC614 plasmid demonstrated an initial exponential phase of growth on glucose as expected, but the cells left the exponential growth phase before glucose had been completely consumed. The cells then continued to grow on glucose at a reduced rate. The HLZ level began initially very high and leveled off at the completion of the exponential phase of growth.

Flow cytometric analysis showed an initial adjustment of the seed culture to the fresh growth medium where the total protein and cell size levels decreased to a minimum level during the exponential phase of growth on glucose as reported elsewhere [7,26,27]. Towards the end of the exponential growth, an increase in both cell size and total protein content was observed. It was important to note that the highest amount of HLZ was produced during the exponential phase of growth.

Batch culture of SEY2102/pMC632 containing the rat α-amylase signal sequence was also performed. The growth behavoir was very similar to that for SEY2102/pMC614. The cells left the exponential growth phase prior to the complete consumption of glucose and continued to grow on glucose at a reduced rate. Since these strains were essentially identical except for the nature of the signal sequence for HLZ, similar growth characteristics were expected. However, both the growth rate and the lysozyme secretion rate for the strain harboring the native yeast signal sequence were higher than the strain with the non-native signal sequence as reported previously [5].

The flow cytometric results were also similar between the two strains, with reduced cell size and protein content following a rapid increase in both of these parameters during the later stages of the exponential phase of growth. The amount of HLZ produced was also similar. It was discovered, however, through localization experiments that there was three to four times more HLZ located within the cells in the strain with the rat α-amylase signal sequence than in the recombinant strain containing the native yeast signal sequence.

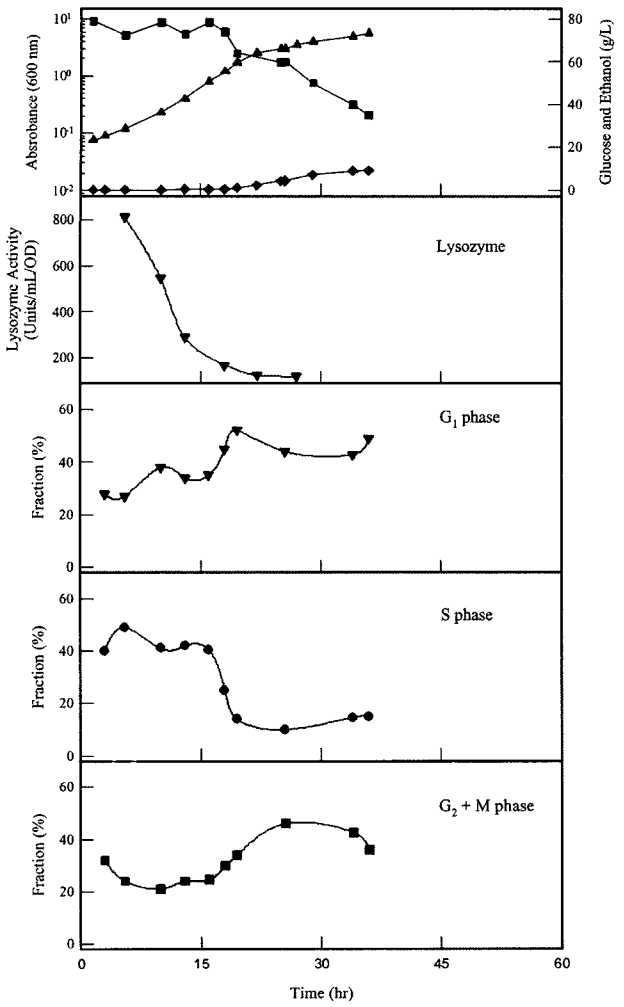

Fig. 1. Profiles of fermentation and cell cycle parameters for aerobic batch culture of recombinant *S. cerevisiae* SEY2102/pMC614 at pH 5.5 and 30°C. Ethanol (◆), glucose (■), and OD_{600} (▲).

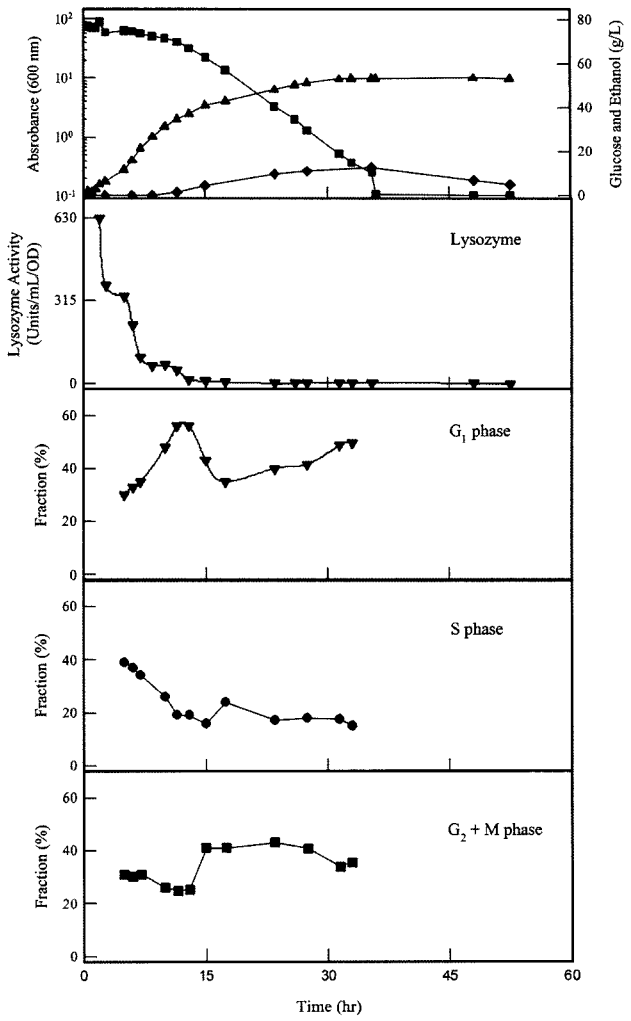

Fig. 2. Profiles of fermentation and cell cycle parameters for aerobic batch culture of recombinant *S. cerevisiae* SEY2102/pMC632 at pH 5.5 and 30°C. Ethanol (◆), glucose (■), and OD_{600} (▲).

The level of lysozyme retained in the intracellular space of the pMC614 strain was only 20 to 30 units/mL at the beginning of the fermentation, while the maximum intracellular lysozyme for the pMC632 strain was 100 units/mL at its highest level. This suggested that the rat α-amylase signal sequence was not efficient for secreting the HLZ protein out of the yeast cell.

To further characterize the production patterns of HLZ in these strains, a cell cycle analysis was performed by staining the cells with a nucleic acid dye, acriflavine. Fig. 1 and 2 show the fermentation behavoir and cell cycle parameters for the recombinant strains SEY2102/pMC614 and SEY2102/pMC632, respectively. A comparison of the characteristics of cell growth and cell-cycle parameters for the two strains demonstrated similar results. During the exponential phase of growth, there were fewer cells in the G_1 phase of the cell cycle as would be expected for rapidly growing cells. More cells were present in the S phase of the cell cycle. Towards the end of the exponential phase, the growth rate slowed down and the number of the cells in the G_1 phase increased while the cells in the S phase decreased. Instead of a drop (24% to 19%) in the number of cells in the G_2+M phase observed in the host strain SEY2102, both of the HLZ producing strains showed an increase in the number of cells in the G_2+M phase of the cell cycle. Suca a difference in cell-cycle parameters was probably due to the secretory nature of the cloned-gene product HLZ. Even with the efficient secretion of the HLZ product out of the cells, the secretion pathway seemed to be saturated as previously discussed for the recombinant yeast producing cloned invertase [7]. At the stationary phase of the fermentation, the recombinant yeast strains continued to grow at a reduced rate and were able to produce HLZ. The transport of the product protein required the secretory pathway, which

is also used by many important proteins necessary for cell division [8]. Saturation of the secretion pathway might block or hinder the passage of proteins necessary for cell division, leading to low growth rate, concomitantly trapping the cells in the G_2+M phase of the cell cycle.

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REFERENCES

[1] Cho, K. M. and Y. J. Yoo (1999) Novel SSF process for ethanol production from microcrystalline cellulose using the δ-integrated recombinant yeast, *Saccharomyces cerevisiae* L2612δGC. *J. Microbiol. Biotechnol.* 9: 340-345.

[2] Chung, B. H., W. K Kim, K. J. Rao, C. H. Kim, and S. K. Rhee (1999) Downstream processing of recombinant hirudin produced in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* 9: 179-183.

[3] Kim, M. D., Y. J. Yoo, S. K. Rhee, and J. H. Seo (2001) Enhanced transformation efficiency of an anticoagulant hirudin gene into *Saccharomyces cerevisiae* by a double δ-sequence. *J. Microbiol. Biotechnol.* 11: 61-64.

[4] Koo, J. H., S. Y. Kim, Y. C. Park, N. S. Han, and J. H. Seo (1998) Invertase production by fed-batch fermentations of recombinant *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* 8: 203-207.

[5] Marten, M. R., N. S. Han, J. B. Park, and J. H. Seo (1999) Analysis of secretion behavior of human lysozyme from recombinant *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* 9: 576-681.

[6] Park, J. N., D. J. Shin, H. O. Kim, D. H. Kim, H. B. Lee, S. B. Chun, and S. Bai (1999) Expression of *Schwanniomyces occidentalis* α-amylase gene *Saccharomyces cerevisiae* var. *diastaticus*. *J. Microbiol. Biotechnol.* 9: 668-671.

[7] Peterson, M. S., A. Y. Patkar, and J. H. Seo (1992) Flow cytometric analysis of total protein content and size distribution of recombinant *Saccharomyces cerevisiae*. *Biotechnol. Tech.* 6: 203-206.

[8] Romanos, M. A., C. A. Scorer, and J. J. Clare (1992) Foreign gene expression in yeast: A review. *Yeast* 8: 423-488.

[9] Seo, J. H. and J. E. Bailey (1985) Effects of recombinant plasmid content on growth properties and cloned gene product formation in *E. coli*. *Biotechnol. Bioeng.* 27: 1668-1674.

[10] Shapiro, H. M. (1995) *Practical Flow Cytometry*. 3rd ed., p. 339. Wiley-Liss, New York, USA.

[11] Alberghina, L. and D. Porro (1993) Quantitative flow cytometry: analysis of protein distributions in budding yeast. *Yeast* 9: 815-823.

[12] Compagno, C., D. Porro, S. Radice, E. Martegani, and B.M. Ranzi. (1996) Selection of yeast cells with a higher plasmid copy number in a *Saccharomyces cerevisiae* autoselection system. *Yeast* 12: 199-205.

[13] Eitzman, P. D. and F. Srienc (1991) Dynamics of activation of a galactose-inducible promoter in *Saccharomyces cerevisiae*. *J. Biotechnol.* 21: 63-81.

[14] Li, F., P. L. Flanary, D. C. Altieri, and H. G. Dohlman (2000) Cell division regulation by *BIR1*, a member of the inhibitor of apoptosis family in yeast. *J. Biol. Chem.* 275: 6707-6711.

[15] Mateus, C. and S. V. Avery (2000) Destabilized green fluorescence protein for monitoring dynamic changes in yeast gene expression with flow cytometry. *Yeast* 16: 1313-1323.

[16] Porro, D., C. Smeraldi, E. martegani, B. M. Ranzi, and L. Alberghina (1994) Flow-cytometric determination of the respiratory activity in growing *Saccharomyces cerevisiae* populations. *Biotechnol. Prog.* 10: 193-197.

[17] Porro, D., E. Martegani, B. M. Ranzi, and L. Alberghina (1997) Identification of different daughter and parent subpopulations in an asynchronously growing *Saccharomyces cerevisiae* population. *Res. Microbiol.* 148: 205-215.

[18] Rodriguez, F., L. Popolo, M. Vai, E. Lacana, and L. Alberghina (1990) Changes in the protein synthesis pattern during a nutritional shift-down transition in *Saccharomyces cerevisiae*. *Exp. Cell. Res.* 187: 315-319.

[19] Schlesinger, M. B. and T. Formosa (2000) POB3 is required for both transcription and replication in yeast *Saccharomyces cerevisiae*. *Genetics* 155: 1593-1606.

[20] Smeraldi, C., E. Berardi, and D. Porro (1994) Monitoring of peroxisome induction and degradation by flow cytometric analysis of *Hansenula polymorpha* cells growing on methanol and glucose media: cell volume, refractive index and FITC retention. *Microbiology* 140: 3161-3166.

[21] Srienc F. and B. S. Dien. (1992) Kinetics of the cell cycle of *Saccharomyces cerevisiae*. *Ann. NY Acad. Sci.* 665: 59-71.

[22] Vanoni, M., M. Lotti, and L. Alberghina (1989) Expression of cloned *Saccharomyces diastaticus* glucoamylase under natural and inducible promoters. *Biochim. Biophys. Acta* 1008: 168-176.

[23] Blake, C. C. F., D. F. Koenig, G. A. Mair, A. L. T. North, D. C. Philips, and N. R. Sarma (1965) Structure of hen egg white lysozyme. *Nature* 206: 757-761.

[24] Castaman, M. J., W. Spevak, G. R. Adolf, E. Chlebowicz-Sledziewska, and A. Sledziewska (1988) Cloning of human lysozyme gene and expression in the yeast *Saccharomyces cerevisiae*. *Gene* 66: 223-234.

[25] Morsky, P. (1983) Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells: Reexamination of reaction conditions. *Anal. Biochem.* 128: 77-85.

[26] Chapman C. and W. Bartley (1968) The kinetics of enzyme changes in yeast under conditions that cause the loss of mitochondria. *Biochem. J.* 107: 455-459.

[27] Polakis E. S., W. Bartley, and G. A. Meek (1964) Changes in the structure and enzyme activity of *Saccharomyces cerevisiae* in response to changes in the environment. *Biochem. J.* 90: 369-374.

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