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Expression and High-Level Secretion of *Trichoderma reesei* Endoglucanase I in *Yarrowia lipolytica**

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Abstract

The endoglucanase I (EGI) from fungus *Trichoderma reesei* was cloned, expressed, and secreted from *Yarrowia lipolytica* using the *XPR2* promoter. The signal sequence of EGI transferred from *T. reesei* was efficiently processed in the *Y. lipolytica* secretory pathway and directed the secretion of active EGI into the culture medium. However, the recombinant EGI produced from YLCSIn strain was hyperglycosylated and significantly larger than the native enzyme produced by the parent strain. The expression of EGI using *XPR2* preproregion has caused secretion of modified proteins that still retained cellulase activity. This resulted from imprecise processing of the N-terminus of recombinant protein. While the batch culture produced 5 mg EGI/L from YLCSIn strain, the EGI yield was increased approx 20-fold when the fed-batch fermentation process strategy in combination with the high-cell density cultivation technique was employed. These results showed that the *Y. lipolytica* is a useful host organism for production of a large amount of large size heterologous proteins, especially when used in combination with high-cell density and fed-batch culture techniques.

*The abbreviations used in this article are: EGI, endoglucanase; OBR-HEC, hydroxyethylcellulose linked Ostazin Brilliant Red H-3B; AEP, alkaline extracellular protease; ER, endoplasmic reticulum; DNS, dinitrosalicylic acid; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Endo H, endoglycosidase H; Xpr6p, KEX2-like endopeptidase.

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Index Entries: Gene expression; secretion; cellulase; endoglucanase I; high-cell density culture; fed-batch fermentation; *Yarrowia lipolytica*.

Introduction

Yeast has been used widely both as a model system for a study of higher eucaryotes and as a host organism for expression of recombinant proteins because of some advantages that yeast renders. Some benefits of using prokaryotes include simple medium, rapid growth rate, and an option of using a high-cell-density cultivation technique. An important advantage of using yeast is that it takes care of posttranslational processing and modification of heterologous proteins secreted in its subcellular compartments. Yeast is an attractive host organism for heterologous protein expression because extensive and well-established genetic databases are available and molecular biology techniques and tools have been developed for the manipulation of yeast genes *(1–5)*. *Saccharomyces cerevisiae* has been the popular choice for this purpose. However, non-*Saccharomyces* yeast strains such as *Yarrowia lipolytica* have been a new focus of study recently as potential alternative host systems for production of foreign proteins owing to their advantages in terms of greater promoter strength, relatively high secretion capacity, and ease of applying high-cell-density culture technique to production of heterologous proteins *(3,5,6)*.

Y. lipolytica is a dimorphic yeast, being oval-shaped in minimal medium containing glucose and glycerol but forming mycelia in complex medium. This yeast strain has been used for production of single-cell protein, citric acid, erythritol, and mannitol *(7–10)*. *Y. lipolytica* is one of several yeast strains currently considered as alternative hosts for heterologous gene expression, since this strain is capable of secreting 1–2 g/L of an alkaline extracellular protease (AEP) under optimal conditions *(11)*. Pulse-chase experiments with [^{3}H]leucine were used to examine AEP secretion. An analysis of the labeled isotopes incorporated into AEP showed that AEP synthesis accounted for 6.9% of total protein *(12)*. In addition, RNase, several acid proteases, and lipase are secreted from *Y. lipolytica (9)*. These data strongly suggest that *Y. lipolytica* has a high-level secretion capability and a potential for secreting large amounts of heterologous proteins of high molecular weight.

The filamentous fungi *Trichoderma reesei* produces and secretes a cellulase complex that synergistically hydrolyzes cellulose *(13,14)*. Endo-1,4-β-D-glucanase (endoglucanase) is one of the enzymes in the cellulase complex family and is capable of hydrolyzing soluble and substituted cellulose such as carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) internally and randomly *(15,16)*. Several research groups have used *S. cerevisiae* to construct the novel cellulolytic yeast for more efficient brewing, baking, and wine making *(17,18)*. Silva et al. constructed a set of vectors to express and secrete *Clostridium thermocellum* endoglucanase A, and they showed that the endoglucanase may be a suitable reporter enzyme for

monitoring heterologous protein secretion and glycosylation in *S. cerevisiae* because of its simple detection method *(19)*.

In this paper, the expression and secretion of *T. reesei* EGI with heterologous EGI signal sequence in *Y. lipolytica* under the control of strong *XPR2* promoter is described. A significant improvement of EGI productivity and secretion with recombinant *Y. lipolytica* strain using the fed-batch fermentation process strategy is also presented.

Materials and Methods

Strains, Media, and Vectors

Yeast strains and plasmids used in this work are described in Table 1. Plasmid pEndoI carries the 4.2 kb *Trichoderma reesei* endoglucanase I genomic DNA at the *Hin*dIII site of pBR322. The *Escherichia coli* strain DH5α *(20)* was used for expression vector construction and plasmid DNA propagation. A derivative strain of *Yarrowia lipolytica* CX161-1B, SMS397A (*Mat A*, *ade1*, *ura3*, *xpr2*), was used as the host strain for the expression vectors.

Media

The LB medium used for *E. coli* cultivation was prepared as described in Sambrook et al. *(21)*. *Y. lipolytica* culture was maintained on YM medium (0.3% Bacto yeast extract, 0.3% Bacto malt extract, 0.5% Bactopeptone, 1% dextrose, and 2% agar) and cultivated in YPD medium containing yeast extract (10 g/L), Bactopeptone (10 g/L), and glucose (20 g/L). Modified GPP medium *(22)* containing 1.0% glycerol, 0.34% Difco proteose peptone, 50 mg/L adenine, and 0.34% yeast nitrogen base without amino acids and ammonium sulfate was used as the recombinant EGI production medium. For the first selection of Ura^- transformants, minimal medium without uracil (uracil drop-out medium) was used. Selection medium for the transformants producing EGI was a YM plate, which was modified by adding 0.2% hydroxyethylcellulose containing 13.5% (w/w) of covalently linked Ostazin Brilliant Red H-3B (OBR-HEC) (Sigma Chemical Co.) *(23)*.

Determination of Cell Concentration

Cell concentration (gram dry cell weight per liter) was measured with a spectrophotometer (DU-50, Beckman) as the optical density at 600 nm (OD_{600}) and converted to the dry cell weight per liter of whole broth using calibration data (1 OD_{600} unit = 0.52 g cell/L) predetermined for the *Y. lipolytica* strains used.

DNA Manipulation and Transformation

The standard recombinant DNA techniques as described in Sambrook et al. were employed for the DNA manipulation and transformation *(21)*. *E. coli* transformation was performed by the SEM method *(24)*. Yeast transformation was carried out by the lithium acetate method *(25)*.

Table 1
Strains and Plasmids Used in This Work

Designation	Descriptions	Source or reference
E. coli		
DH5α	*supE44 ΔlacU169 (ϕ80lacZΔM15)hsdR17 recA1 endA1 gyrA96*	*20*
Yarrowia lipolytica		
SMS397A	*Mat A, ade1, ura3, xpr2*	D. M. Ogrydziak (UC Davis)
YLCMIn	SMS397A harboring pXCMIn	This work
YLCSIn	SMS397A harboring pXCSIn	This work
Plasmids		
pEndoI	4.2 kb *Trichoderma reesei* endoglucanase I genomic DNA at *Hin*dIII site of pBR322	M. Ward (Genencor)
pIMR52	*XPR2* gene in pUC19	D. M. Ogrydziak (UC Davis)
pIMR100	*XPR2, URA3* gene in pBR322	D. M. Ogrydziak (UC Davis)
pXCMIn	*XPR2* promoter::*XPR2* prepro:endoglucanase I in pIMR100	This work
pXCSIn	*XPR2* promoter::endoglucanase I pre::endoglucanaseI in pIMR100	This work
pXCMIn(myc)	*XPR2* promoter::*XPR2* prepro::endoglucanase I::myc epitope in pIMR100	This work
pXCSIn(myc)	*XPR2* promoter::endoglucanase I pre::endoglucanaseI::myc epitope in pIMR100	This work

Selection of Transformants Producing T. reesei *Endoglucanase I*

For the first selection, yeast cells transformed with expression vectors were grown on uracil drop-out medium. The selected Ura^+ transformants were transferred onto OBR-HEC plates containing YM for the second selection. After 2 d of incubation at 28°C, the transformants producing recombinant *T. reesei* EGI and showing halos around the colonies were selected.

Construction of Expression Vectors

The modified SOE PCR *(26)* method was utilized to eliminate the two introns contained in the *T. reesei* EGI genomic DNA of pEndoI and to construct fusion between the *XPR2* promoter/preproregion and the EGI coding sequence.

The primers used for the PCR are listed below.

```
Primer  XA1: 5'-CATCCACCGGCTAGCGGAACACAG-3'
                          NheI
Primer ED M1: 5'-GGTACCCGGTTGCTGTCGCTTGGCATTAGAAGAAGCAGG-3'
Primer ED M2: 5'-TCTAATGCCAAGCGACAGCAACCGGGTACCAGCACCC-3'
Primer ED S1: 5'-AACTGAGGGCGCCATTGTTGGATTGGAGGATTGGAT-3'
Primer ED S2: 5'-TCCTCCAATCCAACAATGGCGCCCTCAGTTACACTGCCG-3'
Primer ED In1: 5'-TCCGGGGCCGTAGTAGCTTTTGTAGCCGCTGCCATAGGG-3'
Primer ED In2: 5'-GGCAGCGGCTACAAAAGCTACTACGGCCCCGGAGATACC-3'
Primer ED In3: 5'-AAGCTTCTAAAGGCATTGCGAGTAGTAGTCGTTGCTATAC
                  TGGCACGT-3'
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To remove the two introns from the EGI genomic DNA, primers ED In1, In2, and In3 were used. The primers ED S1 and ED S2 were employed to construct the fusion between *XPR2* promoter and EGI signal sequence, and the primers ED M1 and M2 to construct the fusion between *XPR2* preproregion and EGI coding sequence. The resulting PCR fragments were cloned into pUC19 with blunt end ligation and constructed pUCM, pUCS, and pUCCIn. The insert of pUCM contains precise fusion between the *XPR2* preproregion and EGI coding sequence and the first exon of EGI. However, pUCS carries the fusion between *XPR2* promoter and EGI signal peptide. The plasmid pUCCIn now holds the EGI gene devoid of its two introns.

One micromole of each primer and 5 U of Vent polymerase (New England Biolabs, Beverly, MA) were used for each PCR reaction in 100 µL total volume. The PCR reactions were performed for 25 cycles of 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C, followed by 7 min incubation at 72°C.

Construction of Expression and Integration Vectors for T. reesei *EGI*

The fusion vector (pXCS) containing the *XPR2* promoter, EGI signal peptide, EGI coding sequence, and *XPR2* terminator was constructed by ligating the large fragment of the pIMR52 *Nhe*I-*Kpn*I digest, the small fragment of pUCS digested with *Nhe*I-*Eag*I, and the small *Eag*I-*Kpn*I restriction fragment of pUCCIn. Finally, the *URA3* gene of pIMR100 was inserted into pXCS as a selectable marker to create the integration vector, pXCSIn.

To construct the second expression vector, pUCM was used instead of pUCS, resulting in the plasmid, pXCMIn, containing the *XPR2* prepro-region instead of the EGI signal peptide. Both vectors were transformed into *Y. lipolytica* SMS397A by using the lithium acetate method *(25)* after linearizing it with *Mlu*I.

c-myc Epitope Tagging

The c-myc epitope tagging method was used to detect the recombinant EGI in the Western blot analysis. The c-myc epitope sequence of pTrcHis2 (Invitrogen, San Diego, CA) was transferred to the C-terminus of the EGI coding sequence in pXCSIn and pXCMIn using the standard PCR method. After confirming the right constructs by DNA sequencing, the resulting plasmids were named pXCSIn(myc) and pXCMIn(myc).

SDS-PAGE and Western Blot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli *(27)*. After separation by SDS-PAGE, proteins were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) in ice-cold transferring buffer (15.6 m*M* Tris, 120 m*M* glycine, and 20% methanol, pH 8.3) at 100 V for 1 h. The first antibody for Western blot analysis was anti-c-myc antibody that was purchased from Invitrogen. The second antibody was anti-mouse IgG antibody conjugated with peroxidase that was purchased from the Phototope-HRP Western Blot Detection Kit (New England Biolabs). The manufacturer's recommended procedure was followed for the detection.

Endoglucanase Activity (CMCase activity)

The CMCase activity of the recombinant EGI was determined by measuring reducing sugar concentration using the dinitrosalicylic acid (DNS) method *(14,28)*. The reaction was initiated by adding 0.2 mL of enzyme solution to the 1.8 mL of reaction solution containing 1% carboxymethylcellulose substrate in 50 m*M* sodium citrate at pH 4.8. After 20 min incubation at 50°C, 3 mL of DNS solution was added to terminate the reaction. Following 5 min boiling, the absorbance at 540 nm was determined. As a standard, glucose solution was used. One enzyme unit corresponds to the amount of enzyme required to produce 1 μ*M* of reducing sugar from the substrate per minute. One unit of enzyme activity used is equivalent to approximately 16.67 nanokatal.

Purification of Recombinant Endoglucanase I by Affinity Chromatography

Purification of recombinant EGI secreted by *Y. lipolytica* was performed by using the method described in Owolabi et al. *(29)*. One gram Avicel sample (microcrystalline cellulose, Merck, Germany) was washed

in 20 mL distilled water and recovered by centrifugation (at 5000*g* for 5 min). The three times washed microcrystalline cellulose was resuspended in the same volume of distilled water and autoclaved for 30 min at 121°C and 15 psig. The aqueous phase was replaced with buffer (4 mL/g of 50 m*M* sodium citrate, pH 4.8, with 0.02% sodium azide) after washing the Avicel twice and the Avicel was equilibrated overnight at 4°C. An equal volume of yeast culture supernatant containing EGI and ice-cold microcrystalline cellulose solution were mixed and kept on ice for 3 h.

After removing the unadsorbed material by filtering twice with buffer, the Avicel–enzyme complex was packed into a column. Recombinant EGI bound to microcrystalline cellulose was eluted with distilled water under gravity flow at room temperature. Endoglucanase activity was measured for each fraction as described above.

Fed-Batch Fermentation

Fed-batch culture experiment was conducted in a 1.5 L jar fermenter with glycerol as a limiting substrate. The temperature and pH of the culture were maintained at 28°C and 6.8, respectively. The aeration and agitation rates were varied from 1 to 2 vvm and 600 to 900 rpm, respectively, commensurate with the oxygen requirement of the culture. The fermenter containing 700 mL growth medium was inoculated with 30 mL seed culture and initially operated under batch conditions. When the carbon source was depleted, the operation was switched to a fed-batch culture mode. When glycerol was completely depleted, approx 20 h had elapsed under the batch culture condition, the feeding of growth medium to the fermenter was initiated by turning on a peristaltic pump. Once a desired cell concentration was obtained, the growth medium was changed to the production medium so that recombinant EGI gene expression could be induced. In order to maintain a high cell concentration, 25% oxygen enriched air was supplied to the fermenter.

To control and maintain a constant cell growth rate, we used a stepwise feeding control strategy based on the following working equation:

$$F = 1.5\ \mu XV / S_f Y_{x/s}$$

where the coefficient, 1.5, is the empirical constant for the experimental system and operating conditions used, μ is the specific growth rate (controlled at 0.1 h^{-1}), X is the cell concentration, V is the working culture volume, S_f is the limiting substrate concentration in the feed medium, $Y_{x/s}$ is the cell yield coefficient (0.5, for the given set of experimental conditions and the yeast recombinant strain used), and F is the medium feed rate.

Results and Discussion

Expression and Secretion of EGI in Y. lipolytica

The transformants were initially selected using uracil dropout plates and then patching onto OBR-HEC-containing YM plates *(23)*. The forma-

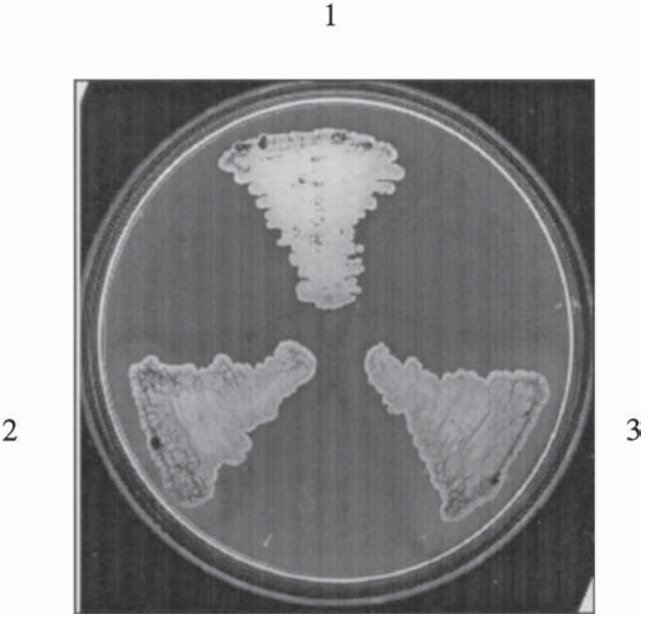

Fig. 1. *Y. lipolytica* host and transformants grown on OBR-HEC-containing YM plate. 1, Host *Y. lipolytica* (SMS397A); 2, YLCSIn; 3, YLCMIn.

tion of halos around the colonies after 1 or 2 d of incubation was used as a confirmatory test for the endoglucanase activity (Fig. 1). The integration of expression vector into genomic DNA of recombinant strains was confirmed by Southern blot analysis (data not shown). Figure 1 shows that the transformants harboring either pXCSIn or pXCMIn produced and secreted the active form of the recombinant enzyme EGI. This result was also confirmed by detecting endoglucanase activity in the supernatant of shake flask culture. Transformants containing pXCSIn or pXCMIn were named YLCSIn (*Y. lipolytica* harboring pXCSIn) and YLCMIn, respectively. The plate assay showed that the YLCMIn strain produced less recombinant EGI than YLCSIn (Figs. 1 and 2). Determination of endoglucanase activity in flask culture revealed that YLCSIn (1.18 U/mL) produced approximately three times the recombinant EGI than YLCMIn (0.44 U/mL) (Fig. 2). The specific production rate of EGI was estimated as 15 and 7.5 U of enzyme per g dry cell per hour for YLCSIn and YLCMIn, respectively.

This result shows that the native EGI signal peptide is recognized in the secretory pathway of *Y. lipolytica* and the EGI is efficiently secreted. In addition, it was found that the native signal peptides of heterologous proteins (EGI and rice α-amylase) have lead to a significantly higher secretion in *Y. lipolytica* than the *XPR2* preproregion *(30)*. Based on these results, the expression and secretion of heterologous proteins with these signal peptides in *Y. lipolytica* were confirmed.

The promoter and preproregion of alkaline extracellular protease (AEP) encoded by the *XPR2* gene were used to secrete heterologous pro-

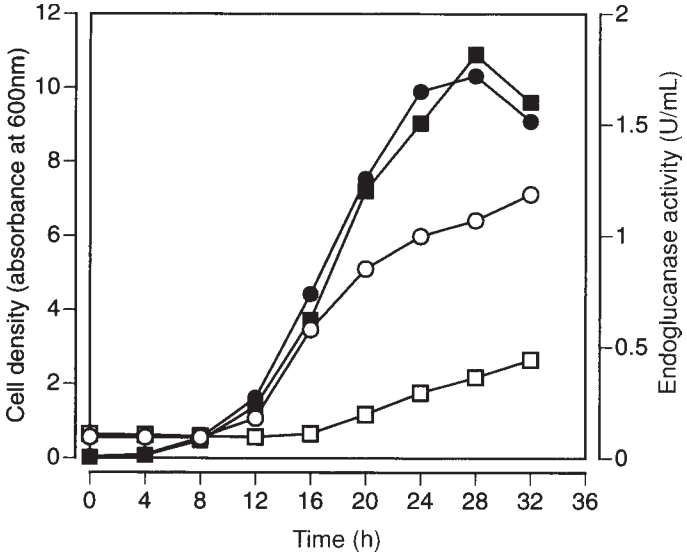

Fig. 2. Growth and production of endoglucanase I by recombinant *Y. lipolytica* in the batch culture. (■) cell density (absorbance at 600 nm) for YLCMIn strain; (●) cell density (absorbance at 600 nm) for YLCSIn strain enzyme activity; (□) endoglucanase activity (U/mL) for YLCMIn strain; (○) endoglucanase activity (U/mL) for YLCSIn strain.

teins in *Y. lipolytica (30–34)*. Because the *XPR2* signal peptide is not effective in directing secretion of some foreign proteins in *Y. lipolytica (30,33)*, two expression vectors for *T. reesei* EGI secretion were constructed: (1) *XPR2* promoter-*XPR2* preproregion-EGI coding sequence-*XPR2* terminator and (2) *XPR2* promoter-EGI signal peptide and coding sequence-*XPR2* terminator. It was necessary to remove two introns from the *T. reesei* EGI genomic DNA (4.2 kb insert at *Hin*dIII site of pEndoI) for protein expression and secretion.

Although the *XPR2* preproregion leads to EGI secretion, the recombinant protein (45 kDa) produced was different from that secreted from YLCSIn strain. Since the myc epitope was tagged at the C-terminus of EGI, this fragment cannot be the proteolytic product cleaved at the C-terminus of this protein. Instead, an altered processing may account for the difference between the recombinant EGI secreted by YLCSIn and YLCMIn. The fusion protein between *XPR2* preproregion with rice α-amylase and other foreign protein caused imprecise processing at the Kex2p cleavage site and led to the wrong N-terminal amino acid in the secreted heterologous protein *(30,33)*.

Morphology

When *T. reesei* EGI was expressed in *S. cerevisiae*, the cell morphology in terms of cell shape and volume was altered. The cell volume of recombinant was approximately three times greater and the cell shape was irregular as compared to the control strain, SMS397A *(35)*. However, no

significant difference in growth rate, cell shape, or cell volume between the EGI producing strains (YLCSIn and YLCMIn) and the control strain of *Y. lipolytica* was detected in the production medium.

Characterization of the Recombinant EGI

T. reesei endoglucanase I (EGI) was successfully expressed and secreted in *Y. lipolytica* using the signal peptide of EGI. However, the secreted EGI has a higher molecular weight compared to the native enzyme from *T. reesei*, but similar to the recombinant EGI in *S. cerevisiae*. The main band was detected at 66 kDa with faint smears above the band, indicating a hyperglycosylated protein was also produced (Figs. 3 and 4). The endo-H treatment removed hyperglycosylation and altered the molecular weight of the protein from 66 kDa to 55 kDa (Fig. 3, lane 4). These results suggest the difference in the degree of glycosylation between native and recombinant EGI. When *T. reesei* EGI was expressed in *S. cerevisiae*, Van Arsdell et al. and Penttila et al. observed two main bands of about 70 kDa with heterogeneous smears and these bands were retained after endo-H treatment *(35,36)*. These results indicated that there were alternate sites of glycosylation in the EGI enzyme that have different susceptibilities to endo-H. However, the recombinant EGI produced from YLCSIn did not show two heterogeneous bands (Fig. 3). This observation suggests that there exist similarities in hyperglycosylation and differences in the types of glycosylation between *Y. lipolytica* and *S. cerevisiae* in their secretory pathways.

The recombinant EGI produced by YLCSIn was purified using cellulose (Avicel) affinity chromatography. Figure 3 shows that the molecular weight of secreted recombinant EGI is about 66 kDa, which is larger than the native EGI enzyme (55 kDa) secreted by *T. reesei (35)*. However, it is similar to the recombinant EGI produced by *S. cerevisiae*, which showed two thick bands at approximately 70 kDa with a smear indicating a hyperglycosylation *(35,36)*. After removal of N-linked glycosylation using Endo-H treatment, the EGI band was shifted from 66 kDa to 55 kDa (Fig. 3, lane 4). In addition, SDS-PAGE revealed a smear above the recombinant EGI band (Fig. 3). To determine whether this was due to hyperglycosylation of EGI or an experimental background, Western blot analysis was performed. It has been reported that the construction of epitope-tagged protein for immunolocalization and immunopurification in yeast system was observed *(37)*. We have applied myc epitope tagging to detect the recombinant EGI in the Western blot analysis. The vectors prepared, pXCSIn(myc) and pXCMIn(myc), were transformed into *Y. lipolytica*, and enzyme assays of recombinant strains showed that the myc epitope did not affect endoglucanase activity (data not shown). The hyperglycosylation of recombinant EGI produced from YLCSIn was determined using Western blot analysis. It revealed a smear above the 66 kDa band (Fig. 4, lane 2).

A Western blot analysis of supernatant sample from the YLCMIn culture revealed a 45 kDa band. A 48 kDa band was observed in the supernatant sample of EGI-producing *S. cerevisiae* culture *(35)*. Penttila et al.

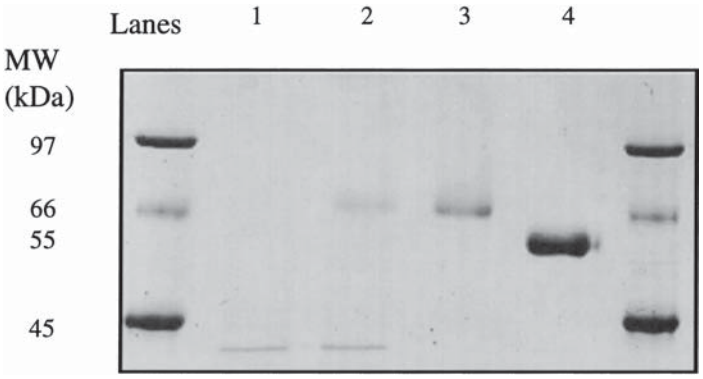

Fig. 3. SDS-PAGE analyses of recombinant endoglucanase I produced by YLASIn. MW, molecular weight marker; lane 1, culture supernatant of host strain; lane 2, culture supernatant of recombinant strain (YLCSIn); lane 3, affinity purified recombinant fungal endoglucanase I secreted by YLCSIn; lane 4, endo-H treated recombinant endoglucanase I. In lanes 1 and 2, samples were prepared by concentrating the culture supernatant 10-fold with TCA precipitation before loading (10 µL). In lanes 3 and 4, approximately, 0.65 mg samples were loaded.

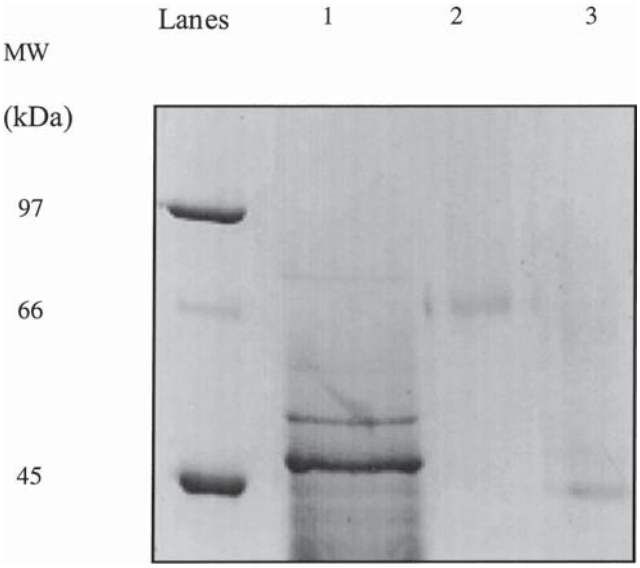

Fig. 4. Western blot analysis of recombinant endoglucanase I secreted by YLCSIn and YLCMIn. Lane 1, culture supernatant of SMS397A; lane 2, culture supernatant of YLCSIn; lane 3, culture supernatant of YLCMIn. 10 µL of 10-fold concentrated culture supernatant was loaded in each lane.

assumed that this band was the proteolytic product of EGI that was homologous to cellobiohydrolase I (CBHI) and contains a site susceptible to proteolytic cleavage in the proximity to C-terminus *(35)*. However, this is unlikely in *Y. lipolytica* because the myc epitope was tagged at the end of

the C-terminus of EGI. If the proteolytic cleavage occurred at this site, the recombinant EGI cannot be detected with Western blot analysis. Although several attempts were made to analyze the amino acid sequence of the N-terminus of this fragment to determine imprecise cleavage between *XPR2* proregion and EGI coding sequence, the sequence analysis could not be carried out since it was blocked just as some proteins of plant origin are. Gaillardin et al. and Park et al. suggested an imprecise cleavage between *XPR2* preproregion and some heterologous proteins due to its improper posttranslational processing in the secretion pathway. A different N-terminal amino acid sequence of secreted protein from *Y. lipolytica* was reported *(30,33)*.

Production of EGI with Batch and Fed-Batch Fermentation

The profiles of cell growth and EGI productivity in terms of the CMCase activity for YLCSIn and YLCMIn recombinant strains were compared using shake flasks. Figure 2 shows that YLCSIn secreted EGI 3 times more than YLCMIn. This result and our previous data *(30)* showed that the *XPR2* preproregion is somewhat less efficient in directing recombinant protein secretion in *Y. lipolytica* than heterologous protein signal peptides (e.g., rice α-amylase and *T. reesei* EGI).

Chang et al. successfully improved the recombinant rice α-amylase production by 28-fold in *Y. lipolytica* using the fed-batch fermentation technique *(6)*. To test the feasibility of improving EGI productivity, performance of fed-batch culture with the EGI producing recombinant YLCSIn was evaluated. During the growth phase up to about 36 h, the EGI production level was less than 1 U/mL, since the *XPR2* promoter was repressed by a nitrogen source, $(NH_4)_2SO_4$ (Fig. 5). When the cell concentration reached 30 g/L, approx 36 h culture time, the growth medium was switched to the production (or induction) medium and fed-batch mode of operation started for production of recombinant EGI. After the induction, EGI production continued at a constant rate for about 1 d fed-batch cultivation period. The final cell concentration and EGI activity obtained were 86 g/L and 22.6 U/mL, respectively. Compared with batch culture, the EGI productivity in terms of the final enzyme concentration was increased by 19-fold. The specific production rates of EGI from YLCSIn in the batch and fed-batch cultures were estimated and found comparable, 15.0 and 15.3 U of enzyme per g dry cell per hour, respectively. The result demonstrated that the fed-batch fermentation technique can be employed to significantly increase the productivity of heterologous protein in recombinant *Y. lipolytica*.

With YLCSIn, about 5 and 100 mg/L of recombinant EGI was produced in shake-flask culture and in fed-batch culture, respectively. The EGI productivity in *Y. lipolytica* is significantly higher than that observed in *S. cerevisiae*, which secreted about 0.66 mg/L of EGI with 2 μ-based vector *(35)*. Three kinds of vectors have been developed to express heterologous genes in *Y. lipolytica*: autonomously replicating sequence (ARS)-based, single copy integration, and multi-copy integration vector *(33,38)*. When

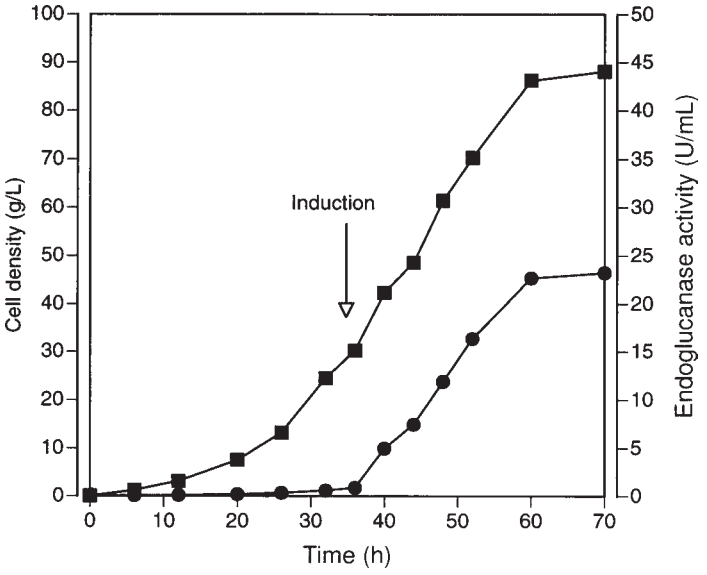

Fig. 5. High-cell-density fed-batch culture for *Y. lipolytica* (YLCSIn) producing *T. reesei* endoglucanase I. Detailed techniques are described in Materials and Methods. (■) cell density (g/L); (●) enzyme activity (U/mL).

vectors containing the ARS sequence were used to produce alkaline extracellular protease (AEP), porcine α1-interferon, and bovine prochymosin under the control of the *XPR2* promoter, a two- to sixfold increase in protein production was observed as compared to using a single copy integration vector system *(34)*. Recently, Ledall et al. developed several integration vectors that have average copy numbers of 5, 12, or 25–60 *(38)*. Since the YLCSIn strain has a single copy integration vector, there is a strong possibility of improving EGI production by employing the ARS-based replicate or multiple integration vectors.

Conclusion

We have demonstrated a potential feasibility of using *Y. lipolytica* as a yeast host system for foreign protein secretion. As an example, the recombinant EGI is secreted into the culture medium as an active form and detected on the media plates, suggesting that this enzyme can be used as an attractive reporter molecule in *Y. lipolytica*. Also, the fed-batch bioprocess control strategy was successfully applied to significantly increase the productivity of EGI in recombinant *Y. lipolytica*.

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References

1. Buckholz, R. G. and Gleeson, M. A. G. (1991), *Bio/Technology* **9,** 1067–1072.
2. Buckholz, R. G. (1993), *Curr. Opin. Biotechnol.* **4,** 538–542.
3. Gellissen, G., Melber, K., Janowicz, Z. A., Dahlems, U. M., Weydemann, U., Piontek, M., Strasser, A. W., and Hollenberg, C. P. (1992), *Antoine Van Leeuwenhoek* **62,** 79–93.
4. Russell, C., Mawson, J., and Yu, P. L. (1991), *Aust. J. Biotechnol.* **5,** 48–55.
5. Sudbery, P. E. (1996), *Curr. Opin. Biotechnol.* **7,** 517–524.
6. Chang, C. C., Ryu, D. D. Y., Park, C. S., Kim, J. Y., and Ogrydziak, D. M. (1998), *Appl. Microbiol. Biotechnol.* **49,** 531–537.
7. DeZeeuw, J. R. and Tynan, E. J. (1973a), USA Patent No 3736229.
8. DeZeeuw, J. R. and Tynan. E. J. (1973b), USA Patent No 3756917.
9. Heslot, H. (1990), *Adv. Biochem. Eng. Biotech.* **43,** 43–73.
10. Rane, K. D. and Sims, K. A. (1994), *Biotechnol. Bioeng.* **43,** 131–137.
11. Tobe, S., Takami, T., Ikeda, S., and Mitsugi, K. (1976), *Agric. Biol. Chem.* **40,** 1087–1092.
12. Matoba, S., Fukayama, J., Wing, R. A., and Ogrydziak, D. M. (1988), *Mol. Cell. Biol.* **8,** 4904–4916.
13. Harkki, A., Mantyla, A., Penttila, M., Muttilainen, S., Buhler, R., Suominen, P., Knowles, J., and Nevalainen, H. (1991), *Enzyme Microb. Technol.* **13,** 227–233.
14. Niku-Paavola, M.-L., Lappalainen, A., Enari, T.-M., and Nummi, M. (1985), *Biochem. J.* **231,** 75–81.
15. Beguin, P. and Aubert, J.-P. (1994), *FEMS Microbiol. Rev.* **13,** 25–58.
16. Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. (1991), *Microbiol. Rev.* **55,** 303–315.
17. Knowles, J., Pentilla, M., Teeri, T., Andre, L., Lehtovaara, P., and Salovuori, I. (1987), in *Biological Research on Industrial Yeasts*, Steward, G. G. et al., ed., CRC Press, Boca Raton, pp. 189–199.
18. Wong, W. K. R., Curry, C., Parekh, R. S., Parekh, S. R., Wayman, M., Davies, R. W., Kilburn, D. G., and Skipper, N. (1988), *Bio/Technology* **6,** 713–719.
19. Silva, A., Benitez, J., and Hollenberg, C. P. (1991), *Anal. Biochem.* **197,** 290–295
20. Hanahan, D. (1983), *J. Mol. Biol.* **166,** 557–580.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual*, 2nd ed, Cold Spring Harbor Laboratory, New York.
22. Enderlin, C. S. and Ogrydziak, D. M. (1994), *Yeast* **10,** 67–79.
23. Farkas, V., Liskova, M., and Biely, P. (1985), *FEMS Microbiol. Lett.* **28,** 137–140.
24. Inoue, H., Nojima, H., and Okayama, H. (1990), *Gene* **96,** 22–28.
25. Gaillardin, C., Ribet, A. M., and Heslot, H. (1985), *Curr. Genet.* **10,** 49–58.
26. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989), *Gene* **77,** 61–68.
27. Laemmli, U. K. (1970), *Nature (London)* **227,** 680–685.
28. Bailey, M. J. and Nevalainen, K. M. H. (1981), *Enzyme Microb. Technol.* **3,** 153–157.
29. Owolabi, J. B., Beguin, P., Kilburn, D. G., Miller, R. C., and Warren, R. A. J. (1988), *Appl. Environ. Microbiol.* **54,** 518–523.
30. Park, C. S., Chang, C. C., Kim, J. Y., Ogrydziak, D. M., and Ryu, D. D. Y. (1997), *J. Biol. Chem.* **272,** 6876–6881.
31. Franke, A. E., Kaczmarek, F. S., Eisenhard, M. E., Geoghegan, K. F., Danley, D. E., DeZeeuw, J. R., O'Donnell, M. M., Gollaher, M. G., Jr., and Davidow, L. S. (1988), *Dev. Ind. Microbiol.* **29,** 43–57.
32. Tharaud, C., Ribet, A.-M., Costes, C., and Gaillardin, C. (1992), *Gene* **121,** 111–119.
33. Gaillardin, C., Fournier, C. P., Nicaud, J. M., He, F., Fabre, E., Lopez, M. C., and Tharaud, C. (1990), in *Proceedings of the 6th International Symposium on Genetics of*

Industrial Microorganisms, Vol. 2, Heslot, H., Davies, J., Florent, J., Bobichon, L., Durand, G., and Penasse, L., eds., Société Française de Microbiologìe, Strasbourg, pp. 509–518.

34. Nicaud, J. M., Fournier, P., Bonnardiere, C. L., Chasles, M., and Gaillardin, C. (1991), *J. Biotechnol.* **19,** 259–270.
35. Penttila, M. E., Andre, L., Saloheimo, M., Lehtovaara, P., and Knowles, J. K. (1987), *Yeast* **3,** 175–185.
36. Van Arsdell, J. N., Kwok, S., Schweickart, V. L., Ladner, M. B., Gelfand, D. H., and Innis, M. A. (1987), *Bio/Technology* **5,** 60–64.
37. Lafontaine, D. and Tollervey, D. (1996), *Nucleic Acids Res.* **24,** 3469–3472.
38. Ledall, M. T., Nicaud, J. M., and Gaillardin, C. (1994), *Curr. Genet.* **26,** 38–44.