

Construction of a new recombinant protein expression system in the basidiomycetous yeast *Cryptococcus* sp. strain S-2 and enhancement of the production of a cutinase-like enzyme

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Abstract Yeast host–vector systems have been very successful in expressing recombinant proteins. However, because there are some proteins that cannot be expressed with existing systems, there is a need for new yeast expression systems. Here we describe a new host–vector system based on the basidiomycetous yeast *Cryptococcus* sp. strain S-2 (S-2). Two advantages of S-2 are that it naturally produces some very useful enzymes, so it would be a good system for expressing multiple copies of some of its genes, and that, it is a nonhazardous species. The orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) gene (*URA5*) was selected as a selectable marker for transformation in the new host–vector system. *URA5* was isolated and introduced into a uracil auxotroph of S-2 by electroporation. To demonstrate the S-2 system, we selected one of its unique enzymes, a plastic-degrading cutinase-like enzyme (CLE). We were able to insert multiple copies of the CLE gene (*CLE1*) into the chromosomes in a high fraction of the targeted cells. Under optimal conditions, one transformant exhibited 3.5 times higher CLE activity than the wild type. Expression vectors, including an inducible promoter (the promoter for the xylanase or α -amylase gene), were constructed for recombi-

nant protein production, and green fluorescent protein was expressed under the control of these promoters. The xylanase promoter was more tightly controlled. Furthermore, putting *CLE1* under the control of the xylanase promoter, which is induced by xylose, increased CLE activity of the culture medium to approximately 15 times greater than that of the wild type.

Keywords Recombinant protein production · Host–vector system · Basidiomycetous yeast · Inducible promoter · Cutinase

Introduction

Yeast host–vector systems are widely used to produce recombinant proteins. Some of the best known systems are based on *Schizosaccharomyces pombe* (Beach and Nurse 1981), *Pichia pastoris* (Cregg et al. 1985), *Kluyveromyces lactis* (Das and Hollenberg 1982), *Saccharomyces cerevisiae* (Hinnen et al. 1978), and *Candida maltosa* (Takagi et al. 1986). Such systems can be used for the production of recombinant heterologous enzymes or to enhance the ability of yeast to produce homologous enzymes. For the production of recombinant proteins, most yeast host–vector systems, like the ones mentioned above, use ascomycetous yeasts (Böer et al. 2007; Buckholz and Gleeson 1991; Domínguez et al. 1998). However, 1,312 yeast species, including 464 basidiomycetous yeast species, have been identified so far (Kurtzman et al. 2011), so there may be many other yeast expression systems that are better than the present ones. Basidiomycetous yeasts tend to have higher GC contents than ascomycetous yeasts. Thus, they also tend to have different codon usage than ascomycetous

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yeasts and might be better suited for expressing some recombinant proteins. Two species of basidiomycetous yeasts, *Pseudozyma flocculosa* and *Pseudozyma antarctica*, have recently been used to construct host–vector systems for heterologous gene expression using either heterologous promoters (heat shock protein 70, glyceraldehyde-3-phosphate dehydrogenase promoters from *Ustilago maydis* and α -glucosidase promoter from *Pseudozyma tsukubaensis*) or a homologous promoter (actin promoter) (Avis et al. 2005, 2008; Cheng et al. 2008; Neveu et al. 2007).

Although many recombinant protein production systems have been developed, none are universally applicable. Furthermore, synthesis of several enzymes by existing protein expression systems is difficult. Since the efficiency of protein production can vary considerably depending on the host or production system, different approaches are used for optimization. Modification of the host strain is one such strategy (Gasser et al. 2007; Valkonen et al. 2003). Another strategy is to construct a new host–vector system in a new microorganism for recombinant protein production. Mass production of enzymes has many uses in industry and applied microbiology. Therefore, the development of new systems for recombinant protein production should accelerate the application of enzymatic technology.

The basidiomycetous yeast *Cryptococcus* sp. strain S-2 (S-2) was isolated from the air in Japan and stored in our laboratory (Iefuji et al. 1994). S-2 produces certain unique extracellular enzymes, for example, acid cellulase (Thongekkaew et al. 2008), acid xylanase (Iefuji et al. 1996a), raw-starch-degrading α -amylase (Iefuji et al. 1996b), and plastic-degrading cutinase-like enzyme (CLE) (Kawai et al. 2011; Kodama et al. 2009; Masaki et al. 2005). Each of these enzymes was expressed and secreted into culture media under their respective optimum conditions. CLE has attracted much interest because of its potential use in industry and environmental preservation, e.g., in biodiesel production (Kamini and Iefuji 2001), as an additive in laundry detergent (Thirunavukarasu et al. 2008), and in plastic degradation (Kawai et al. 2011; Masaki et al. 2005). Because of the usefulness of CLE, we wished to genetically modify S-2 to produce more CLE in this study.

Safe handling of host strain to produce enzymes is also important in industrial use. S-2 appears to be a safe strain to work with because it cannot grow at 37°C (Iefuji et al. 1994) and feeding to mice produced no ill effects (H. Yamaguchi and K. Uchida, personal communication). Currently, 70 species in the genus of *Cryptococcus* have been described (Fonseca et al. 2011). The ITS1 and ITS2 sequences of S-2 (119 and 172 bp) are 100% and 97%, identical, respectively, to those of *Cryptococcus flavus*. On the other hand, the two species have different GC content (67% and 55%) (Iefuji et al. 1994). In a phylogenetic analysis of 33 species of *Cryptococcus* and related genera

(Takashima and Nakase 1999), *C. flavus* was obviously different from *Cryptococcus neoformans* and *Cryptococcus gattii* that are known as pathogenetic yeasts. S-2 is thought to be closely related to *C. flavus* based on their physiological, biochemical, and chemotaxonomic properties (Iefuji et al. 1994) as well as their ITS sequences. In this paper, we report the construction of a new host–vector system that uses the basidiomycetous yeast S-2 and one of its powerful promoters for recombinant protein expression.

Materials and methods

Strains, vectors, and media

The wild-type *Cryptococcus* sp. strain S-2 (S-2) is deposited in the International Patent Organism Depository (accession number: FERM ABP-10961). S-2 was cultured at 25 °C in liquid or solid (2.0% agar) yeast malt medium (YM) containing 1.0% glucose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract, unless otherwise stated. Uracil-free synthetic complete media, SC(–ura), contains 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, USA), 2% dextrose, and 0.077% dropout supplements without uracil (Clontech, Mountain View, USA). *Escherichia coli* K12 strain DH5 α (Nippon gene, Tokyo, Japan) was used as the host for cloning and plasmid amplification. pUC19 was used for construction of the yeast transformation system. The vector pGEM-T Easy (Promega, Madison, USA) was used for subcloning and nucleotide sequencing. The green fluorescent protein (GFP) gene was amplified from pEGFP (Clontech, Mountain View, USA).

Isolation of the uracil auxotroph and sequencing of the orotate phosphoribosyltransferase (OPRTase) gene (*URA5*) of S-2

To prepare host strains for a transformation system for this yeast, uracil auxotrophs were obtained by using 5-fluoroorotic acid (5-FOA) (Boeke et al. 1984). Spontaneous 5-FOA-resistant mutants were isolated from the medium containing 5-FOA. The isolated mutant U-5 was inoculated in minimum medium containing orotic acid or orotidine. To sequence the orotate phosphoribosyltransferase (OPRTase) gene (*URA5*) by PCR, four degenerate primers were designed from highly conserved regions of 10 OPRTase genes from the following microbes: *C. neoformans* (CAD88274); *S. cerevisiae* (P13298); *P. pastoris* (AAQ72470); *S. pombe* (O94331); *K. lactis* (CAA04694); *Yarrowia lipolytica* (CAA80294); *E. coli* (AAG58786); *Azotobacter vinelandii* (COG0461); *Pseudomonas aeruginosa* (AAC44427); and *Vibrio cholerae* (AAF93387). (Protein IDs from DDBJ/EMBL/Swiss-Prot

follow microbe names.) Two sense degenerate primers, 5'-CCHTAYTTCTTYAAYKYBGG-3' (primer-1F) and 5'-TTYGGYCCBGCYTAYAARGG-3' (primer-2F), and two antisense degenerate primers, 5'-CCDGMRGTSK VACRTCRTC-3' (primer-1R) and 5'-AKRBTDCDC CYTCRCRTG-3' (primer-2R), were used to derive a part of *URA5* of S-2. PCR was performed using these primers, ExTaq (Takara Bio, Shiga, Japan) and genomic DNA from S-2. An approximately 400-bp fragment amplified by primer-1F and primer-2R was purified using QIAquick Gel extraction kit (Qiagen, Hilden, Germany), and the sequence was analyzed using ABI PRISM 310 (Applied Biosystems, Foster City, USA). The acquired sequence was used for subsequent primer designs.

Total RNA was extracted from yeast that was cultured at 25°C in uracil-free medium by the hot phenol method (Köhler and Domdey 1991). Poly(A)⁺RNA was purified from total RNA using OligotexTM-dT (Super) mRNA purification kit (Takara Bio, Shiga, Japan). After reverse transcription, the full-length cDNA sequence of *URA5* was determined by 5'- and 3'-RACE (rapid amplification of cDNA ends) methods using 5'- and 3'-Full RACE Core Sets (Takara Bio, Shiga, Japan), respectively.

The genomic sequences of the upstream and downstream regions of *URA5* were obtained by inverted PCR (Triglia et al. 1988). The genomic DNA was isolated by gene trapping

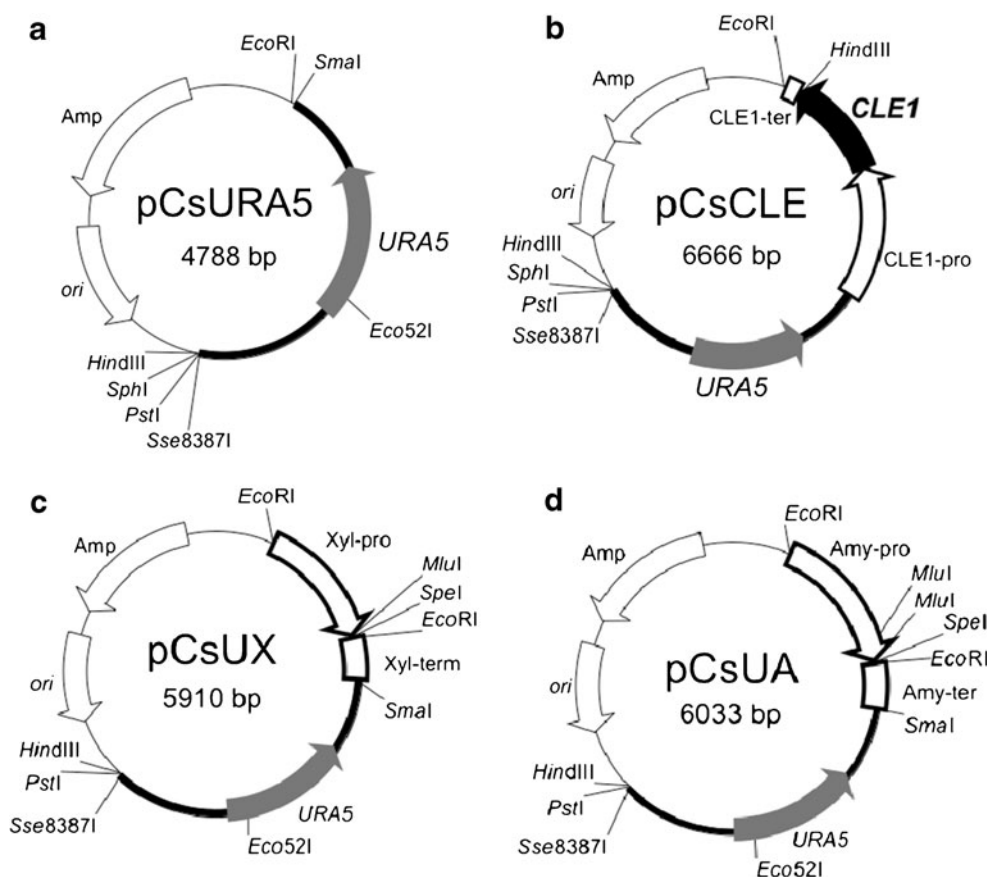
using a Liquid Extraction kit (Takara Bio, Shiga, Japan) and digested by *Pst*I. Using self-ligated genomic DNA as a template, inverted PCR was performed with the following primers: sense primer, 5'-AACCAAATCGACGGTT CAGG-3', and antisense primer, 5'-GGCACTCGAGAT GACACAAG-3'. Amplified fragments were cloned into pGEM-T vectors and sequenced. Genomic DNA of U-5 was also isolated. The DNA fragment containing *URA5* was amplified by PCR, and its sequence was analyzed along with a wild-type strain.

The entire *URA5* gene of S-2 including parts of the 5' and 3' UTRs was amplified by PCR using genomic DNA as template and sense and antisense primers AACCTG CAGGTGCTTCGAGTGCTTGTTACC and AAACCCGGGCTGATGGATTGCAGGTCTGC, which include *Sse*8387I and *Sma*I sites (underlined). The product was digested by *Sse*8387I and *Sma*I and cloned into the multicloning site of pUC19 to construct the plasmid pCsURA5 (Fig. 1a).

Transformations

To transform the uracil auxotroph U-5, cells were cultured overnight in 100 ml of YM broth in a 500-ml baffled Erlenmeyer flask at 25 °C with vigorous aeration in a shaker at 130 rpm. The culture was harvested at an optical

Fig. 1 Schematic representation of the plasmids. **a** The plasmid pCsURA5 containing *URA5* of *Cryptococcus* sp. strain S-2. The 5' flanking region (777 bp) and the 3' flanking region (481 bp) are represented as thick lines. **b** The plasmid pCsUCLE for multiple integration of *CLE1*. *CLE1*, including 1,003 bp of 5' flanking (*CLE*-pro) and 111 bp of 3' flanking (*CLE*-ter) regions, is cloned into pCsURA5. **c** The plasmid pCsUX containing xylanase promoter (*Xyl*-pro; 837 bp) and terminator (*Xyl*-ter; 277 bp) regions. **d** The plasmid pCsUA containing α -amylase promoter (*Amy*-pro; 940 bp) and terminator (*Amy*-ter; 297 bp) regions



density of 0.8–1.0 at 660 nm ($OD_{660\text{ nm}}$) by centrifugation at $2,300\times g$ and 4 °C for 3 min. The harvested cells were washed twice in a wash solution and resuspended in 0.5 ml electroporation solution.

Cells were transformed by three different electroporation methods as previously described for *S. cerevisiae* (Becker and Guarente 1991) (method 1) and *C. neoformans* (Varma et al. 1992) with some modifications (methods 2 and 3). In method 1, the wash and electroporation solutions were 1 M sorbitol. The cell suspension (50 μ l) in the electroporation solution was transferred to 0.2-cm-gap disposable electroporation cuvettes (Bio-Rad, Hercules, USA). After the addition of plasmid DNA (2 μ g), the cells were subjected to an electric pulse from a Gene Pulser (Bio-Rad). The pulse setting was 1.5 kV, 25 μ F, and 200 Ω . In method 2, the wash solution contained 270 mM sucrose, 1 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.6), and 4 mM DTT and the electroporation solution contained 270 mM sucrose, 1 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.6). The pulse setting was 470 kV, 25 μ F, and 1,000 Ω . Method 3 was identical to method 2 except the pulse setting was 470 kV, 25 μ F, and ∞ .

Under all conditions, circular and linear plasmids were applied to transformations. *EcoRI* digested at a multi-cloning site located at the outside of the 3' flanking region of *URA5*. *Eco52I* digested pCsURA5 at an internal region of *URA5* ORF. The amount of plasmid was also changed from 0.1 to 5 μ g for method 3. After electroporation, cells were plated on an SC(–ura) plate, which was incubated at 25°C until colonies appeared. The colonies on the SC(–ura) plates were counted to estimate transformation efficiency under each condition.

Introduction of the CLE gene (*CLE1*) to construct multicopy transformants

Plasmid pCsURA5 was digested by *SmaI* (blunt end digestion) and *EcoRI* and purified using a gel extraction kit (Qiagen, Hilden, Germany). *CLE1* including 1,003 bp of the 5' flanking region and 111 bp of the 3' flanking region was amplified by PCR. *EcoRI* cleaved only the 3' region of the PCR product. This fragment, digested by *EcoRI*, was purified and cloned into pCsURA5. The resulting plasmid, pCsUCLE (Fig. 1b), was linearized by digestion with *EcoRI* and transferred into U-5 by electroporation as described above. Ten transformants were randomly selected and inoculated into 50 ml of CLE production medium (1% yeast extract, 0.5% lactose, 1% KH_2PO_4 , 0.1% $MgSO_4\cdot 7H_2O$, 1% triolein, pH 5.5) in a 200-ml baffled Erlenmeyer flask and cultured at 25°C with vigorous aeration in a shaker at 140 rpm for 7 days (Kamini et al. 2000). The culture supernatant was assayed for CLE activity using *p*-nitrophenyl butyrate (PNPB) as a substrate. An aliquot (10 μ l) of supernatant was used for one well of

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, gels were stained by Coomassie Brilliant Blue using CBB Stain One (Nacalai Tesque, Kyoto, Japan).

CLE activity

CLE activity was assayed by measuring the amount of *p*-nitrophenol released from PNPB. The reaction mixture contained 2.5 mM PNPB and 50 mM sodium acetate (pH 5.5) and 2% (*w/v*) of Triton X-100. The reaction was initiated by adding 50 μ l of the enzyme solution to 950 μ l of the reaction mixture. The reaction was terminated by adding 2 ml of acetone. The concentration of *p*-nitrophenol was determined spectrophotometrically at 410 nm using an extinction coefficient of 0.62 $mM^{-1} cm^{-1}$. One unit of hydrolysis activity was defined as the hydrolysis of 1 μ mol of the substrate per minute, at 37 °C.

Construction of plasmids containing inducible promoters for recombinant protein expression

Plasmid pCsURA5 was digested by *SmaI* and *EcoRI* and purified using a gel extraction kit (Qiagen, Hilden, Germany). The xylanase terminator region (277 bp) was amplified by PCR and also digested by *SmaI* and *EcoRI*. The two restriction enzyme-treated fragments were ligated. The resulting plasmid was digested by *EcoRI* and used directly in the next step. The xylanase promoter region (837 bp) was amplified by PCR, digested by *EcoRI*, and cloned into the constructed plasmid to generate pCsUX (Fig. 1c).

The α -amylase terminator region (297 bp) was also amplified by PCR and digested by *EcoRI* and *SmaI* and cloned into the plasmid pCsURA5, which had previously been digested with *EcoRI* and *SmaI*. The resulting constructed plasmid was digested by *EcoRI* and used directly in the next step. The α -amylase promoter region (940 bp) was amplified by PCR and digested by *EcoRI* and cloned into the constructed plasmid to generate pCsUA (Fig. 1d).

Cloning the GFP gene into pCsUX and pCsUA

The GFP gene (*GFP*) was amplified from a pEGFP DNA template by PCR using a forward primer that contained an *MluI* site and a reverse primer that contained an *SpeI* site. The amplified fragment was digested by *MluI* and *SpeI*, and the resulting fragment was cloned into pCsUX at the same sites to generate plasmid pCsUX.GFP. The amplified *GFP* with *SpeI* sites at the 5' and 3' ends was digested by *SpeI* and cloned into pCsUA at the *SpeI* site to produce plasmid pCsUA.GFP.

These constructed plasmids were transferred into U-5 by electroporation as described above. Integrations of GFP expression cassettes in the transformants were confirmed by colony PCR using primers located in the promoter region and the GFP gene.

Transformants resulting from integration of pCsUX.GFP or pCsUA.GFP were cultured in yeast nitrogen base medium (0.67% yeast nitrogen base without amino acids, 2% carbon source) at 25°C for 24 h. Glucose, xylose, maltose, dextrin, and xylan were used as carbon source. After the cells were harvested by centrifugation and washed with water, expression levels of GFP were observed by fluorescence microscopy (ECLIPSE E600 microscope, Nikon, Tokyo, Japan), using the appropriate filter set.

Cloning *CLE1* into pCsUX and construction of recombinant CLE production yeast

CLE1 from genomic DNA of S-2 was amplified by PCR using a forward and a reverse primer that contained an *MluI* site before the start and stop codons. The amplified fragment was digested by *MluI*, and the resulting fragment was cloned into pCsUX at these same sites to produce plasmid pCsUX.CLE. The constructed plasmid was digested by *Sse8387I* and transferred into U-5 by electroporation as described above. After electroporation, cells were plated on an SC(-ura) plate, which was incubated at 25°C until colonies appeared. Forty transformants were cultured in 5 ml SC(-ura) medium containing 2% xylose as the sole carbon source at 25°C for 3 days. The transformant showing the highest CLE activity was selected and used for subsequent CLE expression. The selected transformant was inoculated into YM medium and cultured for 2 days as a preculture. The preculture (2.5 ml) was inoculated into 50 ml of expression medium consisting of 6% xylose and 1% yeast extract in a 200-ml baffled flask and cultured at 25°C and 140 rpm. The activity of the supernatant from the culture was measured each day for 6 days.

Accession numbers

The accession number of the cDNA sequence of *URA5* is AB470526 in DDBJ. Accession numbers of α -amylase promoter, xylanase promoter, and *CLE1* are D83540, D63381, and AB671329, respectively.

Results

Isolation of the uracil auxotroph of S-2

Spontaneous 5-FOA-resistant mutants were isolated from medium containing 5-FOA. One of them was found to be a

uracil auxotroph. 5-FOA-resistant uracil auxotrophs are usually deficient in OPRTase and/or orotidine-5'-phosphate decarboxylase (OMPdecase, EC 4.1.1.23) because these enzymes catalyze the last two steps in the biosynthesis of pyrimidine (Bai et al. 1999). This isolated mutant could grow in a medium containing orotidine, but not in a medium containing orotic acid. Therefore, the isolated mutant was deficient in OPRTase because orotic acid is converted into orotidine-5'-phosphate by OPRTase. The uracil auxotroph, named U-5, was subsequently used as a host strain for the following host–vector system construction.

Isolation of *URA5* of S-2

An approximately 400-bp fragment of *URA5* encoding OPRTase of S-2 was amplified by degenerate PCR using four degenerate primers, which were designed from highly conserved regions of *URA5* from other microbes. Using the sequence of an isolated genomic DNA fragment, the full cDNA sequence of *URA5* was obtained by 3' and 5' RACE. The upstream and downstream sequences of the *URA5* ORF (1,126 and 522 bp, respectively) were derived by inverted PCR. The *URA5* ORF region of genomic DNA contains three exons and two introns. The OPRTase of S-2 consists of 235 amino acids and has a molecular weight of 25 kDa. Its deduced amino acid sequence exhibits 71% and 43% homology with the OPRTases of *C. neoformans* and *S. cerevisiae*, respectively. One base substitution was detected in *URA5* of U-5, which resulted in a Ser-to-Leu substitution at amino acid position 48.

Transformation by electroporation

URA5 including 777 bp of the 5' flanking region and 481 bp of the 3' flanking region was cloned into the multicloning site between the *SmaI* and *Sse8387I* sites of plasmid pUC19. The resulting plasmid, named pCsURA5 (Fig. 1a), was used to transform U-5 by electroporation. Three forms were prepared: a circular plasmid, a linear plasmid digested by *EcoRI* outside the *URA5* region, and a linear plasmid digested by *Eco52I* within the *URA5* ORF. Three methods that differed in the preparation of competent cells and the electric pulse were compared using each of the three plasmid forms. The highest transformation efficiency was obtained with method 3 using linear DNA digested by *EcoRI* (Table 1).

The optimal concentration of plasmid for transformation by method 3 was also studied. Both circular and linear (*EcoRI* digested) plasmids (0.1–5 μ g) were used for this evaluation. The transformation efficiency was higher for the linear plasmid under all concentrations. Although the transformation efficiency at low concentration (0.1 μ g) was apparently greater than that at higher concentrations

Table 1 Transformation efficiencies of plasmids

Plasmid type	Transformation efficiency (colonies/ μg DNA)		
	Method 1	Method 2	Method 3
Circular	8	12	140
Linearized with <i>EcoRI</i>	37	21	250
Linearized with <i>Eco52I</i>	3	13	79

(1–5 μg plasmid), only a few colonies appeared at low concentration (0.1 μg). Therefore, 2 μg of plasmid was used for the following studies with method 3, which exhibited the highest transformation efficiency using 1–5 μg plasmid.

Introduction of *CLE1* to construct multicopy transformants

We attempted to introduce multiple *CLE1* genes containing its promoter region into U-5 to construct a multicopy transformant. *CLE1* containing 1,003 bp of 5' flanking region and 111 bp of 3' flanking region was cloned into the multicloning site between *SmaI* and *EcoRI* sites of plasmid pCsURA5 to form pCsUCLE (Fig. 1b). Linearized plasmid was introduced into the uracil auxotroph by electroporation and transformants that appeared on an SC(-ura) plate were isolated. Eight of 10 randomly selected transformants showed higher *CLE* activity than the host strain, U-5 (Fig. 2a), indicating that most of the transformants contained *URA5* and *CLE1*. Growth of each transformant, U-5 as well as the wild type, was the same (Fig. 2b). Four transformants exhibited approximately six to nine times higher *CLE* activities than the U-5, and transformants 4 and 6 exhibited approximately 3.5 times higher activity than the wild type. SDS-PAGE of the culture supernatant revealed a strong 21-kDa band of *CLE* in the transformants (Fig. 2c, lanes 4, 5, 6, and 8). This result was consistent with an enzymatic assay (Fig. 2a). Furthermore, *CLE* was a major protein in the culture supernatant of multicopy transformants, and other extracellular proteins were present in very low amounts. Multiple integration of *CLE1* was confirmed by genomic Southern analysis in four transformants with higher *CLE* activity (nos. 4, 5, 6, and 8) (Supplemental Fig. S1). Each transformant showed a different pattern of multiple bands of *CLE1* in the Southern analysis. This result indicated that the transformation resulted in ectopic integration of the gene.

Heterologous recombinant protein expression vectors including inducible promoters

Two different expression plasmids were constructed for the expression of heterologous proteins in S-2 as shown in Fig. 1c (pCsUX) and d (pCsUA). Plasmid pCsUX contained a

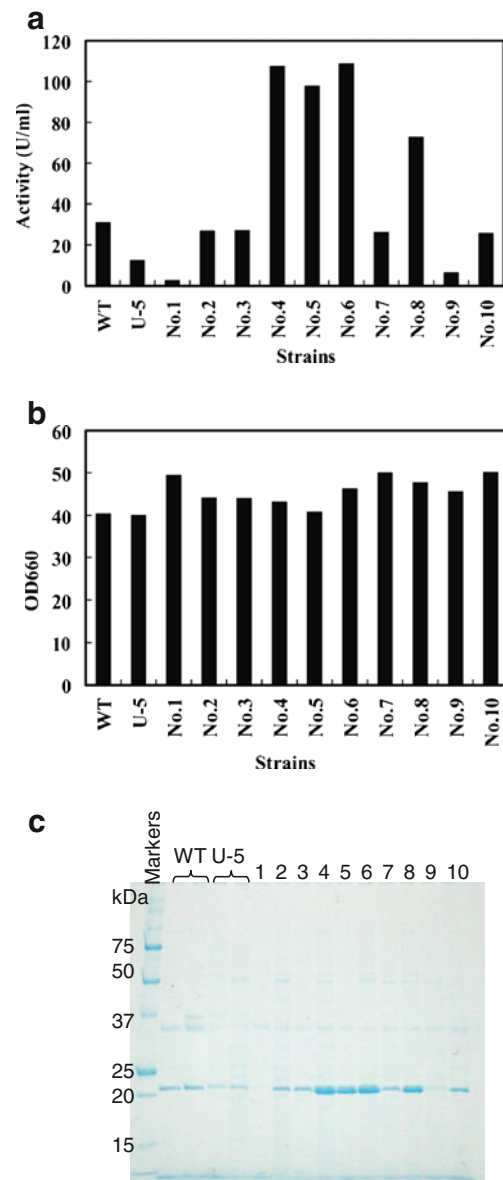
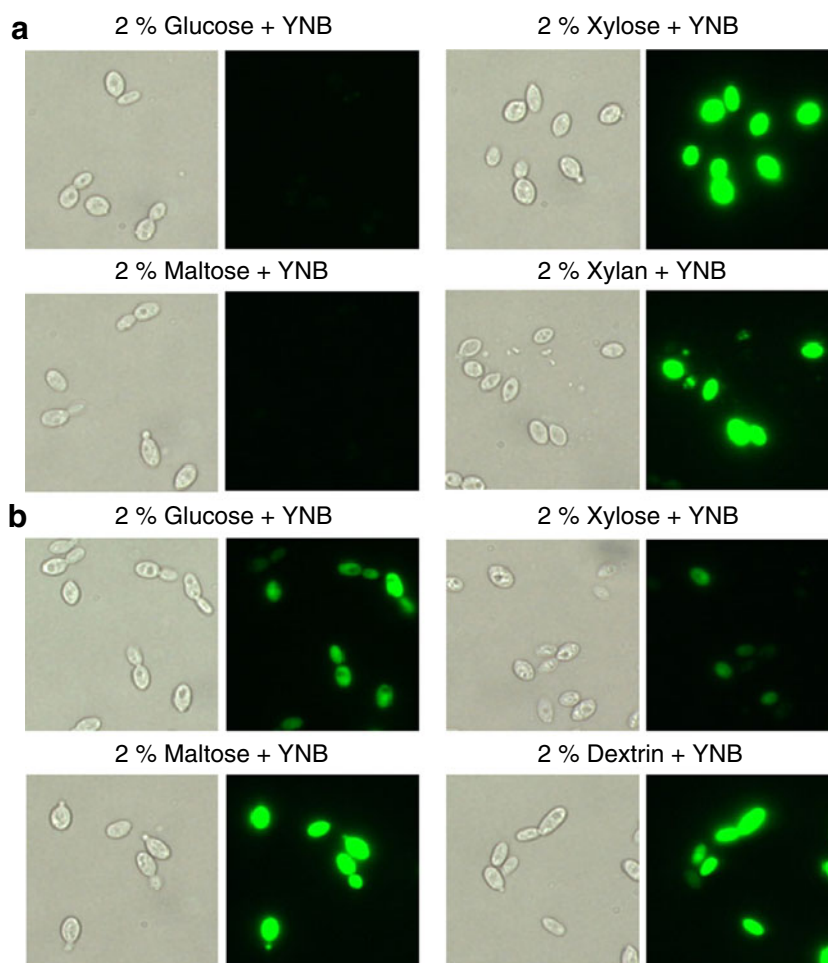


Fig. 2 *CLE* expression from multicopy transformants. Transformants (nos. 1–10) were randomly selected. **a** *CLE* activities of transformant cultures. WT and U-5 represent *CLE* activities of supernatants of the wild-type yeast and uracil auxotroph, respectively. **b** Growth of transformants. **c** SDS-PAGE of supernatants (10 μl) of transformant cultures

xylanase promoter, which is induced by xylan (Iefuji et al. 1996a) and xylose, and the corresponding terminator. Plasmid pCsUA contained an α -amylase promoter, which is induced by maltose (Iefuji et al. 1996b), and the corresponding terminator. Then, a DNA fragment of the desired protein was integrated between the promoter and terminator. GFP expression was investigated with both plasmids. Induction and expression of GFP were observed by fluorescence microscopy. When the xylanase promoter was used, GFP expression was strongly induced by xylose or xylan, but not by glucose or maltose (Fig. 3a). When the α -amylase

Fig. 3 Induction and expression of GFP observed by fluorescence microscopy. Transformants were cultured in media containing the indicated carbon source at 25°C, 24 h. *Left:* differential interference contrast microscopy. *Right:* fluorescence microscopy. **a** Microscope observations of transformants with pCsUX.GFP. Glucose, xylose, maltose, and xylan were used as the carbon source. **b** Transformants with pCsUA.GFP. Glucose, xylose, maltose, and dextrin were used as the carbon source



promoter was used, GFP expression was strongly induced by maltose or dextrin (Fig. 3b). When glucose or xylose was added, only weak fluorescence of GFP was observed.

Recombinant CLE production under inducible conditions of the xylanase promoter

The inducible plasmid for recombinant CLE production was constructed by cloning *CLE1* into pCsUX. The constructed plasmid (pCsUX.CLE) was introduced into U-5 and transformants were screened for CLE production. Most transformants demonstrated higher CLE activities in the culture medium than the wild type. Of 40 transformants examined, the one with the highest CLE was selected. The selected transformant was inoculated into a medium containing 6% xylose to induce the xylanase promoter. CLE activity of the culture medium increased, reaching about 220 U/ml after 6 days (Fig. 4), which was twice the activity of the multicopy transformant in Fig. 2. In the presence of xylose, the wild type produced approximately 15 U/ml of CLE, whereas the transformant produced approximately 15 times this amount.

Discussion

We have constructed a host–vector system of the basidiomycetous yeast *Cryptococcus* sp. strain S-2 cells, using a plasmid that contains the *URA5* and a uracil auxotroph. This system was useful for recombinant CLE expression. In

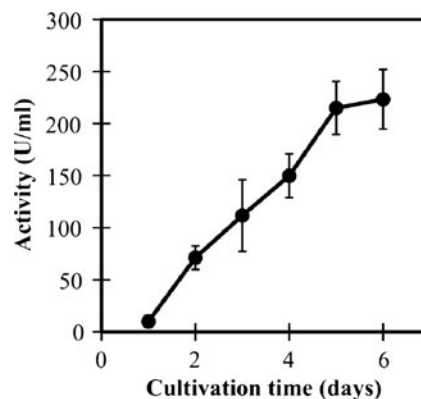


Fig. 4 Recombinant CLE production under the inducible condition of the xylanase promoter

general, yeast expression systems have some advantages for recombinant protein production. For example, a yeast can secrete an own protein in appropriate conformation directly into the medium. Moreover, fewer native proteins are secreted in the culture medium as shown in Fig. 2c, which is also preferred for these applications.

URA5 was used as a marker for the constructed system. In the case of *S. cerevisiae*, 5-FOA-resistant isolates are predominantly defective in *URA3*, which encodes OMPdecase (Boeke et al. 1984), because *S. cerevisiae* has a minor OPRase isozyme (*URA10*) (De Montigny et al. 1990). However, *URA5* has been used as a marker for some yeast host–vector systems. In the case of *C. neoformans*, most of the spontaneous 5-FOA-resistant mutants were OPRase deficient, i.e., *ura5* cells (Edman and Kwon-Chung 1990; Kwon-Chung et al. 1992). In S-2, *URA5* is probably the only gene encoding OPRase. In U-5, the OPRase has a Ser48 to Leu substitution, which could be the cause of OPRase deficiency because Ser48 was found to be conserved in 10 microbial OPRases described in “Materials and methods”.

Method 3 in Table 1, which is optimized for *C. neoformans* (Varma et al. 1992), was most effective for S-2. In addition, using a linearized plasmid increased the transformation frequency, as was also found to be the case with *C. neoformans* (Edman and Kwon-Chung 1990). The optimal transformation efficiency (obtained with method 3; Table 1) was comparable to that obtained with *C. neoformans* (Varma et al. 1992). The plasmid linearized by *EcoRI* was more effective for transformation than the circular plasmid. Although the plasmid digested by *Eco52I* was also linear, its transformation efficiency was not higher than that of the circular plasmid. Because *Eco52I* was used to digest the internal region of *URA5* ORF to use it for transformation, developing a nonmutated *URA5* by homologous recombination at the *ura5* locus in the chromosome of the uracil auxotroph was necessary. On the other hand, *EcoRI* did not digest the *URA5* including its promoter and terminator regions. Therefore, the introduced plasmid digested by *EcoRI* could directly produce an active OPRase.

Multicopy transformants incorporating *CLE1* were generated under the optimized transformation conditions. In general, multicopy transformants express higher quantity of protein than single-copy transformants (Read et al. 2007; Werten et al. 1999). Because the wild-type strain has one copy of the intrinsic *CLE1*, it is expected that most of the transformants have two or more copies of this gene in its genome. Four of 10 randomly selected transformants clearly contained more than two copies of *CLE1* as determined by genomic Southern analysis (Supplemental Fig. S1). The transformants that exhibited nine times higher CLE activities than the host strain were isolated during the screening process that contained only 10 transformants.

High-frequency integration of *CLE1* into the genome may result in multiple integrations. Although the mechanism of multiple integrations has not yet been elucidated, this feature of S-2 is desirable in the host strain for recombinant protein production.

We investigated two inducible promoters for construction of an expression vector. The xylanase promoter of S-2 was induced by xylan as well as xylose. Xylose is produced from xylan and xylooligosaccharides by the xylanase of S-2 (Iefuji et al. 1996a). In another yeast, *Cryptococcus albidus*, endo-1,4- β -xylanase induction by xylose was quite limited, while xylan or xylobiose strongly induced production of the enzyme (Biely et al. 1980; Biely and Petrakova 1984). Xylanase from the basidiomycete *Sclerotium rolfsii* was induced by xylan and xylobiose as well as a broad variety of substrates (Sachslehner et al. 1998). The xylanase promoter in the constructed expression vector was tightly controlled. Glucose and maltose did not induce expression of GFP, whose gene was cloned downstream of the xylanase promoter. On the other hand, the regulation of the α -amylase promoter seemed less constrained than that of the xylanase promoter. The α -amylase promoter was strongly induced by maltose or dextrin. Unexpectedly, xylose also induced the α -amylase promoter but at a lower level than did glucose.

Next, we used the xylanase promoter for recombinant CLE production because this promoter was more effective than the α -amylase promoter, whose efficiency we evaluated. Under inducible conditions of the xylanase promoter, the isolated transformant produced about 220 U/ml of CLE (Fig. 4). Under the same condition (6% xylose and 1% yeast extract), approximately 15 U/ml of CLE was produced by the wild-type yeast, although this condition was not optimal for intrinsic CLE production.

Kamini et al. (2000) determined the optimal conditions for CLE production by the S-2 wild type. As shown in Fig. 2a, the wild-type yeast produced approximately 30 U/ml of CLE under the optimum condition for intrinsic CLE production. Until this study, maximal production has been 30 U/ml. In this study, the production of CLE was highest under the control of the xylanase promoter and was more than seven times higher than the maximum productivity of the wild-type yeast. We have indicated that CLE can effectively degrade plastic (Kawai et al. 2011; Masaki et al. 2005) and the transesterification of triglycerides to fatty acid methyl esters for biodiesel fuel (Kamini and Iefuji 2001). The ability to achieve a higher level of CLE production enhances its overall potential for these and other applications.

We constructed a yeast with an enhanced ability to produce CLE. The basidiomycetous yeast S-2 produces certain unique extracellular enzymes under different conditions. The S-2 system can also be used to increase the production of these enzymes by multicopy transformants

and/or by using a xylanase promoter. Because the marker and the promoters, which are the main parts of this system, are derived from the host yeast, constructing a self-cloning strain that does not contain a heterologous gene and produces desirable homologous enzymes of S-2 is possible.

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