Hue University Journal of Science: Natural Science
Vol. 135, No. 1B, 57–64, 2026

pISSN 1859-1388
eISSN 2615-9678

Saccharomyces cerevisiae's culture conditions for heterologous expression: A detailed investigation using reporter gene eGFP and *Escherichia coli*'s heat-labile toxin B subunit

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(*Received: 24 December 2025; Revised: 11 March 2026; Accepted: 11 March 2026*)

Abstract. The *Saccharomyces cerevisiae* recombinant expression host 2805 has been employed extensively for various applications, ranging from recombinant enzyme production, oral vaccine development, and viral antigen production. For all of its previous applications, recombinant yeast cells have been cultured under a conventional three-stage protocol: after being revived and grown on selective agar plates, (i) cells are seeded in 5 mL Uracil deficient medium (–Ura medium) for two days; (ii) cells are diluted at a 1:8 ratio in the complete medium YEPD and cultured for 16 hours; (iii) flask culture in YEPD for 1 to 5 days at 30 °C. Despite its routine use, the rationale and performance of this culture regime have not been systematically examined. In this study, using a synthetic, yeast codon optimised gene encoding the B subunit of *Escherichia coli* heat-labile toxin (LTB) fused to the reporter enhanced Green Fluorescent Protein (eGFP), we employed flow cytometry to monitor the expression dynamics in the recombinant 2805 yeast strains across all cultural stages. The results show that LTB-eGFP expression peaked on day 1 of the first seeding in the –Ura medium, dropped on day 2, then increased again after being transferred to YEPD, and then declined steadily in flask culture from day 1 to day 5. Across conditions, the percentage of fluorescent positive cells was approximately 45–60%. These results indicate that the re-optimisation of the culture protocol is necessary in order to maximise the expression efficiency.

Keywords: *Saccharomyces cerevisiae*, LTB, eGFP, culture conditions, flow cytometry

1 Introduction

Saccharomyces cerevisiae is a widely utilised host for recombinant protein expression, supporting a broad spectrum of applications, including recombinant enzyme production [1], viral antigen synthesis [2], vaccine development [3], and metabolic engineering [4]. Our previous research extensively employed *S. cerevisiae* strain 2805, a mutant auxotrophic for uracil and several amino acids, such as histidine. This strain is frequently used for heterologous protein expression via transformation with episomal vectors, such as yEP-GDP-TER [5]. Indeed, the 2805/yEP-GPD-TER has demonstrated its consistent success across diverse biotechnological applications [1–4].

Standard protocols for expressing heterologous proteins in this particular system involve a multistage cultivation process: initially, a single colony is inoculated into 5 mL of a uracil dropout liquid medium and cultured for 2 days. This primary culture is subsequently transferred to a complex medium such as YEPD for 16 hours, before being scaled up to a 300 mL flask containing 40 mL of YEPD and incubated in a shaking incubator at 30 °C for 3 to 5 days. However, we have

recently observed that at reduced expression temperatures from 30 to 20 °C, the productivity and the structural assembly of target proteins improved significantly [5]. This finding has been recently applied to the metabolic engineering of yeast for the production of a novel anticancer agent [4].

To expand upon these findings, we investigate the time course expression of a target protein from the moment when a colony is picked from a dropout plate until the 5th day of flask culture. The detailed picture of temporal expression pattern revealed by this study will serve as the foundation for further optimisation of *S. cerevisiae* 2805 yeast expression protocols.

To facilitate the measurement of the expression level of the target protein, we employed a fusion protein consisting of an antigen from *Escherichia coli*, namely, the *E. coli* heat labile toxin B subunit (LTB), fused to a reporter gene, enhanced Green Fluorescent Protein (eGFP) at its C terminal. Given that eGFP is a robust and well-characterised reporter [6, 7] and that LTB expression level is known to directly influence mucosal vaccine efficacy [3, 8, 9], this fusion model serves as an ideal proxy for monitoring system performance. The system could be employed to screen various synthetic LTB genes to identify the best-performing variant.

To our best knowledge, this is the first study that gives a detailed description of the temporal expression pattern of heterologous protein expression in the 2805 yeast by using the episomal vector yEP-GPD-TER.

2 Materials and methods

2.1 Materials

Strains and culture conditions

The *Escherichia coli* (*E. coli*) Top10 (Invitrogen) was employed for all cloning and plasmid propagation. The *Saccharomyces cerevisiae* (*S. cerevisiae*) strain 2805 (MATα pep4::HIS3 prb 1-δ Can1 GAL2 his3 ura3-52) served as the heterologous expression host.

E. coli was maintained in Luria Bertani (LB) broth or agar, supplemented with ampicillin (50 μg/mL) as required. Prior to transformation, *S. cerevisiae* was cultured in the complete medium YEPD (Yeast Extract Peptone Dextrose: peptone 20 g/L, yeast extract 10g/L, glucose 20 g/L). Following transformation with the episomal vector yEP-GPD-TER [10], transformants were selected and maintained in Uracil dropout media (–Ura media) (0.56 yeast nitrogen base with ammonium sulfate; 0.76% KCl, 2% Glucose and 0.14% yeast synthetic dropout; 20 mg/L tryptophan, histidine, leucine, and adenine hemisulfate) [3].

2.2 Methods

Construction of the LTB-eGFP expression vector

A synthetic yeast codon optimised LTB was designed by using Codon Optimisation Online (COOL) [11], synthesised via primer overlapping PCR, and cloned into the pGEMT easy vector (Promega); its sequence was confirmed by using Sanger sequencing. The LTB sequence was deposited on NCBI Genbank with the accession number PX651957.

The LTB-eGFP construct consists of the synthetic LTB at the N-terminal fused to a synthetic eGFP (OP866271.1) via a flexible linker GPGP, which was routinely used in previous works [3, 12]. The fusion construct was assembled by Overlap-Extension PCR (OE-PCR). The forward primer introduced a *Bam*HI restriction site followed by a Kozak sequence while the reverse primer incorporated a *Sal*I site behind a double stop codon (Table 1). The resulting LTB-eGFP fragment was cloned into the pGEMT easy vector for sequence verification.

pISSN 1859-1388
eISSN 2615-9678

Table 1. Primers used in this study

Primer name	Primer sequence (5′→3′)
LTB-*Bam*HI-F	GGATCCAACG**ATG**GCTCCCCAG ACCATC
OF	ATTTCTATGAAGAAC**GGTCCAG GTCCA**ATGGTGAGCAAGGGCGA G
OR	CTCGCCCTTGCTCACCAT**TGGAC CTGGACC**GTTCTTCATAGAAAT
eGFP-*Sal*I-R	GACGAGCTGTACAAG**TAATGA**G TCGAC

Following confirmation of an error-free sequence, the LTB-eGFP cassette was excised and released from the recombinant pGEMT easy vector by restriction digestion with *Bam*HI/*Sal*I and cloned into the yEP-GPD-TER consisting of a constitutive promoter GPD and a Gal7 terminator [3, 8]. The newly constructed vector was designated yEP-GPD-LTB-eGFP.

Yeast transformation and culture conditions

yEP-GPD-LTB-eGFP was transformed into the host *S. cerevisiae* 2805 with the lithium acetate method described in detail in previous work [3]. The recombinant yeasts were selected on –Ura agar plates at 30 °C .

For protein expression, a recombinant yeast colony was selected from the –Ura plate, inoculated into the 5 mL –Ura liquid medium, and cultured for 48 hours in a shaking incubator (30 °C and 220 rpm). This primary culture was diluted at a 1:8 ratio in the 5 mL YEPD medium and cultured for another 16 hours under identical conditions. Subsequently, approximately 5.25 mL of this culture was inoculated into 40 mL of the YEPD medium and cultured at 30 °C and 220 rpm for 5 days.

Expression analyses

The expression level of LTB-eGFP fusion protein in the yeast host was determined qualitatively and relatively via the fluorescent intensity of the recombinant yeast cells by using flow cytometry and Western blot.

Briefly, cells from culture plates in 15 mL Falcon tubes or flasks were harvested and washed three times with a cold PBS buffer (pH 7.4). Subsequently, 10^6 cells were analysed on a BD FACSCanto machine equipped with a 488-nm excitation laser and 530/30-nm emission filter for eGFP detection, and the results were analysed by using FlowJo v10 as follows: first, a yeast cell gate was drawn on a forward scatter-area (FSC-A) versus side scatter area (SSC-A) plot to exclude subcellular debris and large aggregates. From this gate, single cells were selected by plotting forward scatter heights versus forward scatter areas (FSC-H vs FSC-A) and gating on the diagonal population with a near-linear FSC-H/FSC-A relationship. All subsequent analyses were performed on these single-cell populations. GFP fluorescence was measured as compensated FITC-A (Comp-FITC-A). Negative and positive populations were defined by using the recipient 2805 yeast strain as the negative control, grown under identical conditions. A one-dimensional histogram of Comp-FITC-A was generated for the single-cell gate of the negative control, and a fluorescent threshold was placed so that ≥99% of events in the negative control fell within the GFP-negative region. The GFP-positive (Comp-FITC-A^+) gate was defined as all events with Comp-FITC-A values above this threshold. The same gate was applied similarly to all experimental samples. For each sample, the percentage of eGFP-positive cells was calculated relative to the single-cell parent gate, and geometric mean fluorescence intensities were extracted for both the total single-cell population and the GFP-positive subpopulation.

Immunoblotting was carried out as described previously [5]. Briefly, the cell density was determined with a spectrophotometer, and the same number of yeast cells from the flask culture stage were harvested, washed three times with a cold PBS buffer, and lysed via boiling for 15 minutes in a mixed buffer containing a protein extraction buffer [3, 5] and a 6x protein sample loading buffer. The cell debris was removed by centrifugation at 13.000 rpm for 2 minutes, and the supernatant was resolved on two identical (twin) 12% SDS-PAGE gels. Subsequently, one of the gels was blotted onto a nylon membrane and the target protein was probed with mouse polyclonal anti-eGFP antibodies [13], followed by AP-conjugated goat anti-mouse secondary antibodies.

Ten colonies were screened by using FACS to obtain three strains with the highest expression level. These three strains were then cultured for 5 days, and their temporal expression patterns, starting from selective plates until day 5 in flask culture, were determined by using FACS and Western blot.

3 Results and discussion

3.1 Construction of the expression plasmid

The LTB-eGFP construct was successfully synthesised, and its sequence was verified to be error-free. The cassette was subsequently cloned directionally into the yeast episomal vector yEP-GPD-TER. The resulting yEP-GPD-LTB-eGFP has an approximate size of 7 kbp. Fig. 1 illustrates the map of yEP-GPD-LTB-eGFP and the sequencing result of LTB-eGFP around the linker region.

3.2 Yeast transformation and screening of recombinant yeast strains

The lithium acetate transformation of yEP-GPD-LTB-eGFP into the 2805 recipient strain yielded numerous transformants. Ten colonies from these transformants were randomly selected for expression screening. The expression of LTB-eGFP was expressed as the fluorescent intensity of eGFP by the BD FACSCanto machine, with each colony analysed in triplicate (Fig. 2). All of the selected transformants showed the expression of LTB-eGFP compared with the mock transformant control, which is the 2805 yeast transformed with yEP-GPD-TER.

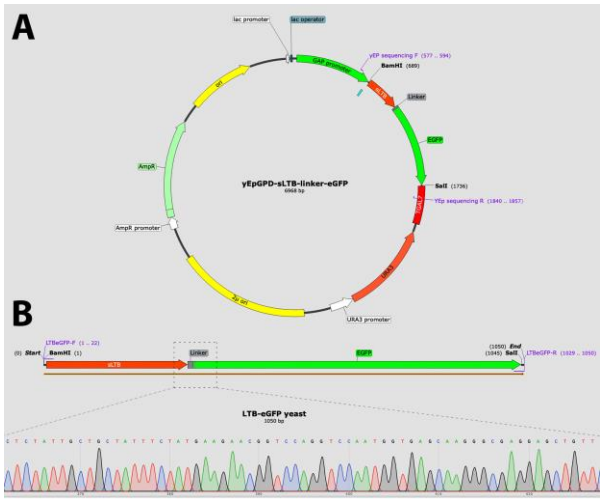

Fig. 1. The map of the yEP-GPD-LTB-eGFP (A) and LTB-eGFP fusion construct (B) as well as the chromatogram of the region around the flexible linker

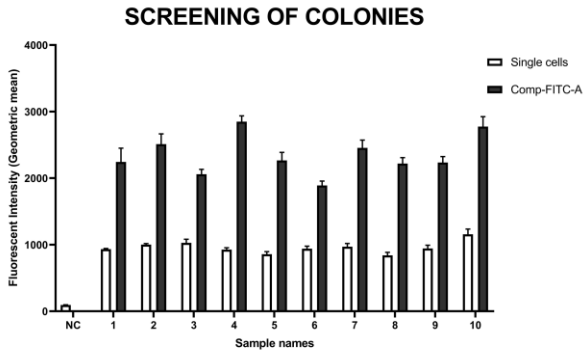

Fig. 2. Measurement of LTB-eGFP expression through eGFP fluorescent intensity by flow cytometry. Mocked transformant was used as the negative control. Comp-FITC-A represented a cell population that was fluorescent positive

While minor variations in the expression levels were observed among the colonies, the differences were insubstantial. This corroborates with previous observations [2, 3, 10], as the copy

pISSN 1859-1388
eISSN 2615-9678

number of yEP-GPD-TER can vary stochastically among transformants with an insignificant level. Based on the results, we selected three transformants with the highest expressed level, namely, transformant 2, 4, and 10, for temporal expression analyses.

3.3 Temporal expression analyses

The temporal expression profiles of the selected transformants were characterised by using flow cytometry and Western blot (Table 2). The data represent the geometric mean and standard deviation across three biological replicates. The Comp-FITC-A$^+$ denotes the percentage of the population exhibiting positive fluorescence, while the mean fluorescent intensity reflects the average protein accumulation within that positive subpopulation.

The results reveal that the proportion of fluorescent positive cells peaked at day 1 in the –Ura medium, then declined in the following day, and increased again after being transferred to YEPD. Less than half of the recombinant yeast cells were positive with green fluorescence. This observation was confirmed in our fluorescent microscope analysis (Supplementary Fig. 1). The ultimate cause of a significant fraction of recombinant yeast cells failing to express LTB-eGFP has never been adequately addressed, but it is relatively common in the *S. cerevisiae* expression system [14]; however, the unusual high fraction of negative cells observed in this study may be partly explained as the instability of the episomal vector. During the flask culture stage, both the percentage of GFP-positive and the MFI peaked on day 1 and progressively declined until day 5. This trend is consistent with our previous results, where yeast cells were cultured at 20 °C . As shown in Fig. 3, the total population's average fluorescence intensity followed a trajectory nearly identical to that of the GFP-positive subpopulation.

In numerous previous studies using this system, there was a standard practice to harvest recombinant yeast on day 3 of flask culture [2, 3, 9, 10]. However, our current results show that by day 3, the expression level of the protein of interest has decreased significantly. This suggests that harvesting at such a late stage may severely limit the yield of target proteins, whether for use as vaccine carriers or as enzyme production hosts.

Table 2. FACS statistics for the three selected transformants

Time point	Comp-FITC-A$^+$	MFI
Plate	33.86 ± 4.7	1711.66 ± 147.75
D1 –Ura	**51 ± 6.4**	**4424.33 ± 1127**
D2 –Ura	44.4 ± 1.15	2159 ± 210.17
D1 YEPD	46.9 ± 1.95	2511.66 ± 388.68
Flask D1	46.36 ± 1.24	1946.66 ± 245.58
Flask D2	44.63 ± 1.66	1843 ± 277.9
Flask D3	37.26 ± 0.83	1600 ± 139.8
Flask D4	33.56 ± 1.5	1547.33 ± 136.3
Flask D5	31.46 ± 2.3	1523.33 ± 159.48

Notes: D1 –ura = Day 1 in –Ura medium; D1 YEPD = Day 1 in YEPD medium; Flask D1 = Day 1 in 300 mL Flask culture; MFI = Mean Fluorescent Intensity

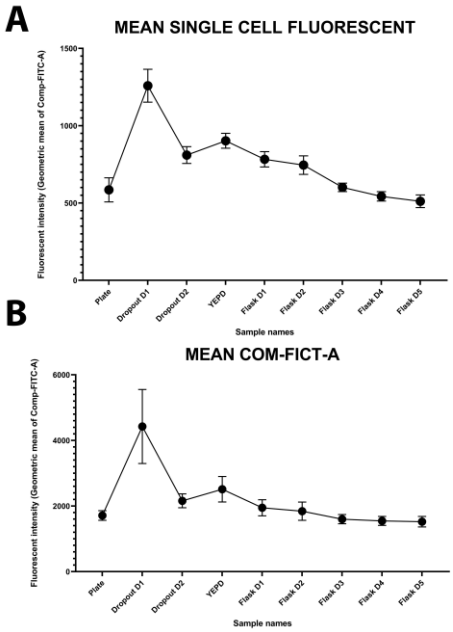

Fig. 3. Temporal expression pattern of yeast expressing LTB-eGFP at 30 °C . (A) is the average fluorescent intensity of the single cell population, while (B) is the mean of the fluorescent intensity of the fluorescent positive cell population

To confirm the flow cytometry results, we carried out Western blot analysis of transformant 10. Fig. 4 shows the Western blot result where LTB-eGFP expression was represented with a band of approximately 43 kDa (upper arrow). Since the theoretical molecular weight of LTB-eGFP is around 39 kDa, we assumed that yeast glycosylation increased the molecular weight. Indeed, a glycosylation prediction of LTB-eGFP using NetNGly 1.0 suggested 7 glycosylation residues. The method we used to lyse yeast cell (boiling) destroys every multimeric form, and we fail to observe any higher molecular assembly of LTB-eGFP. However, it should be noted that a fusion partner protein may not exceed a certain size in order to avoid steric hindrance during multimeric assembly. Indeed, when LTB was fused to ApxIIA, multimeric complexes failed to assemble [9]. An additional band of approximately 27 kDa (lower arrow) was also present, corresponding to eGFP being cleaved from the fusion protein, probably in the lysosome. A previous study showed that eGFP is relatively resistant to protease cleavage in yeast autophagosomes, resulting in intact eGFP that can be detected with Western blot [14]. Overall, the results agreed with our flow cytometry analysis, as the fluorescent intensity resulted from both LTB-eGFP and eGFP itself. Since the total protein was denatured by boiling before SDS-PAGE analysis, the assembly of the LTB-eGFP fusion protein into higher structures cannot be ascertained in this study. The problem of LTB expressed as a fusion, as well as a truncated protein, was observed in a previous study with a much smaller fusion partner [5]. We hypothesised that the GPGP flexible linker was probably too short to prevent steric hindrance between LTB and eGFP, resulting in improper folding and ultimately leading to the cleavage of eGFP from the fusion protein in autophagosomes. eGFP is relatively resistant to autophagosome degradation and even remains in functional form inside autophagosomes, as shown by Torggler et al. [14].

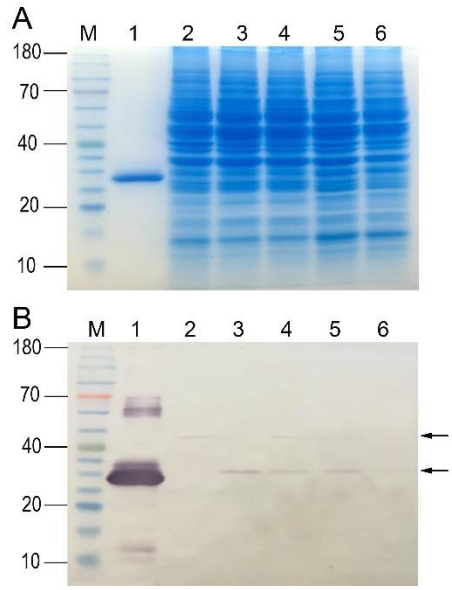

Fig. 4. Time series expression analysis using Western blot. Lane 1: *E. coli* expressed eGFP as positive control. Lane 2 to 6: cells picked from plate, day 1 in -ura, Day 1 in YEPD, Day 1 and Day 3 in flask, respectively. NC was intentionally left out as it never shows any cross-reaction with anti-eGFP antibodies [5, 13]

pISSN 1859-1388
eISSN 2615-9678

3.4 Implications for yeast culture protocol optimisation

While our previous work showed that expression of heterologous protein in yeast by using an episomal vector at a subphysiological temperature (20 °C vs 30 °C) significantly improved the expression level, and that protein expression peaked at day 1 in flask culture and decreased steadily afterwards [5], this study provides critical evidence that the temporal peak of expression remains constant regardless of temperature via additional evidence from yeast expressing LTB-eGFP. Our findings indicate that for the *S. cerevisiae* 2805/yEP-GPD-TER, cells should ideally be harvested at day 1 in flask culture instead of day 3, regardless of the expression temperature. This recommendation is based on the fact that the 2805 yeast growth curve reaches the plateau phase around 24–36 hours post-innoculation [5]. In fact, for oral vaccine application, what matters is the dose delivered, which highly depends on the proportion of cells that actually expresses the target antigen. Furthermore, the results also suggest that for LTB-based fusion constructs intended for a mucosal vaccine, the flexible linker should be carefully designed. A longer linker may be necessary to avoid steric hindrance and facilitate the proper assembly of the LTB pentamer, which is essential for its function as a mucosal adjuvant.

4 Conclusion

This study characterised the expression dynamics of an LTB-eGFP fusion construct in the *S. cerevisiae* 2805 strain by using the episomal vector yEP-GPD-TER. Using flow cytometry and Western blot, we showed that the expression level peaked significantly earlier than previously assumed. Specifically, expression reached its maximum when cells were initially transferred from solid selective media into the liquid –Ura medium, and subsequently peaked on day 1 in the 300 mL flask culture at 30 °C. The expression level peaked at day 1 before decreasing steadily until day 5. These findings necessitate a strategic revision of the standard practice for *S. cerevisiae* 2805 harvesting for expression analysis, as well as practical applications such as vaccine development and enzyme expression. The results also demonstrated a critical problem associated with the GPGP linker, which is probably too short to avoid spatial crowding between LTB and its fusion partner.

Acknowledgement

This work was supported by the Hue University research grant No DHH2023-01-210. The authors thanked Biotechnology laboratory at the Department of Biology, College of Sciences for providing access to facilities to carry out the experiments.

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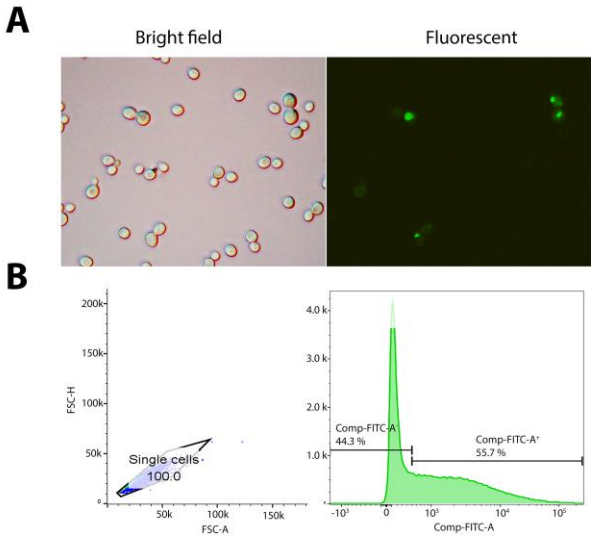

Supplementary Fig. 1. (A) Fluorescent microscope image of yeast expressing LTB-eGFP at 400 time magnification; (B) FACS analysis using FlowJo version 10 showing the single cells population and the histogram of cell populations exhibiting postive and negative fluorescence.