

4 Chapter IV: Expression and secretion studies of GUS^{Eco} and GUS^{Ssp} in yeast

4.1 Introduction

The suitability of GUS^{Eco} as a reporter in the yeast *Saccharomyces cerevisiae* was first demonstrated by Schmitz et al. (1990), showing that yeast does not have endogenous GUS activity, and that expression of GUS^{Eco} does not have adverse effects on yeast growth and viability. Since then, the use of GUS as a reporter has been extended to other yeasts, such as *Schizosaccharomyces pombe* (Pobjecky et al. 1990; Hirt et al. 1990), *Yarrowia lipolytica* (Bauer et al. 1993), *Pichia pastoris* (Sears et al. 1998; Ayra-Pardo et al. 1999), *Kluyveromyces lactis* (Saliola et al. 1999).

In yeast, GUS^{Eco} has been used as a colour selection marker for identifying yeast transformants, or identifying yeast strains in fermentation industries (Petering et al. 1991). It has also been used in various gene fusion experiments to study regulation of gene expression, and protein targeting and transport (Schmitz et al. 1990; Saliola et al. 1999). In addition, GUS^{Eco} has been used as a reporter to study protein-DNA, and protein-protein interactions (Serebriiskii et al. 2000; Setzer et al. 2001).

Protein secretion in yeast is a profound topic for both mechanistic and applied research. Many aspects of the eukaryotic secretory pathway have been elucidated using yeast as a model system, for example, the identification of the translocon (sec61p complex) on the ER membrane (Deshaies & Schekman 1987). Similarly, the large number of thermosensitive *sec* mutants of yeast, which are blocked at different stages of the secretory route, have been very useful in the analysis of various processes along this pathway (Herrero & Sentandreu 1988). The secretion of many recombinant proteins in yeast has also been studied extensively in fermentation industry. For some reviews of general protein secretion in yeast, see Herrero &

Sentandreu (1988); Cleves & Bankaitis (1992).

Unlike in the plant system where secretion of GUS^{Eco} has been studied quite extensively (see section 5.1), no study of GUS^{Eco} secretion in yeast has been recorded. There have been a few reports about the secretion of β -galactosidase, a similar enzyme (Emr et al. 1984; Das et al. 1989).

Invertase and α -factor are two of the most extensively studied secretory proteins in yeast. As a result, their signal peptides are often used to direct foreign proteins into yeast secretion pathways (Moir & Davidow 1991). α -factor is a 13-amino-acid peptide secreted into the culture medium by MAT α cells (Duntze et al. 1970). It has a pre-pro-leader sequence of 83 amino acids. Secreted invertase is a glycoprotein localized in the periplasm, and has a signal sequence of 19 amino acids (Carlson et al. 1983). The cleavage mechanisms of these two signal peptides are different. While cleavage of invertase signal peptide requires only signal peptidase, the processing of α -factor fusions requires two proteases: KEX2 gene product to remove the prosegment and STE13 gene product to remove the initial Glu-Ala-Glu-Ala segment (Kurjan & Herskowitz 1982).

The α -factor prepro peptide has been used for secretion of many proteins, including β -galactosidase (Das et al. 1989). The invertase signal sequence, on the other hand, has been shown incapable of transporting β -galactosidase out of the ER (Emr et al. 1984). The invertase signal sequence, however, can remain functional despite various changes in its exact amino acid sequence (Kaiser et al. 1987). It is also subjected to the simpler canonical signal peptide cleavage mechanism. In our work, yeast modular vectors containing both signal peptides were constructed for secretion studies.

4.2 Materials and Methods

4.2.1 Yeast strain and transformation protocols

Yeast strain InvSc1 (*MAT α* , *his3*, *leu2*, *trp1*, *ura3*) was purchased from Invitrogen (CA, USA). Yeast transformation was done either with the method described by Gietz et al. (1992), or with the EZII-Yeast transformation kit from Bio 101 (CA, USA) according to the manufacturer's recommendation.

4.2.2 DNA constructs

Yeast plasmids are derivatives of the stable and low copy yeast centromeric plasmid (YCp). The plasmids are mitotically stabilized by autonomously replicating sequences (ARS/CEN region), and can be shuttled between yeast and *E. coli*. They contain a functional *ura3* gene for selection of transformants on uracil deficient media when transformed into *ura* defective yeast strains. Cloned genes were under the control of the GAL1 promoter, repressed in media containing glucose, and expressed in media containing galactose.

The invertase and α -factor signal peptides were previously cloned into the vectors by A. Kilian et al. (unpublished). GUS^{Eco} used in this experiment was an N-glycosylation deficient N358Q variant, which is now used as standard GUS^{Eco} reporter in all pCAMBIA vectors. For GUS^{Ssp}, the native protein, and two of its variants, N11Q and C499A, were used.

All the corresponding *gusA* genes were cloned into the BglII/NheI sites of the vector, both with and without the invertase and *mat α* signal peptides. However, fusions to the *mat α* signal peptide were later found to contain incomplete spacer sequence essential for the correct cleavage of the signal peptide, and therefore were not further analysed. All subsequent analyses were focused on the invertase signal peptide.

4.2.3 Media and growth conditions

Yeast was grown at 29°C on standard synthetic media. Media for plates was supplemented with 2% agar or 0.3% phytigel. Selection media was supplemented with 2% glucose, to suppress expression by GAL1 promoter. Expression media contains 1% galactose and 1% raffinose. A complete supplement mixture without uracil (CSM-URA) from Bio 101 (CA, USA) was used for selection of yeast transformed with plasmids containing the uracil auxotrophic marker gene.

4.2.4 Indicator plates

Indicator plates were prepared with expression media containing 100 ug/mL X-glcA. The media (pH about 4.1) was buffered with 100 mM NaPO₄ pH=6.5 and 7.0 as appropriate.

4.2.5 Hot-agarose overlay assay

The assay was modified from Petering et al. (1991). A molten solution of 0.7% agarose containing 100 ug/ml of X-glcA in GUS extraction buffer (50 mM NaPO₄, pH=7.0, 10 mM DTT, 1 mM Na₂EDTA, 0.1% sodium lauryl sarkosyl and 0.1% Triton X-100) was poured over yeast colonies on replicated plates and allowed to set. The plates were incubated at 37°C until blue color is clearly visible.

4.2.6 GUS secretion assay

Yeast secretion assay was developed based on Guarente (1983) and Wilson et al. (1992). Overnight yeast cultures were separated by centrifugation into supernatant and pellet fractions. The pellets were then resuspended in an extraction buffer (50 mM NaPO₄ (pH=7.0), 10 mM DTT, 1 mM Na₂EDTA). Half of the resuspended culture was permeabilized with chloroform and SDS (addition of 40 uL of chloroform and 20 uL 10% SDS to 500 uL of resuspended cells, followed by vigorous vortexing for 10 seconds).

GUS activity was measured in the supernatant (S fraction), the intact-cells suspension, and the permeabilized-cells suspension. In principle, GUS activity measured with intact cells represents the amount of GUS present in the periplasmic space (P fraction), while GUS activity measured with permeabilized cells represents the total amount of GUS present in the cytoplasm and the periplasm (C+P fraction). The secretion index (SI) was calculated as the ratio of secreted (S+P) over total (S+P+C) GUS activity.

4.3 Results and Discussions

4.3.1 Establishment of yeast indicator plate assay

GUS^{Eco} (N358Q variant) and GUS^{Ssp}, with or without the invertase signal peptide, were expressed in yeast *Saccharomyces cerevisiae* strain InvSc1 (figure 4.1).

It was reasoned that if yeast cells are unable to take up GUS substrates, then a plate assay for GUS secretion could be developed, where GUS-secreting yeast colonies would be blue, and non-secreting colonies would be white. However, all transformed yeast cells, even those with signal peptide, remained white on expression media containing X-glcA (figure 4.2).

At that time, it was postulated that both enzymes failed to secrete with the invertase signal peptide, or that the secreted forms of the enzymes were not active. Therefore, the project was expanded considerably to investigate factor(s) that could potentially interfere with secretion or activity of the enzymes, for example, 1) the use of tunicamycin, a drug used to inhibit the synthesis of all N-linked oligosaccharides; or 2) the use of acetanilide, an organophosphate that could dissociate possible egasyn-like interactions between GUS and other ER-resident proteins. However, the results of these experiments became rather irrelevant, and therefore are not presented here, when it was later found out that the white yeast colonies were, in fact, false negatives (see below).

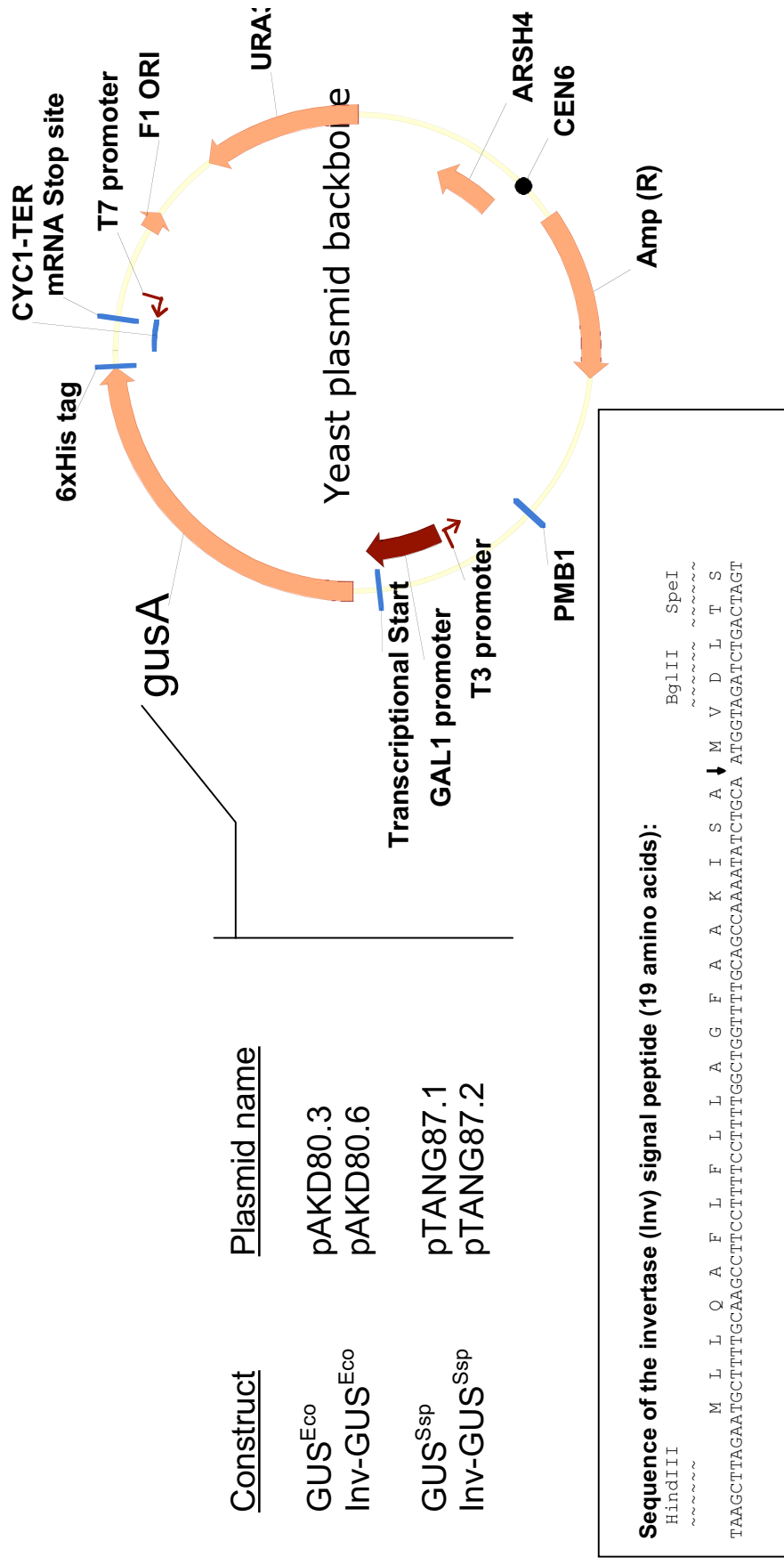


Figure 4.1. DNA constructs used in yeast expression and secretion experiments. Both GUS^{Eco} and GUS^{Ssp}, with and without the invertase (Inv) signal peptide, were constructed. All constructs were based on pYES2 backbone, with the corresponding genes driven by GAL 1 promoter.

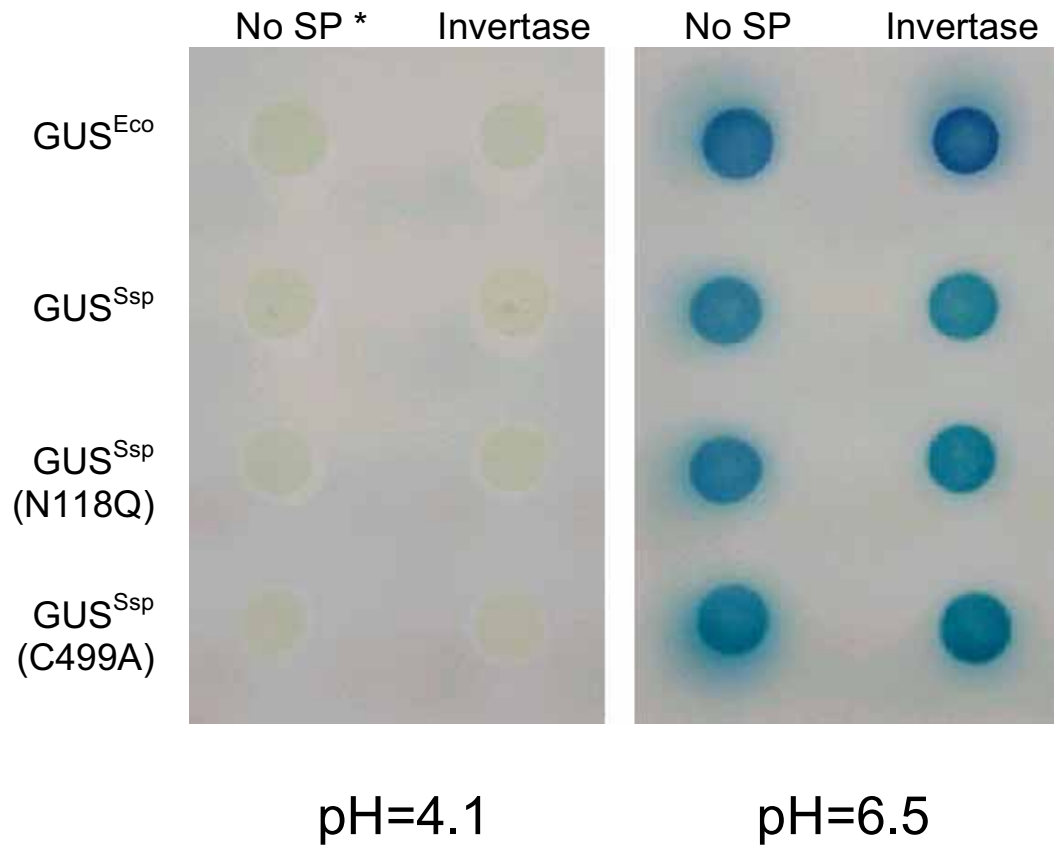


Figure 4.2. Establishment of yeast indicator plate assay. On expression media containing X-glcA (100 ug/mL), all constructs produce white yeast if plated on non-buffered media (pH=4.1), and blue yeast if plated on buffered media (pH=6.5).

* SP = signal peptide

A hot-agarose overlay assay adapted from Petering et al. (1991) was routinely used for quick confirmation of GUS activity in transformed yeast. With this assay, blue color was detected for all constructs (figure 4.2). If the GUS extraction buffer used in the assay was replaced by water, then no blue color was detected. Various assays using “drop-out” GUS extraction buffer were therefore carried out to determine which element(s) in the buffer was responsible: phosphate buffer (pH=7.0), reducing agent, or detergents. It was clear that blue color was only observed if the overlay agarose was buffered (data not shown). We then further confirmed that the hot-agarose overlay assay was in fact not necessary, since yeast cells expressing GUS were readily blue on buffered media (figure 4.2). Phosphate buffer at pH=6.5 was found to give very good color intensity without affecting yeast growth, and solubility of the components in the growth media.

It has been known that yeast cells expressing β -galactosidase are white on media containing X-gal (Rose & Botstein 1983). Similar observation was also known for yeast expressing GUS on media containing X-glcA (Schmitz et al. 1990). Although it was mentioned in these early works that the problem was due to the acidic pH of the medium, various later assay methods somehow were focused more on permeabilization or lysis of yeast cells (using hot agarose, chloroform, or detergents, for example) to obtain adequate blue color reproduction on the indicator plates (Hirt 1991; Petering et al. 1991; Duttweiler 1996).

Our results clearly confirm that the acidic pH of the media is the cause for yeast cells being white on media containing X-glcA, as correctly noticed by Rose & Botstein (1983) and Schmitz et al. (1990). The low pH, about 4.1 in our case, could have inhibited GUS activity, and/or prevented the formation of the blue color product (see Guivarc'h et al. (1996) for details of the reactions). The latter case would be easily confirmed by the use of a different substrate whose detection is not affected by acidic pH (note that all the current common GUS substrates only give good signal detection in neutral or alkaline environment). The answer for this would be applicable for β -galactosidase

assay as well, since X-glcA and X-gal share the same reactions to form the blue color precipitate.

By simply buffering the pH of the media, the indicator plate assay allows *in vivo* detection of GUS expressing yeast colonies, omitting the need for inconvenient work-around solutions proposed by previous authors (Hirt 1991; Petering et al. 1991; Duttweiler 1996; Schneider et al. 1996). Such *in vivo* detection will be very useful, especially for the screening of yeast transformants and GUS-tagged yeast strains. It should be noted that the assay method has not been extended to other yeast strains and promoters, etc. It is conceivable that indicator plate assay methods used by other authors would be better in some circumstances, since cell permeabilization or lysis would significantly increase enzyme-substrate contact.

Given that a yeast colony took several days to become clearly visible, the amount of cellular leakage due to cell death could be significant. Therefore, it was not very surprising that yeast colonies expressing non-secreted GUS were blue. However, this means that a simple blue/white screening for GUS secretion in yeast is not possible. Instead, a quantitative assay for GUS secretion was developed (section 4.2.6).

4.3.2 Both GUS^{Eco} and GUS^{Ssp} are efficiently secreted with the invertase signal sequence

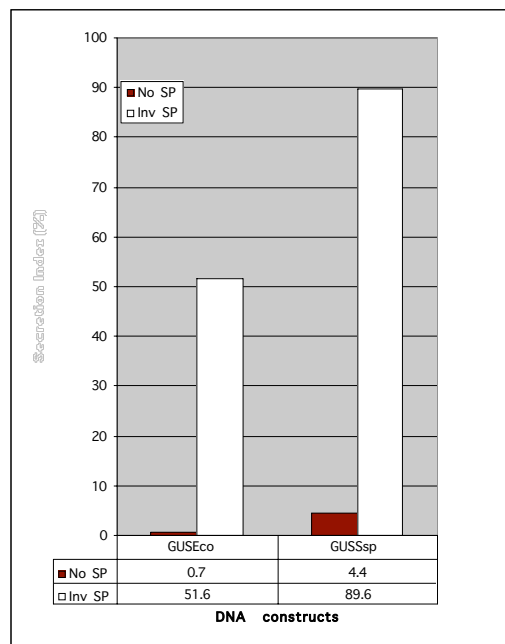
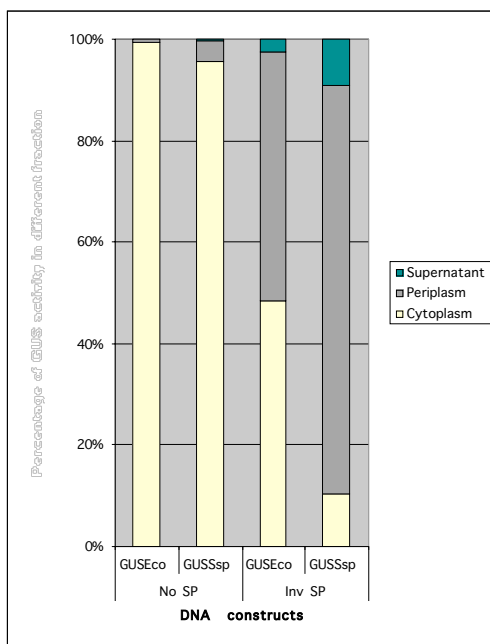
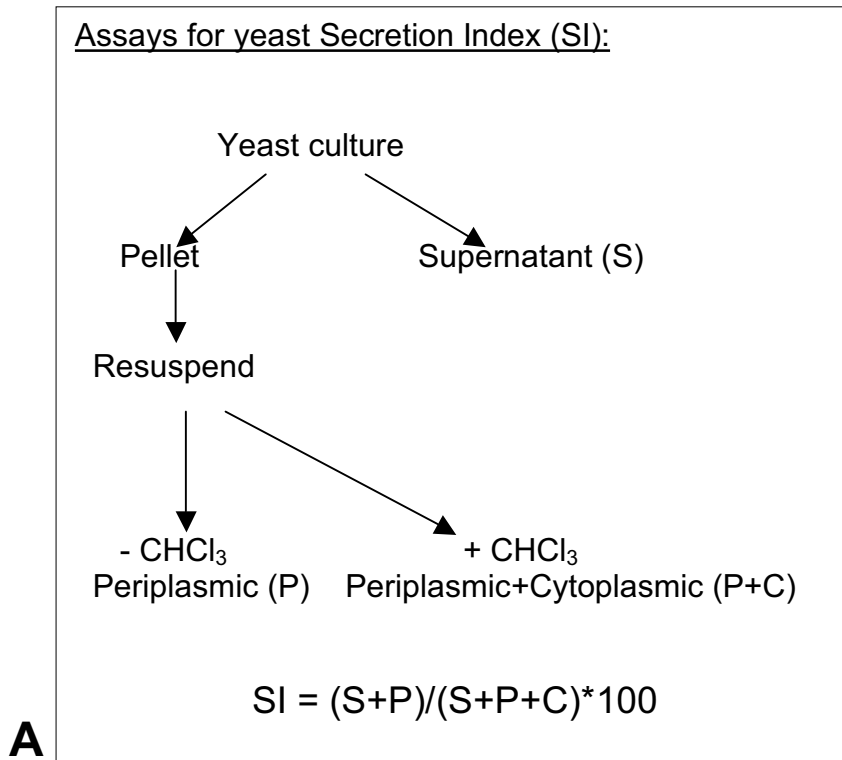
A quantitative assay for GUS secretion was developed. In this assay, GUS activity in various yeast compartments was determined, and a secretion index, i.e. the ratio of secreted over total GUS activity, established (figure 4.3.A).

As seen in figures 4.3.B and 4.3.C, both enzymes GUS^{Eco} (N358Q variant) and GUS^{Ssp} were efficiently secreted. Without signal peptide, the majority of both enzymes was located in the cytoplasm. With the invertase signal peptide, 52% of GUS^{Eco} and 90% of GUS^{Ssp} were secreted and located mainly in the periplasm.

The fact that the N358Q variant of GUS^{Eco} can be quite efficiently secreted in yeast is a very interesting and unanticipated result. To our knowledge, this is the first report on secretion studies of GUS^{Eco} in yeast. Secretion of β -galactosidase in yeast using the α -factor leader sequence was previously reported (Das et al. 1989). In plants, however, there have been various secretion studies of GUS^{Eco} (see section 5.1), including those reported in this thesis (chapter V and VI), all concluded that the enzyme is not secreted when engineered.

These results clearly emphasize the differences in the secretion machineries that operate in yeast and plants – the differences that have become more and more recognized (Bassham & Raikhel 2000). In this regard, the use of GUS^{Eco} as a marker for further investigations on secretion machineries of yeast and plants should be a very interesting line of future research.

The secretion index for GUS^{Ssp} was higher than that of GUS^{Eco} (90% and 52%, respectively). This result is expected, considering that GUS^{Ssp} is a naturally secreted enzyme in *Staphylococcus* sp., while the GUS^{Eco} is a natural cytosolic enzyme in *E. coli*.



B **C**

Figure 4.3. Secretion of GUS^{Eco} and GUS^{Ssp} in yeast

- Schematic representation of yeast secretion index assay.
- Distribution of GUS activity in different cellular fractions. Without signal peptide, both GUS^{Eco} and GUS^{Ssp} were localized exclusively in the cytoplasm. With the invertase signal peptide, both proteins were mainly localized in the periplasm.
- Secretion Index of GUS^{Eco} and GUS^{Ssp}.

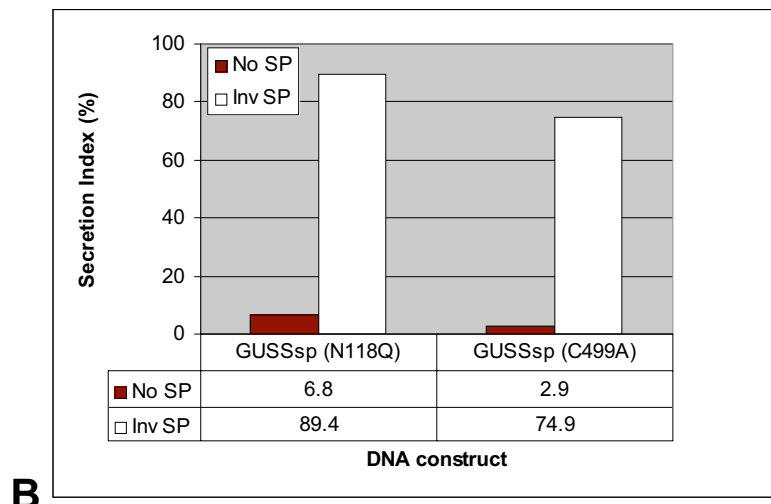
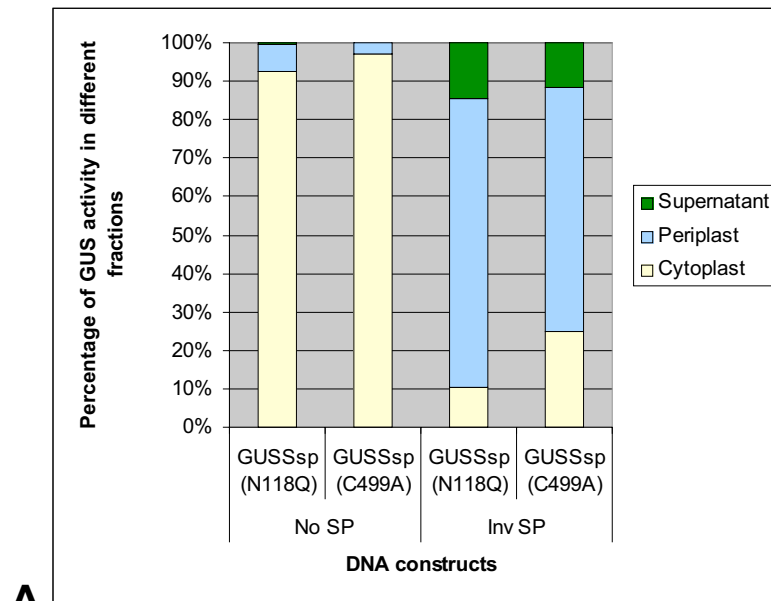


Figure 4.4. Secretion of N118Q and C499A variants of GUS^{Ssp} in yeast.

- A. Distribution of GUS activity in different cellular fractions. Without signal peptide, both variants were localized exclusively in the cytoplasm. With the invertase signal peptide, both proteins were mainly localized in the periplasm.
- B. Secretion Index of N118Q and C499A variants of GUS^{Ssp}.

4.3.3 C499A and N118Q variants of GUS^{Ssp} are also efficiently secreted

GUS^{Ssp} has one potential N-glycosylation site (N118) and one cysteine residue (C499). Therefore, if the protein is targeted into the secretion pathway, it could be subjected to modifications such as the addition of an N-glycan, or the formation of disulfide linkage. These modifications could potentially interfere with GUS^{Ssp} secretion and/or activity.

To investigate such possibilities, variants of GUS^{Ssp} without the potential N-glycosylation site (N118Q), and without the cysteine amino acid (C499A), were expressed in yeast, either with or without the invertase signal peptide. All constructs were able to produce blue yeast colonies on X-glcA-containing expression media buffered to pH=6.5 (figure 4.2).

Quantitative assays for GUS secretion showed that, similar to the wildtype GUS^{Ssp}, these two variants were also efficiently secreted, with secretion index of 89% and 75% for N118Q and C499A, respectively (figure 4.4).

It should be noted that construction and testing of these GUS^{Ssp} variants in yeast was highly relevant when the appropriate yeast indicator plate assay has not been established, causing wrong interpretations about secretion of GUS^{Eco} and wildtype GUS^{Ssp} (section 4.3.1). Considering that the N358Q variant of GUS^{Eco} (with nine cysteines and no N-glycosylation site), and the wildtype GUS^{Ssp} (with one cysteine and one potential N-glycosylation site) are both efficiently secreted in yeast (section 4.3.2), the efficient secretion of N118Q and C499A variants of GUS^{Ssp} is an anticipated result.

With the data presented here and in section 4.3.2, it is postulated that at least in yeast, secretion of GUS^{Eco} and GUS^{Ssp} is not distinctly affected by potential issues, such as cysteine-mediated crosslinking and subsequent retention, or N-glycosylation-mediated mistargeting. Many interesting and important questions, however, remain to be answered. For example, we do not know if cleavage of signal peptide, or formation of

disulfide bonds or N-glycans – on relevant variants of GUS^{Eco} and GUS^{Ssp}, takes place, as these proteins are *en route* to their destination. Confirmation of the cleavage of the invertase signal peptide using N-terminal sequencing, and biochemical analysis of potential post-translational modifications, are important remaining experiments. The availability of GUS^{Eco} and GUS^{Ssp} variants, and of antibodies and methods for immunodetection of various GUSes (chapter III), would facilitate and speed up such studies.

A significant amount of time was spent troubleshooting and establishing an effective indicator plate assay, and assay for the determination of secretion index. These protocols will be useful for future yeast secretion studies of various GUSes from other sources, and can be adapted to secretion studies of other proteins in yeast as well.

