Monitoring chitin deposition during septum assembly in budding yeast.

Arcones, Irene and Roncero Cesar*.

IBFG and Department of Microbiology and Genetics. CSIC/USAL

C/Zacarias Gonzalez 1. 37007 Salamanca (Spain).

*crm@usal.es

Running title

Chitin synthesis during cytokinesis

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SUMMARY

The synthesis of the septum is a critical step during cytokinesis in the fungal cell. Moreover, in *Saccharomyces cerevisiae* septum assembly depends mostly on the proper synthesis and deposition of chitin and, accordingly, on the timely regulation of chitin synthases. In this manuscript we will see how to follow chitin synthesis by two complementary approaches: monitoring chitin deposition *in vivo* at the septum by calcofluor staining and fluorescence microscopy, and measuring the chitin synthase activities responsible for this synthesis.

1. INTRODUCTION

In fungal cells the process of cytokinesis is necessarily coordinated with the synthesis and assembly of new cell wall (CW) components into a septum, which physically separates mother and daughter cells. *S.cerevisiae* has proved to be a fairly straightforward model to study such coordination because septum synthesis mostly depends on a single CW component, chitin [1]. This chitin, later linked to other CW components [2], assembles into a primary septum (PS) that physically separates mother and daughter cells at the time of cytokinesis. The PS, together with the rest of chitin and other cell wall components, forms a conspicuous structure, the bud scar, which remains in the mother cells after cell separation. This relies on the enzymatic action of chitinases, whose exclusive expression from the daughter cell side is directed by the cell cycle-controlled RAM network (review in [3]).

In *S.cerevisiae* chitin synthesis depends on three different chitin synthase (CS) activities, whose coordinated action results in proper septum formation. CSIII synthesizes the majority of cellular chitin [4,5], this forming a ring that performs scaffold functions in the assembly of the septum. Although not essential, the chitin ring has a critical homeostatic function against any alteration in septum assembly [6,7]. CSII performs an essential function in the synthesis of the primary septum (PS) and its activity has been linked directly to the proper contraction of the actomyosin ring [8,9]. This coordination between PS synthesis and AMR contraction is probably conserved across fungi. The additional CSI activity seems to be involved in repair functions at the time of cytokinesis by synthesizing minutes amount of chitin, but its exact function remains poorly understood [10].

Chitin is an extremely insoluble polymer that needs to be synthesized directly into the periplasmic space from the plasma membrane (PM) [11]. Accordingly, CS activity is tightly regulated, mostly at the postranslational level [12]. The three Chs1, Chs2 and Chs3 chitin synthase proteins are transported to the yeast PM and activated in a timely fashion, and hence

several different methods need to be combined in order to study chitin synthesis regulation. Chs2 and Chs3 localize to the neck during cytokinesis, but the protocols for the visualization of proteins during cytokinesis are extensively covered in the different chapters of this book and will not be discussed here. However, the precise localization of these proteins only gives a partial view of the story since it does not necessarily reflect the levels of their activities. In order to address this issue, here we shall describe two different experimental approaches to monitor chitin synthesis, either *in vivo* or *in vitro*, by means of determining chitin levels or chitin synthase activity respectively.

It should be recalled that in *S.cerevisiae* three different CS activities with different functions coexist. CSIII is responsible for the synthesis of more that 90% of the cellular chitin *in vivo*, while *in vitro* CSI activity accounts for more than 90% of the enzymatic activity measured, despite its almost null contribution to chitin synthesis. The protocols developed over the years will allow CS redundancy to be circumvented, providing consistent experimental results *in vivo* and *in vitro*.

2. MATERIALS

All reagents are dissolved in water.

1. Calcofluor White MR2 (10 mg/ml)[1](Fluorescent brightener 28, Sigma) (see Note 1)
2. Formaldehyde 16% (Polysciences INC)
3. Glass beads (0.45-0.6 mm, Sigma)
4. 50 mM Tris-HCl pH 7.5
5. 50 mM Tris-HCl pH 8.0
6. 50 mM Tris-HCl pH 6.5.
7. 0.8 M N-Acetylglucosamine (Sigma)
8. Co^{2+} 50mM ($CoCl_2$, Sigma)
9. Ni^{2+} 50 mM ($(CH_3COO)_2Ni\text{-}4H_2O$, Merck)
10. Mg^{2+} 40 mM ($MgCl_2$, Sigma)
11. Trypsin (10 mg/ml) (Sigma)
12. Trypsin Inhibitor (10mg/ml) (Sigma)
13. Trichloracetic Acid (TCA) 10% (Merck)
14. Borosilicate tubes (10x70 mm)
15. 25mm Glass microfiber filters GF/C (Whatman)

16. [^{14}C] 10mM UDP-N-Acetylglucosamine (Substrate). For preparation of 1ml of substrate: 200µl 50mM UDP-N-Acetylglucosamine (Sigma), 72µl [^{14}C] UDP-N-Acetylglucosamine (Amershan 235mCi/mmol) and 728µl water. The radioactive substrate should contain around 20.000 cpm/5µl (400 cpm/nmol), however precise calibration should be performed in each substrate batch by measuring cpm levels directly in 5 µl of substrate.
17. Yeast growth media:
    YEPD medium (1% yeast extract, 2% peptone, 2% glucose/dextrose)
    SD medium (0.67% Yeast Nitrogen Base w/o aminoacids, 2% glucose, required aminoacids)
18. Superspeed centrifuge. (Sorvall RC5 or equivalent).
19. Filtration tower equipment.
20. Scintillation counter.
21. Fluorescence microscope with a UV filter (Chroma 4900-ET-DAPI or equivalent).

3. METHODS

3.1. QUALITATIVE DETERMINATION OF CHITIN SYNTHESIS BY CALCOFLUOR STAINING.

Calcofluor binds rather specifically to β(1-4) glucans, and hence over the years it has been used for the specific staining of chitin along the yeast and fungal cell walls [13,14]. In *S.cerevisiae*, calcofluor can be used directly on fixed cells, staining pre-existing chitin [13]. However, if added to growing cells the dye will bind much more efficiently to nascent chitin chains, providing much stronger staining at the sites where chitin is being actively synthesized [14]. The use of both protocols provides complementary answers to obtain a more detailed picture of the *in vivo* chitin synthesis occurring in a specific strain (see Figure 1).

The two protocols described will mainly stain the chitin ring and any additional accumulation of chitin formed by CSIII activity [4,15], thereby masking the staining of chitin accumulated at the primary septa which is synthesized by CSII. In order to observe PS staining directly it is necessary to use strains lacking CSIII activity [16] or inhibiting this activity by nikkomycin [6]. The use of *chs3*Δ cells allows the precise study of PS formation during cytokinesis, including potential alterations caused by the de-regulation of CSII (Figure 1; [17]; Sanchez-Diaz and Roncero unpublished observations).

3.1.1. Calcofluor staining on fixed cells:

1- Grow cells in your favourite medium to early logarithmic phase (1-2 x 10^7 cells/ml).

2- Take 2ml of culture and fix cells in by adding 500µl of 16% formaldehyde directly. Incubate at room temperature (RT) for 30 minutes in a roller.

4- Collect cells by centrifugation at 3500xg for 2 minutes and wash them with 2 ml of water.

5- Resuspend them in 0.5 ml of 50 µg/ml of calcofluor in water; then incubate for 5 minutes in a roller.

6- Spin cells down and wash cells with 2 ml of water. Resuspend them in 100 µl of water. Cells can be maintained at 4ºC in the darkness.

7- Observe cells under the fluorescence microscope using the appropriate UV filter.

3.1.2.Calcofluor staining on growing cells (life cell staining):

1- Add calcofluor at a final concentration of 50µg/ml to a YEPD culture of yeast cells growing in the early logarithmic phase (see Note 2).

2- Incubate for an additional 90 minutes and concentrate cells by centrifugation if necessary. Observe cells directly under the microscope with the appropriate UV filter.

3.2.3. Imagining processes.

Calcofluor staining can be used as a semi-quantitative way of measuring chitin synthesis levels by carefully adjusting the microscopic/imaging processes. To do so:

1- Short exposure times should be used, since calcofluor provides a very strong degree of staining (see Note 3).

2- Use identical exposure times for all images, regardless of the staining intensity.

3- Process all images obtained in parallel in order to preserve the relative intensity values.

4- Avoid automatic machine set-ups for exposure times or brightness/contrast levels.

The images shown in Figure 1 compare both experimental protocols for staining WT and *chs3*Δ cells. Note the different level of staining in fixed cells (A) or after life cell staining (B), even after the 20-fold longer exposure times in the fixed-cell images. The images highlight the alterations in chitin deposition after the blockade of endocytosis, and how it affects both the assembly of the chitin ring and the PS.

3.2.- MEASUREMENT OF CHITIN SYNTHASE ACTIVITY.

Chitin synthase activity in yeast was described many years ago [18] and soon after it was recognized as an enzymatic activity associated with cellular membranes [11]. Accordingly, CS determination should include detailed protocols for the isolation of cellular membranes. However, *S.cerevisiae* contains three different CS activities encoded by three different genes [12] and the precise measurements of CS awaited the construction of individual and double mutants in the CS genes in order to design specific protocols for the assay of the different CS activities [19]. The protocols described here are based in the seminal work carried out at Enrico Cabib´s Lab and rely in the use of different cations and pHs and in the zymogenic nature of CSI and CSII, but not of CSIII, activity.

3.2.1. Preparation of membrane extracts.

Membranes were prepared essentially as described by Orlean [20] with slight modifications (see Note 4).

Samples and reagents should be maintained continuously on ice.

1- Grow a 200-300 ml culture at 28º C to a cell density of 1.5-2.0 10^7cells/ml. Chill the culture down on ice.
2- Pellet cells by centrifuging them for 7 minutes at 6000xg at 4ºC. Discard the supernatant.
3- Wash cells with 20ml of cold 50 mM Tris-HCl pH 7.5. Centrifuge for 7 minutes at 5000rpm. Discard the supernatant.
4- Resuspend cells in 200-500µl of 50 mM Tris-HCl pH 7.5 and transfer them to 1.5 ml microcentifuge tubes..
5- Add glass beads (0.45–0.6 mm) until the liquid is almost covered.
6- Break up the cells. Cells can be broken by different protocols. In a Fast-Prep (MPBio), using 15-second pulses at a speed of 5.5. Repeat three times, placing the tubes in ice after each interval. By Vortex, using glass tubes. Typically 8 pulses of 30 seconds at maximum speed. The tubes should be cooled down for 30 seconds between vortex pulses. Alternatively, a Braun homogenizer or a French Press can be used to break yeast cells as long as the temperature of the samples is maintained around 4ºC.

Regardless of the method used, the degree of cell breakage should be checked by light microscopy.

7- Make a hole in the bottom of each microcentrifuge tube with a hot needle and place the tubes on top of new tubes. Spin for 15 seconds at 6000xg in a benchtop centrifuge at 4ºC in order to harvest cell extracts

8- Dilute cell extracts up to 8 ml of 50mM Tris-HCl pH 7.5. Centrifuge for 7 minutes at 6000xg.

9- Transfer the supernatant to new centrifuge bottles. Centrifuge for 35 minutes at 40000xg maintaining, temperature between 0-6ºC Discard all the supernatant.

10- Resuspend the membranes in 200-800 μl of cold 50 mM Tris-HCl pH 7.5, 33% glycerol with the aid of a glass stick. Absolute homogenization is required in order to obtain reproducible results. Measure protein concentration by the Bradfford method. The final extract should contain between 7-12 mg/ml of protein.

Freeze extracts at -80ºC or proceed with chitin synthase reactions.

3.2.2. Chitin synthase assay.

The general principle for CS measurement is based on the incorporation of a fully soluble radioactive precursor, [^{14}C] UDP-N-Acetylglucosamine, into a rather insoluble product: [^{14}C] chitin. Selective precipitation of chitin will allow the amount of radioactivity incorporated during the reaction time to be detected (see Note 5).

Owing to the *in vitro* zymogenic nature of some CSs, measurements are always performed under two different conditions, with and without trypsin, in order to measure basal and total CS activity respectively. The proteolytic activation of CS by trypsin was reported many years ago [21].

Reactions are always performed in 50μl mixtures placed in 10x70 mm borosilicate tubes for effective temperature transfer. Mixtures are maintained continuously on ice just after the incubation period, performed at 30ºC in a water bath. All reactions, including blanks, should be performed in duplicate. The reaction conditions are based directly on the work of Cabib´s Lab [19], but adapted over the years. We present three different chitin synthase assays to determine CSIII, CSII and CSI.

CSIII determination.

This is based on the fact that CSIII activity is maximum at pH 8.0, using Co^{2+} as the activating cation. In addition, CSIII activity is not inhibited by Ni^{2+}. Thus, with these conditions only CSIII activity should be measured, since the potential contaminant CSII will be inhibited by the addition of Ni^{2+}.

Reaction A, without trypsin (Basal activity)

Each tube should contain:

3µl of 50 mM TrisHCl pH 8.0.
5µl of 50 mM Co^{2+} ($CoCl_2$)
2µl of 0.8 M N-Acetylglucosamine
5µl of 10mM [^{14}C] UDP-N-Acetylglucosamine.
5µl of 50mM Ni^{2+} ($(CH_3COO)_2Ni\text{-}4H_2O$)
10µl of water.
20µl of membrane extracts (see Section 3.2.1.).

Incubate 90 minutes at 30ºC. Stop the reaction adding 2 ml of TCA.

Reaction B, with trypsin (Total activity):

Reaction is performed in two consecutive steps. You should need to prepare three different tubes (reaction **B.1, B.2 and B.3**) per sample containing:

3µl of 50 mM TrisHCl pH=8.0.
5µl of 50 mM Co^{2+} ($CoCl_2$)
5µl of 10mM [^{14} C] UDP-N-Acetylglucosamine.
6µl of H_2O.
5µl of 50mM Ni^{2+} ($(CH_3COO)_2Ni\text{-}4H_2O$) .
20µl of membrane extracts.

Add trypsin as follow:
Reaction **B.1)** 2µl of 1mg/ml Trypsin.
Reaction **B.2)** 2µl of 2mg/ml Trypsin.
Reaction **B.3)** 2µl of 3mg/ml Trypsin.

Incubate tubes for 15 minutes at 30ºC.

Add to each tube:
Reaction **B.1)** 2µl of 1.5mg/ml Trypsin Inhibitor.
Reaction **B.2)** 2µl of 3mg/ml Trypsin Inhibitor.
Reaction **B.3)** 2µl of 4.5mg/ml Trypsin Inhibitor.
Finally, 2µl of 0.8M N-Acetylglucosamine to all of them.

Incubate 90 minutes at 30ºC. Stop the reaction adding 2 ml of TCA.

CSII determination.

There are no specific reaction conditions for measuring CSII alone, and hence the joint CSIII/CSII activity is measured first, as indicated below, and CSII activity is determined by subtracting the CSIII activity measured as describe above from this value.

CSIII/CSII activity is measured in exactly the same way as CSIII but omitting Ni^{2+} in the assays and adding the corresponding volume of water. When using any strain devoid of CSIII activity, such as *chs3*Δ mutants, these reaction conditions will provide CSII activity directly.

Reaction A, without trypsin (Basal activity)

Each tube should contain:

3µl of 50 mM TrisHCl pH 8.0.
5 µl of 50 mM Co^{2+} ($CoCl_2$).
2 µl of 0.8 M N-Acetylglucosamine
5 µl of 10mM [^{14}C] UDP-N-Acetylglucosamine.
15µl of water.
20 µl of membrane extracts.

Incubate for 90 minutes at 30ºC. Stop the reaction by adding 2 ml of TCA.

Reaction B, with trypsin (Total activity):

3µl of 50 mM TrisHCl pH 8.0.
5 µl of 50 mM Co^{2+} ($CoCl_2$).
5 µl of 10mM [^{14} C] UDP-N-Acetylglucosamine.
11µl of water.
20 µl membrane extracts.

Add trypsin as follow:
Reaction **B.1)** 2µl of 1mg/ml Trypsin.
Reaction **B.2)** 2µl of 2mg/ml Trypsin.
Reaction **B.3)** 2µl of 3mg/ml Trypsin.

Incubate tubes for15 minutes at 30ºC.

Add to each tube:
Reaction **B.1)** 2µl of 1.5mg/ml Trypsin Inhibitor.
Reaction **B.2)** 2µl of 3mg/ml Trypsin Inhibitor.
Reaction **B.3)** 2µl of 4.5mg/ml Trypsin Inhibitor.
Finally, 2µl of 0.8M N-Acetylglucosamine to all of them.

Incubate 90 minutes at 30ºC. Stop the reaction adding 2 ml of TCA

CSI determination.

The protocol is identical to that described above but using different cations and buffers. Note that the differences are underlined.

Reaction A, without trypsin (Basal activity)

Each tube should contain:

3µl of 50 mM TrisHCl pH 6.5.
5 µ of 40 mM Mg^{2+} ($MgCl_2$)
2 µl of 0.8 M N-Acetylglucosamine
5 µl of 10 mM [^{14} C] UDP-N-Acetylglucosamine.
15µl of water.
20 µl of membrane extracts.

Incubate for 30 minutes at 30ºC. Stop the reaction by adding 2 ml of TCA

Reaction B, with trypsin (Total activity):

3µl of 50 mM TrisHCl pH 6.5.
5 µl of 40 mM Mg^{2+} ($MgCl_2$).
5 µl of 10 mM [^{14} C] UDP-N-Acetylglucosamine.
11µl of water.
20 µl of membrane extracts.

Add trypsin as follow:
Reaction **B.1)** 2µl of 1mg/ml Trypsin.
Reaction **B.2)** 2µl of 2mg/ml Trypsin.
Reaction **B.3)** 2µl of 3mg/ml Trypsin.

Incubate tubes for15 minutes at 30ºC.

Add to each tube:
Reaction **B.1)** 2µl of 1.5mg/ml Trypsin Inhibitor.
Reaction **B.2)** 2µl 3mg/ml Trypsin Inhibitor.
Reaction **B.3)** 2µl 4.5mg/ml Trypsin Inhibitor.
Finally, 2µl of 0.8M N-Acetylglucosamine to all of them.

Incubate for 30 minutes at 30ºC. Stop the reaction by adding 2 ml of TCA

3.2.3. Filtration procedures

Incorporated radioactivity is measured after filtration of TCA precipitates into glass fibre filters. To do so the content of each tube containing the CS reaction is poured on a 25 mm GF/C filter placed in a filter tower equipment (see Figure 2). After application of vacuum, the filter is washed twice with 2 ml of 10% TCA and once with 100% ethanol. Filters are later retired from the tower and dried at RT before adding scintillation liquid for radioactivity measurements in a scintillation counter.

For the final calculation of CS activity, the cpm counts determined as indicated are transformed into nanomoles of N-Acetylglucosamine incorporated into the insoluble material, after which CS activity is usually expressed in mU (incorporated nmoles/hour/mg protein). For a standard CSIII reaction using 20µl of membrane and 90 minutes of incubation you can calculate CS activity as indicated in Figure 3:

An alternative method for CS measurement. A non-radioactive protocol has been described for CS measurement [22]. This protocol has been used in HTS assays [23], but its use in the characterization of CS has not been extensively tested. In our experience it does not work as well as the radioactive method described here, lacking sufficient sensitivity for use in the measurement of the CSII activity involved in PS formation (Foltman and Sanchez-Diaz, unpublished results).

Multiple adaptations of the different protocols described here have been used to measure chitin synthase activity in different fungi such as *Candida albicans* or *Aspergillus sp*. However, to date the multiplicity of chitin synthase genes present in fungi has made it extremely difficult to link *in vitro* CS activities to chitin synthesis *in vivo* in any organism other than *S.cerevisiae*.

The physiological relevance of CS measurements.

The *in vitro* zymogenic nature of some CS makes it difficult to interpret the levels of CS activities in physiological terms. It is unclear whether basal or total activities correspond to the real action of the CS in the cell. This interpretation has proved to be even more complicated since the physiological activator/s of the different CSs has not been defined.

CSIII is only modestly zymogenic, because trypsin treatment only increases activity by a factor of 1.7. Moreover, increases in the levels of Chs3 at the PM have been correlated with higher basal activities and increases in chitin synthesis [15,24]. Therefore it can be assumed that CSIII basal activity correspond with the actual levels of chitin synthase in vivo.

CSII is clearly zymogenic, and trypsin treatment increases CSII activity several-fold. Only very recently has it been possible to link the increase in CS basal activity to a higher *in vivo* functionality of CSII [25,17]. Although the molecular mechanism that lead to CSII remains largely unknown, these results strongly support a direct relationship between CSII basal activity and the *in vivo* synthesis of chitin at the PS, expanding our understanding of how CSII might be regulated at the time of cytokinesis.

NOTES:

1. Calcofluor preparation: Calcofluor is prepared as a stock solution at 10 mg/ml concentration in water. Only the salt form of calcofluor is soluble in water, and hence depending on the manufacturer small amounts of NaOH should be added to the solution for complete dissolution. Calcofluor should be sterilized by filtration though a 0.22μm filter. Once clear, the solution can be maintained at 4ºC in the dark for months. Sigma provides calcofluor under the name of fluorescent brightener 28. This compound is fully soluble in water at 10 mg/ml and does not require the addition of NaOH.

2. Life cell staining with Calcofluor: Growth of yeast cells in YEPD medium is preferred for life staining because a much more uniform staining of the cells can be achieved. However, if selective media is required , the SD medium should be buffered with 50mM Phthalate, pH 6.3, in order to prevent calcofluor precipitation at acidic pH. Longer or shorter incubation times with calcofluor can be used if desired. Life cell staining provides a much higher levels of fluorescence than fixed staining, highlighting the sites of active chitin synthesis (see Figure 1).

3. Imaging procedures: Be careful during images capturing, image overexposure is relatively frequent in un-experienced observers. If some of the images are overexposed you should not be able to compare relative intensity values. Exposure times should be adjusted depending in the microscopy equipment used.

4. Measurement of CS activity: The preparation of membrane extracts is the most critical step in chitin synthase measurement since partial inactivation of the samples may occur during processing. It is therefore recommended to include control cells in each experiment as a reference. Cells extracts are stable for weeks If maintained frozen at -80ºC.

Alternative methods for preparation of crude cell extracts have been used for the measurement of CS activities. These methods rely on the direct permeabilization of cells, either by detergent treatment [26] or osmotic shock [27]. While these methods allow efficient measurement of CS activities, they would alter CS properties either directly or by altering CS

intracellular distribution [28,15]. Accordingly, permeabilized cells should be used with caution in order to translate the *in vitro* results to cell physiology.

Researchers should be aware that CS activities change significantly, depending on the different phases of cellular growth and on the growth medium used. Therefore, it must always be assured that cells growing in the early logarithmic phase are used. The use of synchronized cultures in not practical owing to the large amount of cells required in extract.

Changes in the growth medium would alter not only CS activity but also, indirectly, other parameters able to influence CS measurement. For example, growth in galactose medium alters yeast cell wall composition, rendering cells highly resistant to mechanical rupture. Therefore, extended pulse times are required in either the vortexing step or the fast-prep breakage protocols.

5. Chitin synthase assay: The protocol described make use of a radioactive substrate, therefore you should be aware of the security rules regarding the use of this type of compounds, including not only the protection of the user but also the proper disposal of contaminated material.

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FIGURES

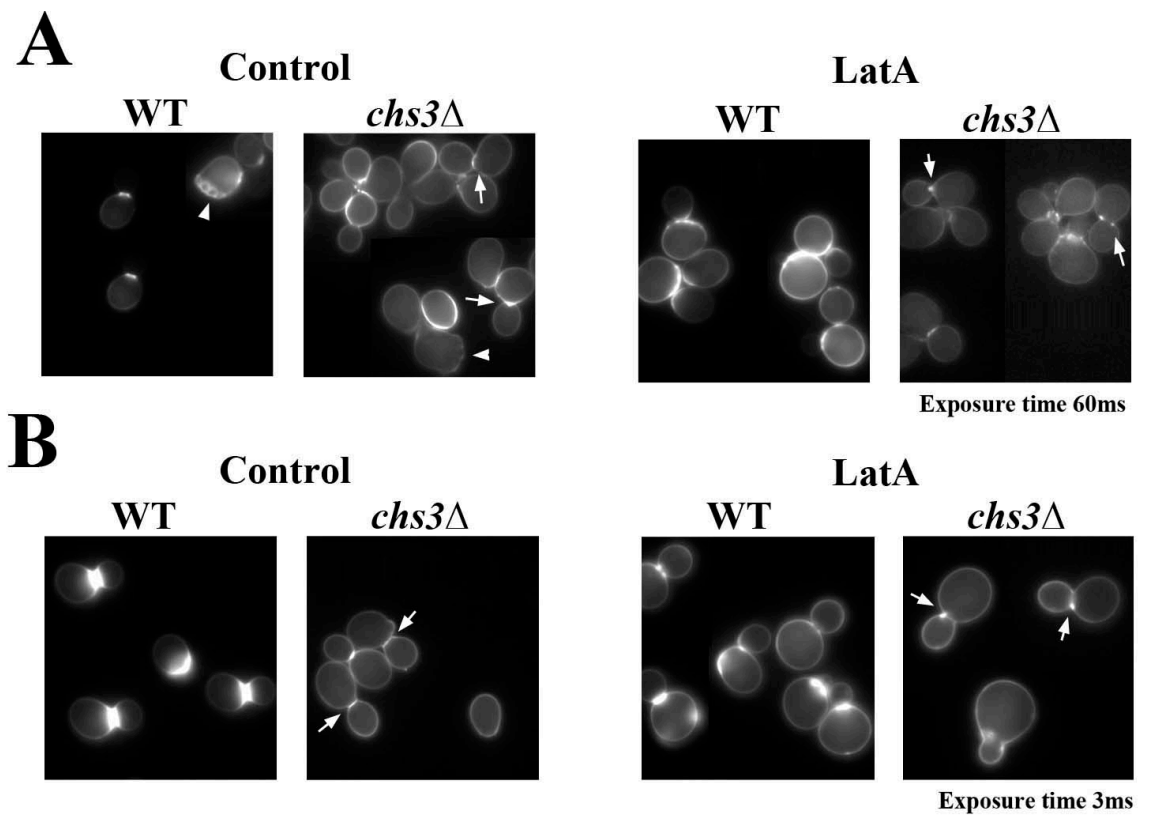

Figure 1. Calcofluor staining of the indicated strains performed following the experimental protocols described in the text: (A) Calcofluor staining on fixed cells, (B) Calcofluor life cell staining. All images were processed in parallel to preserve relative fluorescence levels. However, note the 20-fold longer exposure time used on fixed cells. The WT images highlight the chitin derived from CSIII activity and accordingly the chitin rings are visible in some images (arrowheads). However, appreciate the absence of chitin rings in the bud scars of the

*chs3*Δ mutant cells (arrowheads). Moreover, the images obtained from the *chs3*Δ strain highlighted the chitin associated with CSII activity and therefore chitin deposition at the PS (arrows). Latrunculin A was added directly to cell cultures at a concentration of 30mM and cells were collected after 120 minutes. For life cell staining, Calcofluor was added 30 minutes after the addition of LatA. Note the alteration in chitin deposition after the blockade of endocytosis, including the collapse of some PSs in the *chs3*Δ strain (arrows).

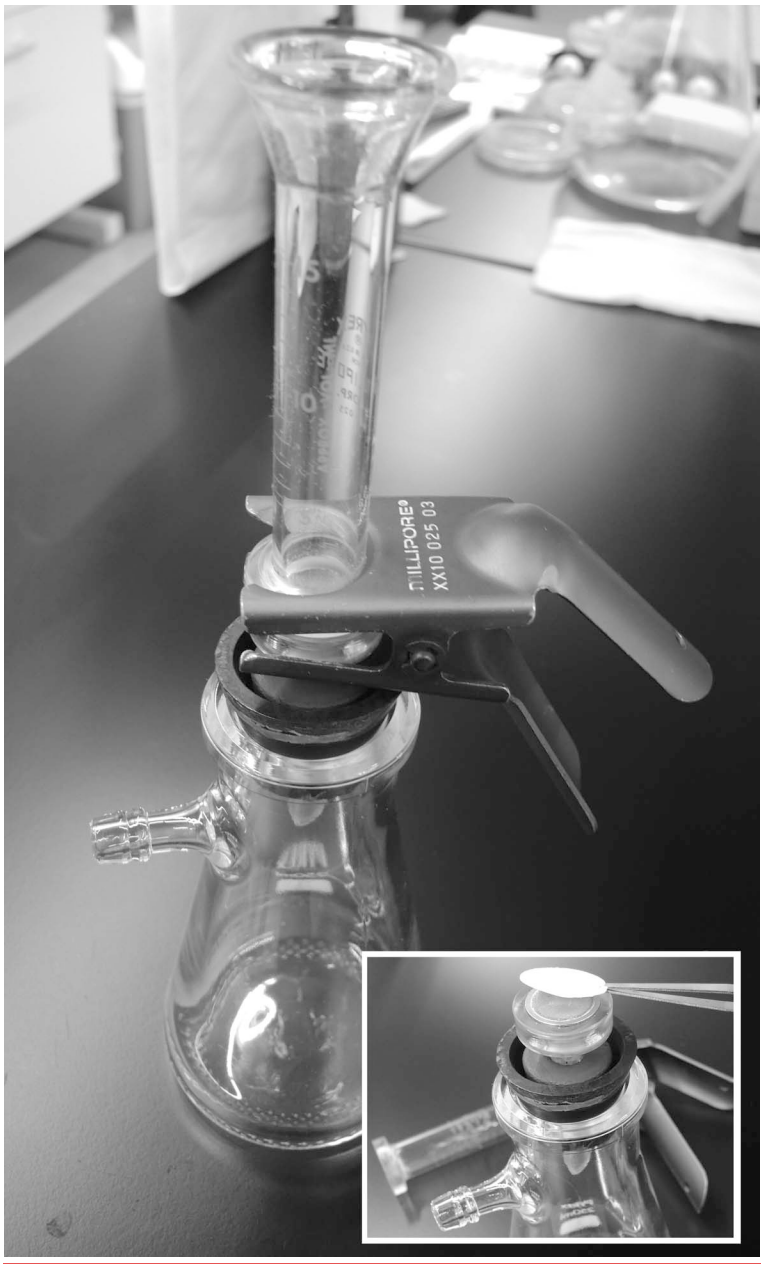

Figure 2. Filtration equipment. Individual samples can be processed using a simple filtration equipment as depicted in the Figure. 25 mm Fiber glass filters are routinely used.

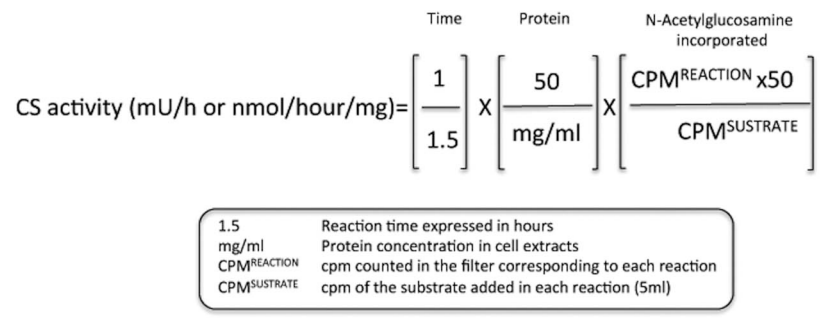

Figure 3. Calculation of chitin synthase activity