# Regulation of Schizophyllan in *Schizophyllum commune*

**Sophie van Velzen**

8260605

*Schizophyllum commune* by Russell McCulley

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Supervisor: Fleur Kleijburg

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# Abstract

Schizophyllan (SPG) is an exopolysaccharide β-(1,3)-(1,6)-glucan produced by the fungus *Schizophyllum commune*. Despite its extensive applications in medicine and industry, its biological function remains unknown. This study aimed to clarify the role of SPG in *S. commune*. SPG production is known to be influenced by various factors, such as pH, medium composition, shear stress and temperature. Here, *S. commune was* exposed to various abiotic growth conditions. This study found the SPG production of *S. commune* was reduced when grown when grown in a highly buffered medium compared to low-buffered medium. Additionally, SPG production was increased when under heat stress. SPG production did not differ significantly between biofilm-like growth and shaken conditions. Possibly SPG acts as a physical barrier protecting *S. commune* against environmental stressors like acid and heat. Furthermore, this study found that deletion of *fks1* (Schco3|2628724), the gene encoding the catalytic subunit of β-(1,3)-glucan synthase, only partially affected SPG production. Solid-state NMR analysis of the cell wall of the Δ*fks1* mutant showed that the reduction in SPG production was less pronounced compared to the reduction in β-(1,3)-(1,6)-glucan content in the cell wall. Possible reasons for this discrepancy include functional redundancy by the orthologue Fks2 or compensatory mechanisms within the fungus.

# Introduction

The fungal kingdom is made up of a diverse array of organisms that play a crucial role in nutrient cycling. The fungal cell wall is a critical factor contributing to the ability of fungi to thrive in various environments and adapt to changing conditions (Gow et al., 2023). These cell walls are robust, intricate, and dynamic structures that provide mechanical strength and elasticity, enabling fungi to withstand environmental stress. Additionally, the cell wall facilitates interactions between the fungus and its environment, serving as a signaling center in response to environmental stimuli (Bowman and Free, 2006). The composition of the cell wall is precisely regulated in response to environmental cues and external pressures. It consists of a resilient core composite matrix of fibrous and gel-like carbohydrate polymers, to which proteins and other superficial components are bound (Gow et al., 2017). The basidiomycete and saprotrophic fungus *Schizophyllum commune* has a cell wall composed of a structured inner core consisting of highly cross-linked α- and β-(1,3)-glucan, β-(1,3)-(1,6)-glucan, branched β-(1,4)-chitin and polymeric fucose and mannose (Ehren et al., 2020). The outer layer is more flexible and is made up of β-(1,3)-glucan, β-(1,3)-(1,6)-glucan, β-(1,6)-glucan, polymeric mannose, and polysaccharides containing N-acetyl galactosamine and D-galactosamine (Ehren et al., 2020).

Additionally, β-(1,3)-(1,6)-glucan is secreted by *S. commune* as the exopolysaccharide called schizophyllan (SPG). SPG is composed of a β-(1,3)-linked glucan backbone with β-1,6-glucose side chains at approximately every third main chain residue (Zang et al., 2013). SPG is a non-ionic, water-soluble polysaccharide with a molecular weight ranging from 6 to 12 x $10^6$ g/mol. It is secreted as a mucilaginous layer on the hyphal surface (Sietsma and Wessels, 1977). The specific functions of SPG in *S. commune* are still not fully understood. SPG has been found to aid hyphal attachment by helping the formation of hydrophobin coatings (Scholtmeijer et al., 2009). Moreover, it has antioxidative properties that could contribute to preserving the cell wall against harmful radicals (Chen et al., 2020). Exopolysaccharides, including β-glucans, are also crucial for maintaining the integrity of the extracellular matrix in biofilms produced by filamentous fungi (Ansari et al., 2021). Various factors, such as pH, medium composition, shear stress and temperature, influence SPG production (Rau et al., 1992; Kumari et al., 2008; Hamidi et al., 2022).

The biosynthesis pathway of SPG in *S. commune* is currently unknown. However, research on the biosynthesis of β-(1,3)-(1,6)-glucan in *Saccharomyces cerevisiae* provides some insights. By analogy to *S. cerevisiae*, the biosynthesis in *S. commune* is initiated intracellularly, with monomeric glucose precursors being transported to the cell surface (Ruiz-Herrera & Ortiz-Castellanos, 2019). A transmembrane glucan synthase complex, consisting of catalytic subunits Fks1 or Fks2 and regulatory subunit Rho1, then binds the monomers to β-(1,3)-glucan chains. The linear polysaccharide is extruded into the cell wall through the β-(1,3)-glucan synthase channel (Gow et al., 2017). In S. cerevisiae, Kre6 is essential for β-(1,6)-glucan synthesis (Kurita et al., 2011). *S. commune* possesses homologs of Rho1, Fks1, Fks2 and Kre6 (Ohm et al., 2010).

This study aimed to clarify when and how SPG is produced by *S. commune*. While much remains unknown about SPG, it is used in the pharmaceutical, cosmetic, and food industries due to its unique properties as a biocompatible and non-toxic biopolymer with immunomodulatory and anti-inflammatory effects. Therefore, this research contributes to the promotion of sustainable industrial processes by leveraging SPG's unique properties for various applications, ultimately benefiting society through enhanced pharmaceutical development and environmental conservation. In this study, *S. commune was* exposed to various abiotic growth conditions to better understand when SPG is

produced. This study found the SPG production of *S. commune* was reduced when grown when grown in a highly buffered medium compared to low-buffered medium. Additionally, SPG production was increased when under heat stress. SPG production did not differ significantly between biofilm-like growth and shaken conditions. Additionally, a phenotypic analysis was conducted on a *fks1* deletion mutant. This study found that deletion of *fks1* (Schco3|2628724) only limitedly affected SPG production while showing a large reduction in β-(1,3)-(1,6)-glucan in the rigid part of the cell wall.

# Materials and Methods

## Strains and culture conditions

*Schizophyllum commune* dikaryon strains UU-139, UU-176, UU-351 and UU-367 from the Utrecht University collection, and H4-8A ($matA_{43}matB_{41}$; Fowler et al. 1999), its derivatie strain H4.8A::Δku80 (de Jong et al., 2010), and its compatible strain H4-8B ($matA_{43}matB_{41}$; Fowler et al. 1999) were grown on ammonium minimal medium with or without 15 g $L^{-1}$ agar (MM-N or MM-NA, respectively; Kleijburg et al., 2023) for 7 days in the dark. *Pseudomonas helmanticensis* was grown on 10 ml Lysogeny broth (LB; Bertani, 1951) at 30 °C and 200 rpm for 24 hours and cell density was measured by $OD_{600}$ in a 1 ml cuvette in a DU 800 spectrophotometer (Beckman Coulter, www.beckmancoulter.com).

## Quantification of SPG production in liquid shaking cultures

Liquid shaking cultures were grown in MM-N or MM-N with ten times the amount of $PO_4^{3-}$(MM-NPK). Alternatively, ten times $PO_4^{3-}$ or $dH_2O$ (control) were added on day four of incubation (MM-N+PK and MM-N+$dH_2O$, respectively). 50 ml MM-N cultures were grown in 250 ml Erlenmyers or 1200 ml cultures in 2L Erlenmyers at 30 °C and 200 rpm from 0.1 g macerate. Macerate was made by macerating a quarter of a 7-day colony that was grown on MM-NA at 30°C in 50 ml MM-N for 30 sec at 18000 rpm in a Waring 2 Speed Blender (Waring Laboratory Science, https://www.waringlab.com). The homogenate was incubated for 24 h at 200 rpm in a 250 ml Erlenmeyer and macerated again (see above). Mycelium was harvested by centrifugation for 10 minutes at 10,000 rcf and was washed 3 times with 40ml $dH_2O$ and freeze-dried. SPG was precipitated from 25 ml of the spent media supernatant by incubation with a minimal ethanol concentration of 25% for 24 hours at 4°C. After centrifuging at 3200 rcf for 5 min SPG was removed from the liquid. The SPG was washed with 10ml $dH_2O$ for 24 hours on a C1 platform shaker (New Brunswick Scientific, www.eppendorf.com) and precipitated (see above). SPG was removed from the liquid and freeze-dried.

## Quantification of SPG production in plate cultures

Plate culutres were grown on MM-NA, MM-NA with ten-times the amount of $PO_4^{3-}$(MM-NAPK), $Mg^{2+}$ (MM-NAMg), trace elements solution (MM-NATE), $Fe^{3+}$ (MM-NAFe) or 180 mM KOH(MM-NAK), MM-NAPK was acidified with HCl to pH 6.40 (MM-NAPK HCl). A plug of mycelium taken from the outer part of the mycelium of a colony grown on MM-NA was placed on an agar plate with or without a polycarbonate track etched membrane (PCTE; 0.1 $\mu$m pore size, diameter 76 mm) (GvS, www.gvs.com) overlaying the agar medium. Plate cultures were sealed in a plastic bag (20 x 30 cm) and grown at 30 °C or 37°C. Mycelium was harvested and washed with 10 ml dH2O for 24 hours on a shaken platform (see above) to remove the SPG. After removing the mycelium, ethanol was added to a minimal concentration of 25% to the liquid phase and incubated for minimal 24 h at 4 °C. After centrifuging at 3200 rcf for 5 min SPG was removed from the liquid. The SPG was washed with 10ml $dH_2O$ and precipitated (see above). The mycelium and SPG were freeze-dried.

## Quantification of SPG in co-cultures with *S. commune* and *P. helmanticensis*

Co-cultures were grown in 50 ml MM-N in 250 ml Erlenmeyer either with 0.1 g macerate H4-8A and 1000 cells of *P. helmanticensis* or 0.05 g macerate of H4-8A and 0.05 g macerate of UU-139. These were grown at 30 °C and 200 rpm or static. SPG and mycelium were isolated (see above) and freeze-dried.

## Phenotyping of Δ*fks1*

The *ku80* gene was reintroduced before phenotypic analysis. To this end, a wild type H4-8b was crossed with *Ku80*:: Δ*fks1* . F1 that were nourseothricin resistant (indicative of deletion of *fks1*), but hygromycin sensitive (indicative of presence of *ku80*) were selected. The deletion of *fks1* was confirmed with PCR. DNA from plate cultures of H4-8A and Δ*fks1* grown for 7 days at 30°C were washed on a shaking platform to remove SPG (see above) and homogenized by a SK550 1.1 heavy-duty paint shaker (Fast & Fluid, www.fast-fluid.com). To 10 mg of homogenate, 500 ul of extraction buffer was added (Xin et al., 2012) and the suspension was incubated for 30 min at 65 °C. After centrifuging for 5 min at 5000 rfc, 1 volume of dilution buffer (Xin et al., 2012) was added to the supernatant and it was incubated for 30 minutes at 65°C. After centrifuging for 7 minutes at 21300 rcf, the pellet was resuspended in 500 ul of high salt TE (Xin et al., 2012). 500ul isopropanol was added and gDNA precipitated by incubation for 10 min at 4 °C and centrifugion for 7 minutes 21300rcf. The pellet was dissolved in $dH_20$. PCR was performed with forward primer (atcgccacccagccaaacac) annealing in the gDNA upstream of the nourseothricin cassette or *fks1* gene and reverse primer(primer N; tcgtcgtcggggaacacctt) annealing to the nourseothricin cassette or with reverse primer(primer F; cgagacgctcggctgtcaag) annealing to the *fks1* gene. This resulted in products of 500 or 700 bp respectively. Δ*fks1* strain was grown in MM-N in 50 ml and 1200 ml in either a 250 ml or a 2000 ml Erlenmeyer flask and SPG was quantified (see above). Δ*fks1* was grown on plate culture on MM-NA and SPG was quantified (see above). Δ*fks1* and H4-8A grown on MM-NA and MM-NAPK with $^{13}$C-glucose and $^{15}NH_4$ at 30°C. Mycelium was washed with 10 ml for 24 hours on a platform shaker (see above) and freeze-dried. Mycelium was homogenized using SK550 1.1 heavy-duty paint shaker (see above). Solid-state NMR was performed by Adil Safeer.

## Statistics

One-way ANOVAs were performed on replicates of the plate cultures, and two-way ANOVAs were performed on replicates of the liquid cultures, followed by TukeyHSD post hoc tests ($p < 0.05$).

# Results

### Effect of phosphate on SPG production in liquid shaken cultures

Less SPG is produced in pH-static cultivations compared to unadjusted cultivation (Münzer, 1989). To investigate this, *S. commune* was grown in liquid MM-N medium (pH 7.0) or MM-N in which the phosphate buffer was increased 10-fold, called MM-NPK (pH 7.3). SPG and mycelium production were quantified. Less mycelium was produced per liter of medium in MM-NPK (ranging from 0.17±0.11 (H4-8A) g $L^{-1}$ to 1.44±0.47 (UU-351) g $L^{-1}$) than in MM-N (ranging from 1.53±0.36 (UU-139) g $L^{-1}$ to 5.45±1.14 (UU-176) g $L^{-1}$). Previous research has shown a strong correlation between SPG and mycelium production (Rau et al., 1999). To align the mycelium production the phosphate buffer or $dH_2O$ (control) was added on day 4 rather than at the beginning of cultivation (called MM-N+PK and MM-N+$dH_2O$). Strains UU-351 and H4-8A still showed significant differences in the production of mycelium between MM-N+PK (3.05±1.84 g $L^{-1}$ and 2.34±0.39 g $L^{-1}$, respectively; Fig. 1B) and MM-N+dH20 (with 6.44±0.61 g $L^{-1}$ and 9.60±0.23 g $L^{-1}$, respectively). However, strain UU-367 showed no difference in the production of mycelium, but a significant difference in SPG production per gram of mycelium between MM-N+PK (25.00±4.05 mg $g^{-1}$) and MM-N+$dH_2O$ (94.89±50.65 mg $g^{-1}$; Fig. 1A). Additionally, the SPG production of strains UU-139, UU-176, and UU-351 was lower in MM-N+PK ( 24.27±2.58 mg $g^{-1}$, 9.60±0.73 mg $g^{-1}$ and 45.13±2.78 mg $g^{-1}$, respectively) compared to MM-N+$dH_2O$ (48.81±10.47 mg $g^{-1}$, 15.04±0.66 mg $g^{-1}$ and 68.48±38.91 mg $g^{-1}$, respectively), though these differences were not statistically significant. The pH of MM-N+PK after growth was significantly higher for all strains except UU-176, ranging from 5.77±0.10 (UU-139) to 6.05±0.05 (H4-8A), compared to MM-N+$dH_2O$, which ranged from 3.13±0.10 (H4-8A) to 4.70±0.39 (UU-367). The pH after cultivation of UU-176 was 59±0.04 in MM-N+$dH_2O$ and 5.82±0.05 in MM-N+PK (Fig. 1C). Collectively, the data indicate a general trend that in dikaryons, an increase in phosphate buffer concentration results in decreased SPG production.

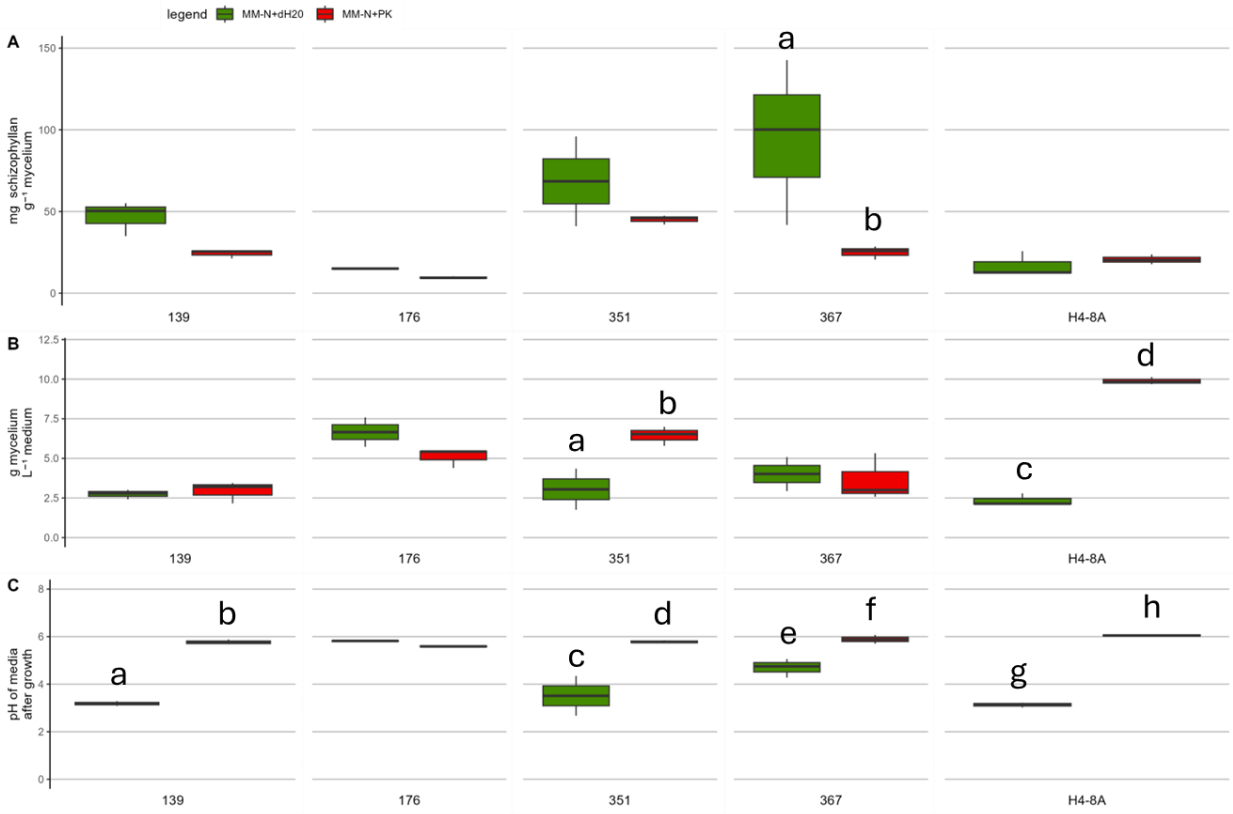


**Figure 1. Production of SPG in liquid shaking cultures.** The production of mg SPG $g^{-1}$ mycelium (A), g $L^{-1}$ mycelium (B) and pH of the medium after growth (C) on MM-N+$dH_2O$ (green) or MM-N+PK (red) were determined for dikaryon strains UU-139, UU-179, UU-351 and UU-367 (column 1 – 4) and monokaryon strain H4-8A (column 5). Letters show significant differences between the media. Strains produced less mg SPG $g^{-1}$ mycelium in MM-N+PK.

## Effect of phosphate on SPG production in plate cultures

Strain H4-8A was grown in plate cultures on MM-N media supplemented with agar, called MM-NA. Additionally, H4-8A was grown on plates with MM-NA including 10-fold the original amount of $PO_4^{3-}$, called MM-NAPK. A reduction in SGP production was observed between MM-NA and MM-NAPK (9.75± 3.36 mg $g^{-1}$ and 0.00±0.00 mg $g^{-1}$, respectively; Fig 2A). The production of mycelium and pH of the medium after growth were 0.17±0.02 g and pH 3.48±0.05 for MM-NA, while these were 0.03±0.01 g and pH 6.62±0.07 for MM-NAPK (Fig. 2BD). The mg mycelium per $cm^2$ was significantly less on MM-NA with 3.21±0.20 compared to MM-NAPK with 5.49±0.34 (Fig. 2C).

The role of pH in the reduction of SPG production was tested by acidifying the MM-NAPK medium with HCl (referred to as MM-NAPK HCl). On MM-NAPK HCl a slight increase in SGP production was observed compared to MM-NAPK (1.14±1.98 mg $g^{-1}$). Additionally, mycelium production also showed a slight increase on MM-NAPK HCl compared to MM-NAPK (0.08±0.02 g). The pH of MM-NAPK HCl was significantly lower from MM-NAPK after growth (pH 5.74±0.09). The mg mycelium per $cm^2$ MM-NAPK HCl with 5.91±0.60 mg $cm^{-2}$ did not differ significantly from MM-NAPK. These results showed an increase in SPG production with a decrease in pH.

The effect of increased concentrations of micronutrients was tested by growing H4-8A on MM-NA with ten-fold increase in potassium, trace elements, iron and magnesium, called MM-NAK, MM-NATE, MM-NAFe and MM-NAMg. H4-8A produced similar amounts of SPG on MM-NAK, MM-NATE, MM-NAFe and MM-NAMg, compared to MM-NA (23.44±9.72 mg $g^{-1}$, 14.87±7.15 mg $g^{-1}$, 19.39±5.4 mg $g^{-1}$, 13.67±7.74 mg $g^{-1}$, respectively). The production of mycelium was significantly less on MM-NAK, MM-NATE, MM-NAFe and MM-NAMg (ranging from 0.04±0.01 (MM-NA) to 0.09±0.03(MM-NAMg)), compared to MM-NA. pH of the media was similar to MM-NA, ranging from 3.52±0.22(MM-NAMg) to 3.88±0.12(MM-NATE). The mg mycelium per $cm^2$ after growth on MM-NAK, MM-NATE, MM-NAFe was significantly lower to that of MM-NA TRIS ranging from 0.84±0.04(MM-NAK) to 2.31±0.54(MM-NATE). That of MM-NAMg was similar to that of MM-NA (3.53±0.63). Overall, no change in SPG production was observed with increased micronutrient concentrations other than phosphate.

Solid-state NMR was performed on mycelium from H4-8A grown on MM-NAPK. The results show the rigid cell wall of MM-NAPK contains 5% β-(1,3)-glucan, 26% chitin and 68% α-(1,3)-glucan (Fig. 10B).

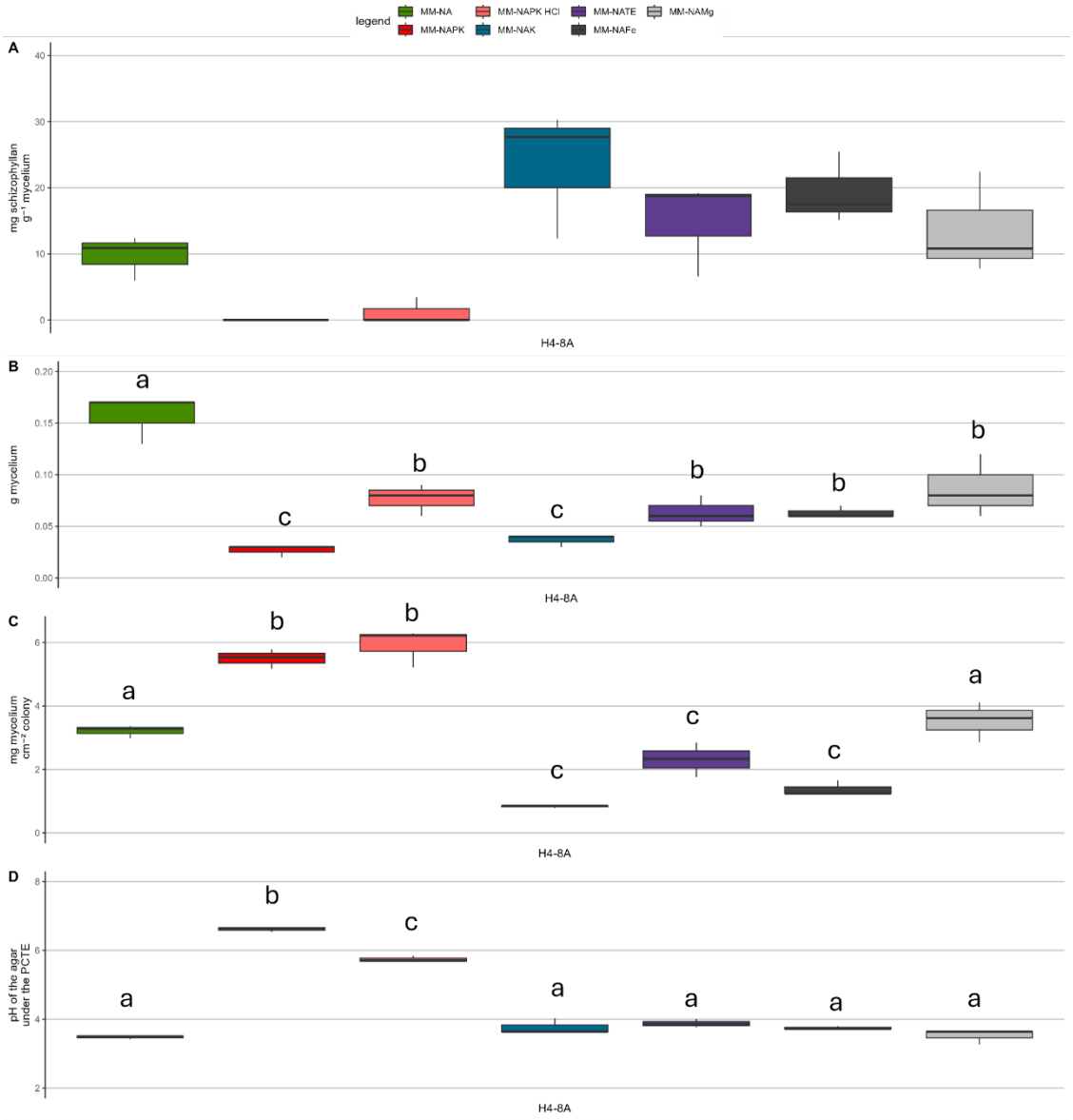


**Figure 2. Production of SPG in plate culture of H4-8A.** The production of mg SPG $g^{-1}$ mycelium (A), g mycelium (B), mg mycelium $cm^{-2}$ colony (C) and pH of the medium after growth under the PCTE (D) on MM-NA (green), MM-NAPK (dark red), MM-NA HCl (light red), MM-NAK (Blue), MM-NATE (purple), MM-NAFe (dark gray) and MM-NAMg (light gray) was determined for strain H4-8A. Letters show significant differences between the media. Strains produced less mg SPG $g^{-1}$ mycelium in MM-NPK and MM-NPK HCl.

## Impact of heat stress on SGP production

To investigate the impact of heat stress on SPG production, H4-8A was cultivated at both 37°C and 30°C on plates. The SPG production of H4-8A grown at 37°C was slightly increased compared to that at 30°C (24.82±8.09 mg $g^{-1}$ and 18.62±7.12 mg $g^{-1}$, respectively; Fig. 3A). H4-8A produced significantly less mycelium at 37°C, with 0.06±0.00 g compared to 0.11±0.01 g (Fig. 3B). Additionally, the mg mycelium per $cm^2$ was significantly higher when grown at 37°C with 2.36± 0.10 compared to 3.71± 0.22 (Fig. 3C). Overall did the increase in temperature result in a increase in SPG production.

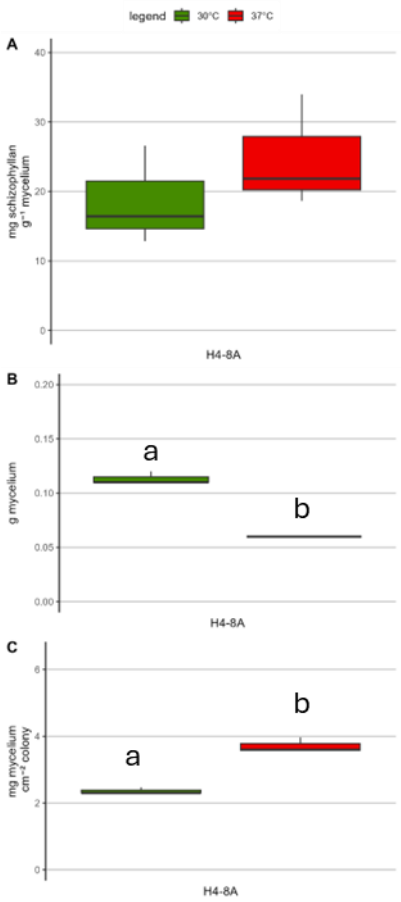


**Figure 3. Production of SPG of H4-8A at 30°C and 37°C.** The production of mg SPG $g^{-1}$ mycelium (A), g mycelium (B), mg mycelium $cm^{-2}$ colony at 30°C (green) and 37°C (red) were determined for strain H4-8A. Letters show significant differences between the media. Cultures at 37°C show a increase in SPG production.

## SPG production in liquid static culture conditions

Biofilm formation was stimulated by growing mono-culture of H4-8A and co-culture of H4-8A with *Pseudomonas helmanticensis* both in liquid shaken culture conditions(control) and liquid static culture conditions. The production of SGP (mg per gram of mycelium), mycelium (gram per liter of medium) and pH of the media after growth were quantified.

The shaken cultures exhibited a pelleted phenotype, whereas the static cultures were more homogeneously distributed (Fig. 4). The production of SPG in the mono-culture of H4-8A was similar in liquid shaken and liquid static conditions (with 60.70±18.07 mg $g^{-1}$ and 42.25±6.40 mg $g^{-1}$ respectively; Fig. 5A). The co-culture of H4-8A + *P. helmanticensis* showed a significant difference in the production of SPG between shaken culture with 77.71±20.56 mg $g^{-1}$ and static culture with 20.45±2.31 mg $g^{-1}$. The mycelium yield was not significantly higher in shaken cultures for H4-8A (2.33±0.16 g $L^{-1}$) and H4-8A + *P. helmanticensis* (2.73±0.76 g $L^{-1}$) compared to static cultures (1.58±0.06 g $L^{-1}$ and 2.13±0.06 g $L^{-1}$, respectively; Fig. 5B). The pH of mono-culture and co-culture was lower in the liquid shaken cultures (with pH 2.96±0.32 and pH 2.78±0.06, respectively), compared to the static cultures (with pH 5.14±0.07 and pH 3.69±0.12, respectively).

Additionally, mono-culture of UU-139 and co-culture of H4-8A with UU-139 were grown both in liquid shaken culture conditions and liquid static culture conditions. The SPG production did not

significantly differ between shaken cultures of UU-139 and H4-8A + UU-139 (with 47.38±12.00 mg $g^{-1}$ and 52.19±9.58 mg $g^{-1}$, respectively) and static cultures (with 56.26±1.35 mg $g^{-1}$ and 54.97±1.66 mg $g^{-1}$, respectively). For UU-139 and H4-8A + UU-139, mycelium production was significantly higher in shaken cultures (2.10±0.19 g $L^{-1}$ and 2.23±0.12 g $L^{-1}$, respectively) compared to static cultures (1.12±0.10 g $L^{-1}$ and 1.20±0.13 g $L^{-1}$, respectively). The pH of the media post-growth was lower in shaken cultures of UU-139 and H4-8A + UU-139 (with pH 3.59±0.03 and 3.07±0.26, respectively), compared to static cultures (with pH 3.44±0.19 and 4.81±0.47, respectively).

Overall, little difference was observed between the liquid shaken and liquid static conditions of *S. commune* monocultures and co-cultures. The co-culture of *S. commune* with *P. helmanticensis* did show a decrease in SPG production compared to the mono-culture of H4-8A during static cultivation, although no phenotypic differences were observed.

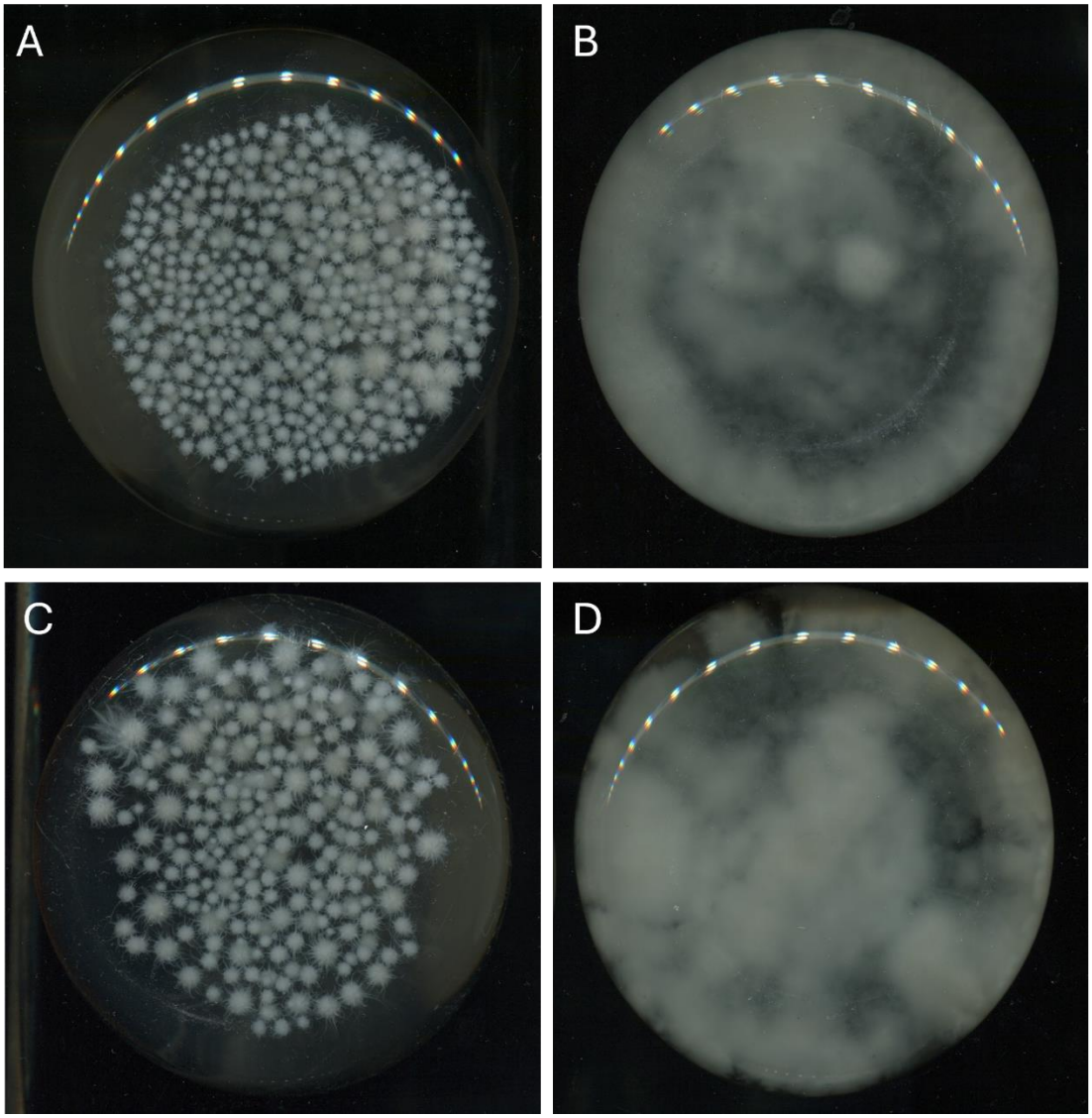


**Figure 4. Liquid shaken and static cultures of H4-8A**. Scans of Erlenmeyer flasks of mono-culture of strain H4-8A in liquid shaken culture (A) and liquid static culture (B) and co-culture of H4-8A + *P. helmanticensis* in liquid shaken culture (C) and liquid static culture (D) after 7 days of growth. The liquid shaken culture demonstrates a pelleted phenotype, while the static culture displays less dense mycelium that is more homogeneously distributed throughout the flask.

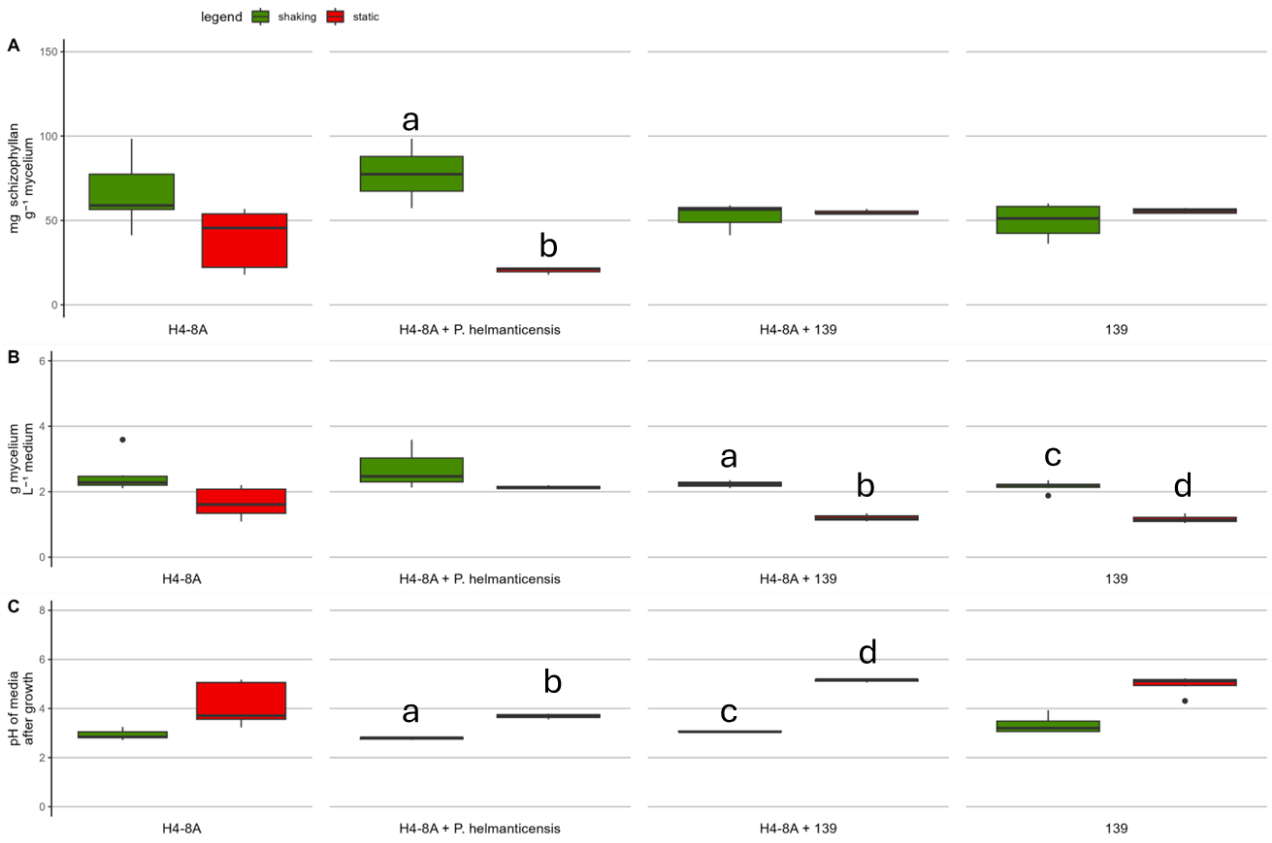


**Figure 5. Production of SPG in liquid shaken cultures and liquid static cultures.** The production of mg SPG $g^{-1}$ mycelium (A), g $L^{-1}$ mycelium (B) and pH of the medium after growth (C) in liquid shaken cultures (green) or liquid static cultures (red) were determined for monokaryon strain H4-8A (column 1), H4-8A together with *Pseudomonas helmanticensis* (column 2), H4-8A together with dikaryon strain UU-139 (column 3) and UU-139 (column 4). Letters show significant differences between the media. The co-culture of H4-8A and *P. helmanticensis* results in increased SPG production under shaken conditions and decreased SPG production under static conditions.

## Phenotyping of Δ*fks1*

Fks1 is part of the β-(1,3)-glucan synthase complex (Ruiz-Herrera & Ortiz- Castellanos, 2019). An Δ*fks1* mutant in a H4-8A::Δ*ku80* background (Schunselaar, 2021) was crossed with H4-8B to reintroduce Ku80 in the genome. Out of 88 spores, 8 showed resistance to nourseothricin (indicative of deletion of *fks1*) but not to hygromycin (indicative of the presence of Ku80). One of the transformants displaying denser mycelium compared to the wild type was chosen (Fig. 6). The Δ*fks1* strain used in this study has mating type C. The deletion was confirmed through a PCR check (Fig. 7).

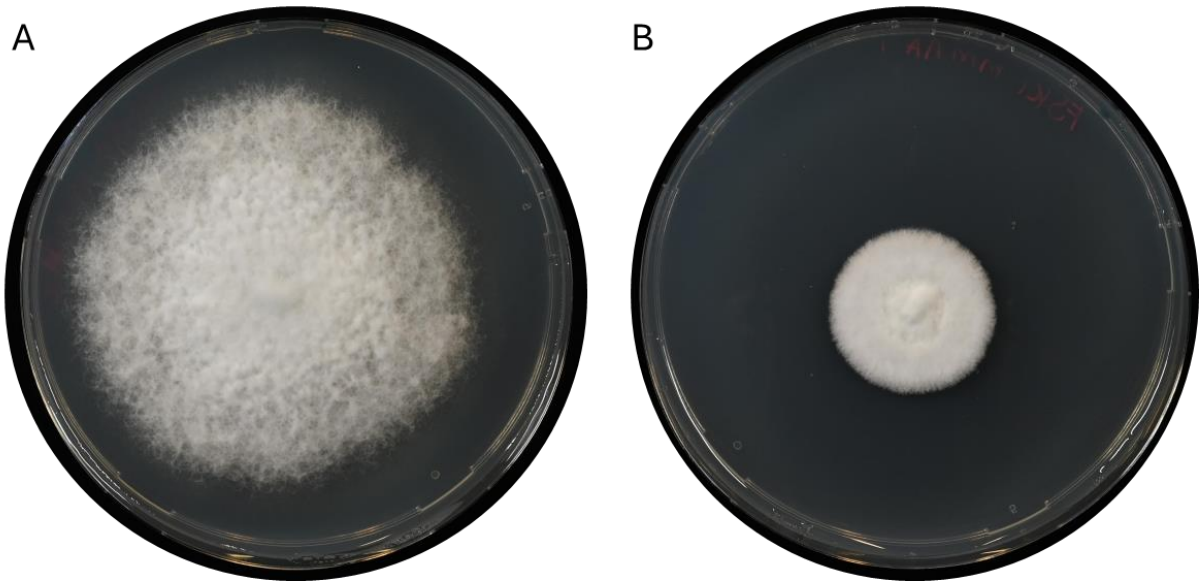


**Figure 6. Plate culture of H4-8A and Δ*fks1***. Scans from plate cultures of H4-8A (A) and Δ*fks1*(B) after 7 days of growth. Δ*fks1* showing a smaller area of growth and denser mycelium after deletion compared to H4-8A.

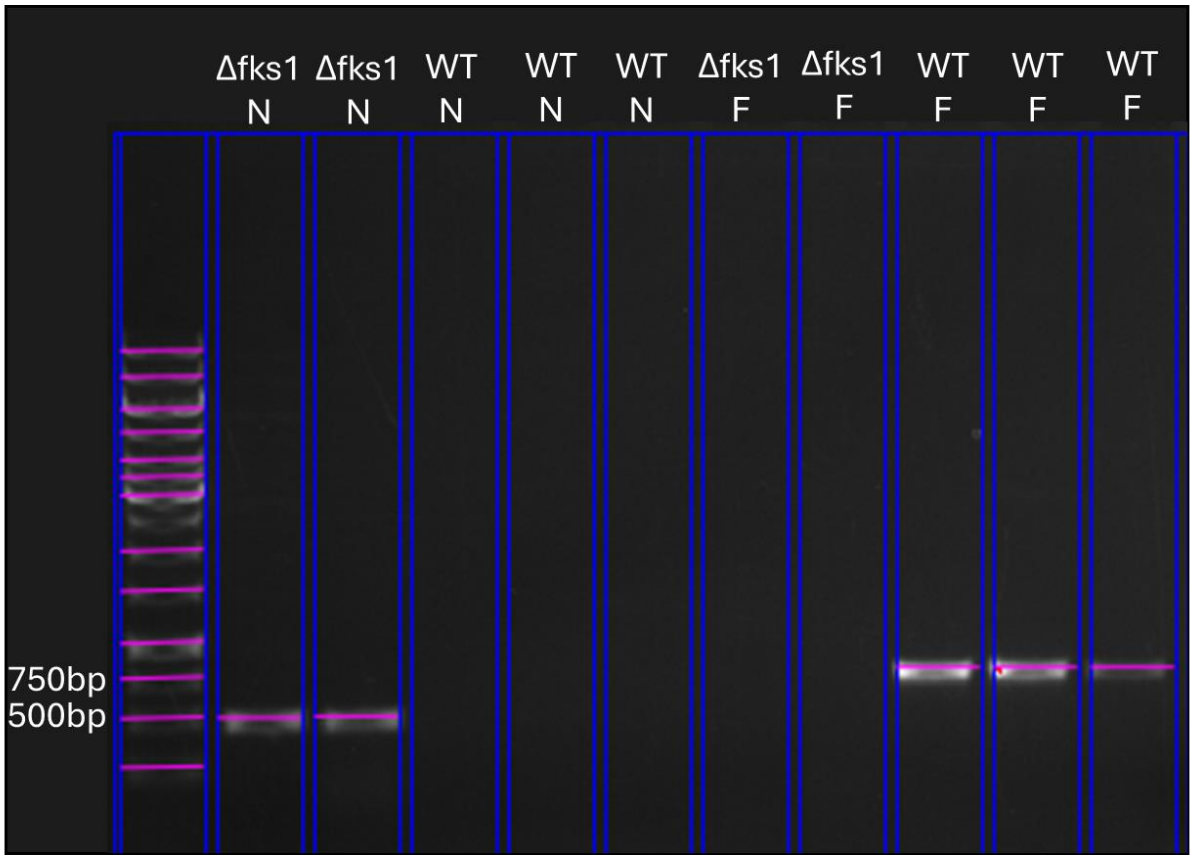


Figure 7, **Agarose gel of check PCR of Δfks1.** PCR using primer sets with reverse primers annealing to the nourseothricin insert (N; 500 bp band) or *fks1* (F; 750 bp band) were used to confirm the absence of *fks1* in gDNA isolated from Δ*fks1* (Δ*fks1*) or wild type (WT) mycelium. The absence of the 750 bp but presence of the 500 bp band confirm the deletion of *fks1*.

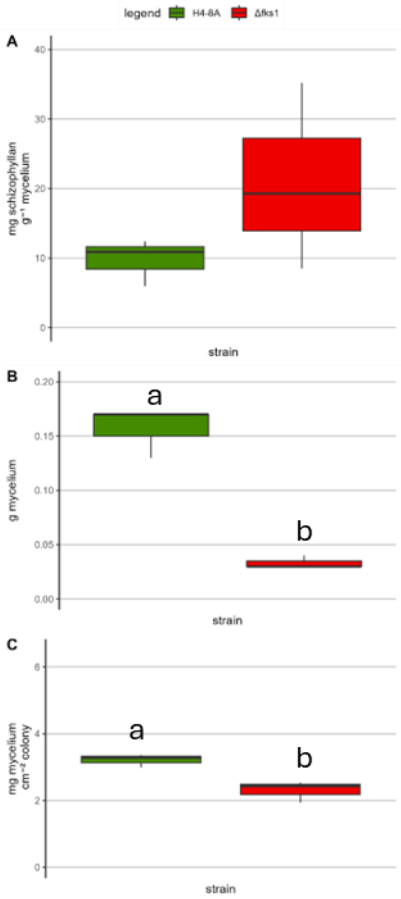


Figure 8. **Production of SPG in Plate culture of H4-8A and *Δfks1*.** The production of mg SPG $g^{-1}$ mycelium (A), g mycelium (B), and mg mycelium $cm^{-2}$ colony were determined for H4-8A (green) and Δfks1 (red). Letters show significant differences between the media. Δ*fks1* does not show a significant difference in SPG production compared to H4-8A.

Both H4-8A and Δ*fks1* were grown on plate and liquid shaken cultures of 50ml and 1200ml. The production of SPG on plate cultures did not show a significant difference, with Δ*fks1* producing 21.00±13.44 mg SPG $g^{-1}$ mycelium and H4-8A producing 9.75±3.36 mg SPG $g^{-1}$ mycelium (Fig. 8A). However, the production of mycelium was significantly less with (0.03±0.01 g (Δ*fks1*) and 0.16±0.02 g (H4-8A); Fig. 8B). Additionally, Δ*fks1* produces less mycelium per $cm^2$ in comparison to H4-8A (2.30±0.32 mg and 3.21±0.20 mg; Fig. 8C). In 50 ml liquid shaken cultures, Δ*fks1* did not produce any SPG (0.00±0.00 mg SPG $g^{-1}$ mycelium), whereas H4-8A produced 53.48±45.66 mg SPG $g^{-1}$ mycelium. In 1200 ml liquid shaken cultures, Δ*fks1* produced a reduced amount of SPG (74.90±10.36 mg SPG $g^{-1}$ mycelium) compared to H4-8A (212.74±12.64 mg SPG $g^{-1}$ mycelium; Fig. 9A). The mycelium yield was also significantly lower for Δ*fks1*, with 1.16±0.06 g $L^{-1}$ in 50 ml cultures and 1.37±0.12 g $L^{-1}$ in 1200 ml cultures, compared to 2.42±0.66 g $L^{-1}$ and 4.93±1.22 g $L^{-1}$ for H4-8A, respectively (Fig. 9B). Overall, Δfks1 showed no significant change in SPG production in plate cultures, however liquid shaken cultures significantly reduced SPG production.

The rigid cell wall composition of Δ*fks1* mycelium was studied using solid-state NMR. The rigid cell wall of Δ*fks1* contains 6 % β-(1,3)-(1,6)-glucan, 12 % lipids, 15% chitin and 65 % α-(1,3)-glucan (Fig.10A).

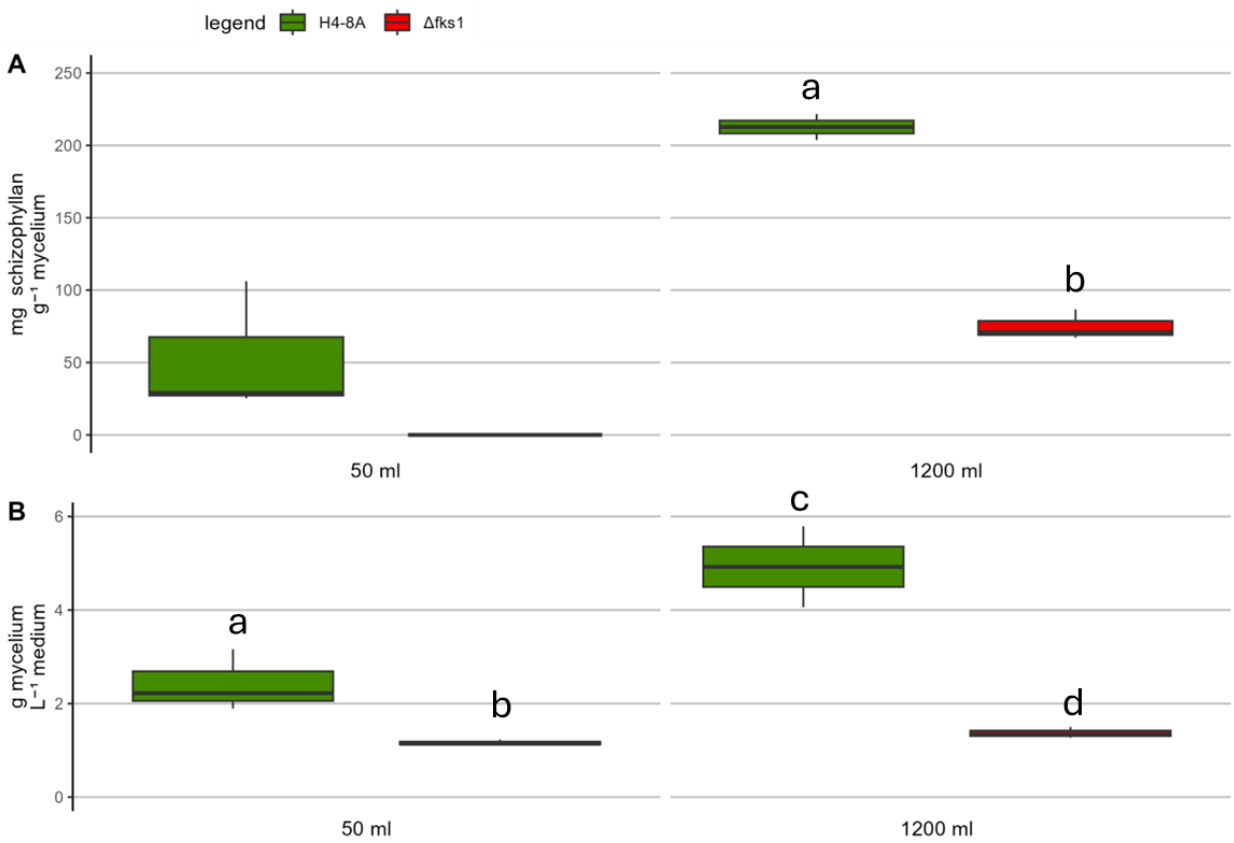


Figure 9. **Production of SPG in liquid shaken culture of H4-8A and Δ*fks1*.** The production of mg SPG $g^{-1}$ mycelium (A), g $L^{-1}$ mycelium (B) were determined for strain H4-8A (green) and Δ*fks1*(red) in 50 mL (column 1) and 1200 mL (column 2). Letters show significant differences between the media. Less SPG is produced by Δ*fks1* compared to H4-8A.

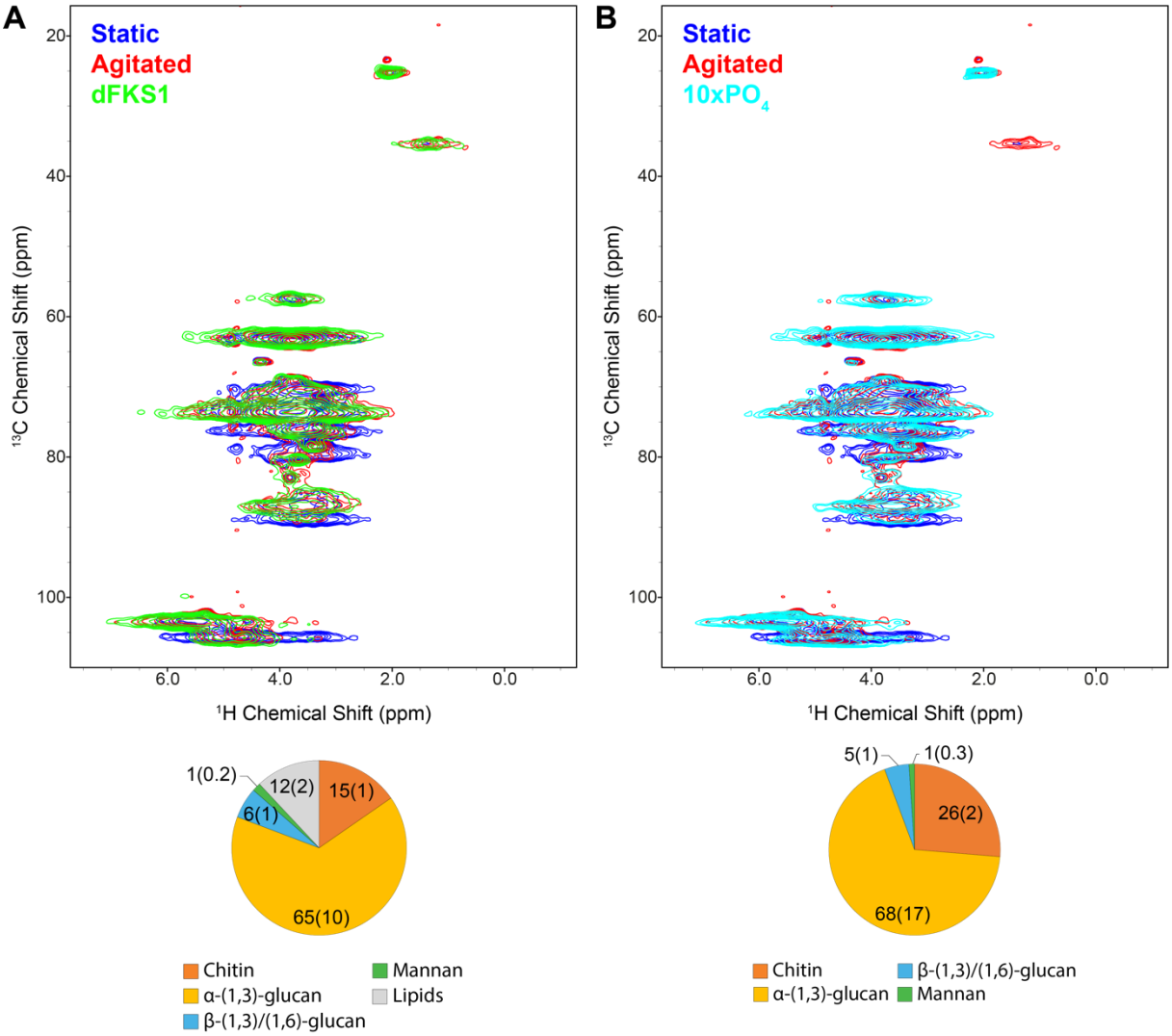


Figure 10. **Dipolar-based (rigid) 1H-detected experiments characterization of the rigid cell walls of *S. commune***. Δ*fks1* (A) and H4-8A grown on MM-NAPK (B), overlaid on agitated(shaken) (red) and static(plate) (blue) *S. commune* H4-8A spectra.

# Discussion

## SPG as a protective barrier against environmental stress

The biological function of SPG produced by *S. commune* is not well understood. Various abiotic factors are known to influence SPG production, although the underlying mechanisms are unclear. One possible hypothesis is that SPG forms a protective barrier, shielding the fungus from environmental stressors. The viscous, gel-like barrier that SPG forms around fungal cells might physically impede the diffusion of harmful substances or help retain a favorable microenvironment, for example during acid-induced stress. SPG production is reduced in pH-static cultivations compared to unadjusted conditions (Münzer, 1989). When the pH of the media is significantly reduced during growth, the fungus can experience acid-induced stress, as $H^+$ ions can diffuse into the cell and disrupt normal cellular activities (Ndukwe et al., 2020). The physical barrier formed by SPG can help stabilize the local pH around fungal cells by creating a gel-like substance that slows the diffusion of hydrogen ions, thereby creating a protective microenvironment (Welin-Neilands et al., 2007). The results of this study showed that both in liquid medium and plate cultures, increased buffering capacity of the media led to a reduction in SPG production. Furthermore, a reduction in pH was shown to induce SPG production, as observed in MM-NAPK HCl medium. This indicates that the pH of the growth medium significantly impacts SPG production, supporting the hypothesis that SPG serves as a protective barrier against pH-induced stress. The level of acid-induced stress experienced by *S. commune* is difficult to determine by the results of this research alone. The high phosphate concentration also seems to induce a stress reaction, as evidenced by the reduction in mycelium production, making it difficult to isolate the effects of acid stress. Additional research on the stress levels experienced by *S. commune* during growth would help confirm this hypothesis. Growth measurements and viability assays could indicate whether *S. commune* is experiencing stress at pH < 4.0.

Additionally, the hypothesis that SPG functions as a protective barrier can explain its increased production under heat stress. Exopolysaccharides are produced in response to heat stress, enabling microorganisms to become more resistant to high temperatures (Prasad and Purohit, 2023). They help maintain an appropriately humid environment around the organism (Shukla et al., 2019; Ophir and Gutnick, 1994). The results show that heat stress induced in H4-8A when grown at 37°C resulted in increased SPG production, suggesting that SPG forms a barrier around fungal cells, protecting them from desiccation and thermal stress.

A significant function of exopolysaccharides in microbial biofilms is protection (López et al., 2010). Even in the absence of immediate stress are the exopolysaccharides still present. The concept of biofilms in filamentous fungi is relatively new and not widely accepted, although *Aspergillus fumigatus* is recognized to form true biofilms (Gutiérrez-Correa et al., 2012). Features associated with biofilm formation by filamentous fungi include structural features and altered gene expression (Harding et al., 2009). Structural features include complex aggregated growth, surface-associated growth of cells, cells embedded in a self-produced and secreted extracellular polymeric matrix. Additionally, altered gene expression resulting in phenotypic changes like changes in enzyme or metabolite production or secretion and physiological changes. The mucilaginous properties of SPG resemble the properties of biofilms and β-glucan has been described as a component of filamentous fungal biofilms (Harding et al., 2009; Ansari et al., 2021).

The static liquid cultures of mono-culture H4-8A and UU-139 and co-cultures of H4-8A+UU-139 and H4-8A+ *P. helmanticensis* of this study exhibited characteristics of biofilms. Showing surface associated growth in bundels and layers and the cells were embedded in a self produced and secreted extracellular matrix. However, no research on altered gene expression was conducted in this study.

The results of this study indicate that SPG is unlikely to play a significant role in biofilm formation in *S. commune*. No significant differences in SPG production were observed between the mono-cultures and co-culture of H4-8A and UU-139 in the liquid static cultures compared to the liquid shaken cultures. However, the co-culture of H4-8A and *P. helmanticensis* showed increased SPG production in liquid-shaken conditions and reduced production in static conditions compared to the H4-8A monoculture. Possible explanations for this result include *P. helmanticensis* inducing metabolic pathways in *S. commune* or differences in oxygen levels affecting SPG production (Shu et al., 2005; Keller et al., 2005).

To further support the hypothesis of SPG as a protective barrier against environmental stress, permeability assays could be conducted to evaluate how effectively SPG acts as a barrier to various substances, including hydrogen ions, under different pH conditions. These assays could involve measuring the diffusion rate of labeled substances (e.g., $H^+$ ions, dyes, or small molecules) through SPG matrices to determine their permeability.

**The biosynthesis pathway of SPG**

The biosynthesis pathway of SPG in *S. commune* was studied by performing solid-state NMR on the cell wall of H4-8A grown on MM-NAPK and Δ*fks1*. On plate cultures with MM-NA, the cell wall of the wild-type strain H4-8A consisted of 71% β-(1,3)-(1,6)-glucan, whereas on MM-NAPK medium, it contained only 5% β-(1,3)-(1,6)-glucan (Safeer, 2024). Additionally, on MM-NAPK, H4-8A did not produce any SPG. Thus, on MM-NAPK, both SPG and β-(1,3)-(1,6)-glucan are reduced tenfold. These results suggest that β-(1,3)-(1,6)-glucan and exopolysaccharide β-(1,3)-(1,6)-glucan are synthesized by the same pathway.

The cell wall of the Δ*fks1* mutant contained 6% β-(1,3)-(1,6)-glucan compared to 71% produced by H4-8A on the same MM-NA medium. Therefore, without Fks1, *S. commune* produces also only a tenth of the β-(1,3)-(1,6)-glucan it normally produces. The deletion of *fks1* did not significantly alter SPG production when grown on plate, suggesting functional redundancy where other genes or compensatory mechanisms might be involved in its biosynthesis. One possibility is that the orthologue of fks1, fks2 (Schco3|2035630), is still able to produce β-(1,3)-glucan and has partial redundancy in their function (Ohm et al., 2010). Another explanation could be degeneracy, where structurally distinct components bear similar or partially overlapping functions, in this case, the biosynthesis of β-(1,3)-(1,6)-glucan (Peng et al., 2012). Given that β-(1,3)-(1,6)-glucan in the cell wall matrix is extremely reduced but SPG is not, it is possible that both are synthesized via different biosynthesis pathways. The disruption of SPG production in liquid shaken culture conditions could be a side effect of the lack of β-(1,3)-glucan production and presence in the cell wall. β-(1,3)-glucan is a critical component of the fungal cell wall, playing a crucial role in maintaining cell structure and integrity (Beauvais and Latgé, 2018). The inability to produce β-(1,3)-glucan would severely compromise the structural integrity, growth and survival of the fungus. In combination with the shear stress of shaken culture methods, this can result in different expression patterns, including SPG production.

Morphological changes of Δ*fks1* were comparable to those observed in fungi treated with caspofungin. Caspofungin is an antifungal agent that inhibits the β-(1,3)-glucan synthese complex (Deresinski et al., 2003). It is a noncompetitive inhibitor of the transmembrane glucan synthase complex of Rho and Fks. Fungi treated with caspofungin exhibit denser growth with more branched mycelium, similar to the Δ*fks1* mutant. This similarity is expected as both are targeted in the same pathway. Additionally, on MM-NAPK, H4-8A showed inhibited growth and more mycelium per $cm^3$ of the colony. Both conditions resulted in a significant reduction in β-glucan in the rigid part of the cell wall. Thus, the lack of β-glucan in the rigid part of the cell wall, reducing the cell's strength, can explain both phenotypes.

# Conclusion

This study showed that SPG production is significantly decreased with increased phosphate buffer in the growth medium. Additionally, it showed that heat stress promoted SPG production and biofilm formation did not seem to affect SPG production. A possible hypothesis as to what the role of SPG is in schizophyllum commune is as a physical protective barrier protecting the fungus against environmental stressors like acid and heat.

Solid-state NMR analysis of the cell wall of the Δ*fks1* mutant showed that the reduction in SPG production was less pronounced compared to the reduction in β-(1,3)-(1,6)-glucan content in the cell wall. Possible reasons for this discrepancy include functional redundancy by the orthologue Fks2 or compensatory mechanisms within the fungus.

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# Layman summary

The fungal kingdom consists of a diverse group of species that are essential for various important ecosystem roles all over the world. The cell walls of fungi are crucial for their survival in extreme places and make up a significant part of the fungus. These cell walls are composed of polysaccharides, lipids, and proteins that form a matrix. *Schizophyllum commune* is a common fungus found globally and grows primarily on dead wood. A large part of its cell wall is made up of the polysaccharide β-(1,3)-(1,6)-glucan. When this polysaccharide is loosely bound or not attached to the cell wall, it is called schizophyllan (SPG). SPG forms a slimy layer on the fungus and makes the growth medium very viscous when dissolved. It has also various unique properties valuable for various industries. However, its function within the fungus itself is still unknown. The production of SPG is influenced by environmental factors such as pH, temperature, and oxygen levels, but how SPG is produced in *S. commune* is still unclear.

In this study, *S. commune* was grown in different conditions to help understand when SPG is produced. First, the phosphate buffer of the growth medium was increased to examine the effect of pH and phosphate on SPG production. It was found that higher pH levels reduced SPG production compared to a normal buffered medium. Additionally, the effect of heat stress on SPG production was tested by growing the fungus at a higher temperature compared to its optimum temperature. This resulted in an increased SPG production. This study also examined if SPG production increases when the fungus forms a biofilm. A biofilm is a community of microorganisms that stick to each other and surfaces, forming a protective, slimy layer. However, SPG production did not increase in biofilms.

Finally, this study looked into the biosynthesis of SPG. Therefore, the gene *fks1*, known to be involved in the production of SPG-like polysaccharides in other fungi, was investigated. The gene was deleted in *S. commune* and the protein Fks1 was therefore not produced in this mutant. Fks1 is important in the production of β-(1,3)-(1,6)-glucan in other fungi. However, SPG production was only slightly reduced in the *fks1* mutant, suggesting that SPG may be produced by an alternative pathway or mechanism.

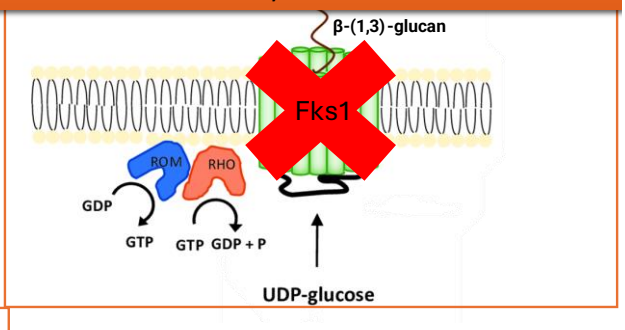
## Graphical abstract

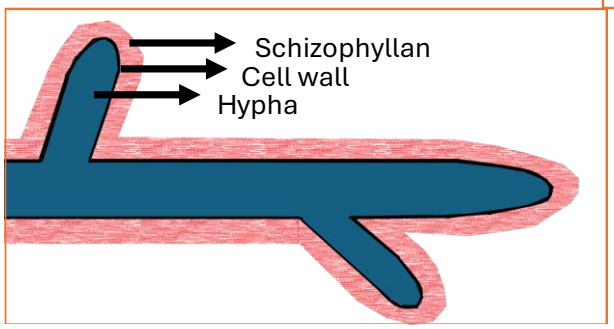
In this study, *Schizophyllum commune was* exposed to various abiotic growth conditions to better understand when schizophyllan is produced. Additionally, a phenotypic analysis was conducted on a *fks1* deletion mutant, targeting β-(1,3)-glucan synthase.

Schizophyllan is a β-(1,3)-(1,6)-glucan exopoly-saccharide secreted by *Schizophyllum commune* that forms a mucilaginous layer on top of the cell wall. It is used in various industries due to its unique properties as a biocompatible and non-toxic biopolymer with immunomodulatory and anti-inflammatory effects.

In this study:

- Schizophyllan production is increased during heat stress
- Schizophyllan production is decreased with increased phosphate buffer in the medium
- Schizophyllan production was not changed during biofilm formation




After the deletion of *fks1*, the reduction in schizophyllan production was less pronounced compared to the reduction in β-(1,3)-glucan content in the cell wall. Possible reasons for this discrepancy include functional redundancy by the orthologue Fks2 or compensatory mechanisms within the fungus.