Heterologous expression of SS1 from potato in Escherichia coli

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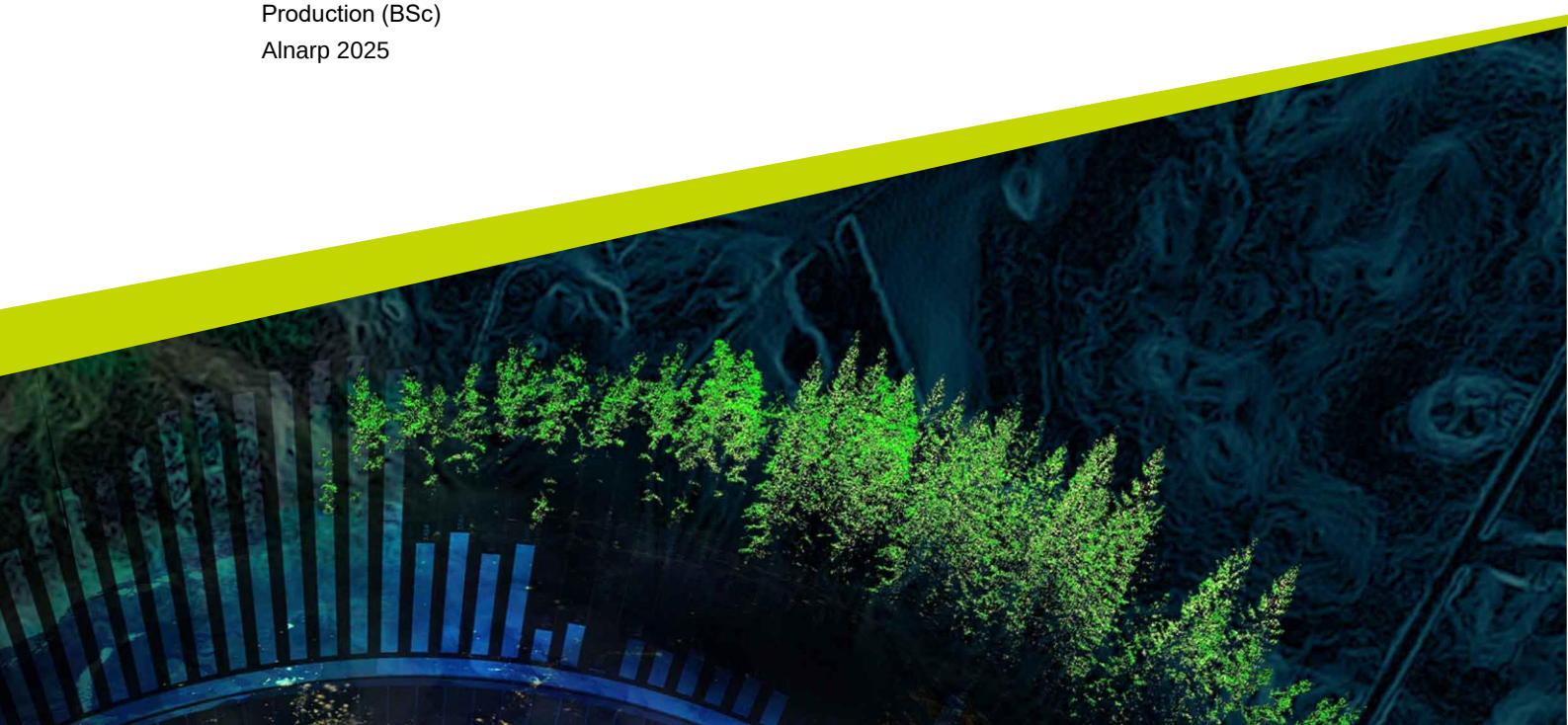

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Abstract

When starch is produced, it is often subjected to chemical treatments in order to achieve desired characteristics. Today, it is possible to genetically modify the potato genome using the CRISPR/Cas9 technique to influence starch synthesis and produce potatoes with a desired starch composition and structure. This enables the production of starch without the need for chemical modification by genetically modifying enzymes involved in starch biosynthesis, such as starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (DBE). To further study these enzymes, one approach is to express them in *Escherichia Coli*. This system allows for a cost-effective production of high protein yields and opens the door for enzyme engineering to tailor or enhance specific catalytic properties. These insights can later be utilised for more accurate editing of the enzymes in potato. This study has utilized a gateway cloning system from Invitrogen, in which SS1 has been transformed into *E. coli* via the donor vector pDON221 and the destination vector pDEST17. Followed by attempts to induce protein expression with IPTG. This study confirmed the insertion of the SS1 in *E. Coli*, but no confirmation of expression was achieved. To express functional proteins in *E. coli*, several optimization steps during the induction of expression need to be taken into consideration. This study provides a solid base for further attempts to express and obtain soluble active SS protein in *E. Coli*.

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Project aim

The aim of this study is to express SS1 from potato in *E. coli* in a soluble and active form, and to establish a reliable expression protocol for the group.

1.1 Project background

This study was conducted at the Swedish University of Agricultural Sciences (SLU), Alnarp, within the Plant Biotechnology Division. It forms part of a larger project focusing on transgene-free targeted mutations in the potato genome using CRISPR/Cas9 technology, aiming to produce high-quality and purpose-specific starch-potato lines without the need for post-harvest modifications.

Many thanks to everyone at the plant biotechnology division for their support and patience. And special thanks to Luboš Říha for your dedication and guidance.

Patrik Lilja

Introduction

Potatoes are among the most important non-cereal food crops, with a production of 359 million tons in 2020, making them the fourth-largest crop in terms of quantity (Tong et al. 2023). Potatoes contain many valuable nutrients such as carbohydrates, proteins, minerals, vitamins, and fiber, making them a valuable food crop. The starch of potatoes has unique qualities that make it useful for various applications, such as the food industry, medical field, and packaging, as seen in Figure 1 (Tong et al., 2023). Starch is an important energy reserve for plants that can be stored and mobilized for growth and development. Starch is an insoluble glucan consisting of two types of glucose polymers: amylopectin and amylose. Both of these polysaccharides are chains built up with 1,4-linked D-glucose. While amylose is considered linear, amylopectin branches through 1,6-linked D-glucose as seen in Figure 1. Amylose has long chains consisting of hundreds or even thousands of glucose units, whereas amylopectin is branched and shorter. This results in a complex molecular structure (Bertoft, 2017). Starch consists of chains of varying length and branching structure. It is the ratio between amylose and amylopectin, along with the branching structure itself, that gives the potato starch its unique qualities (Toinga-Villafuerte et al., 2022). With an increasing demand for starch as a raw material, it is valuable to understand the different pathways of how starch is synthesized (Zeeman et al. 2010).

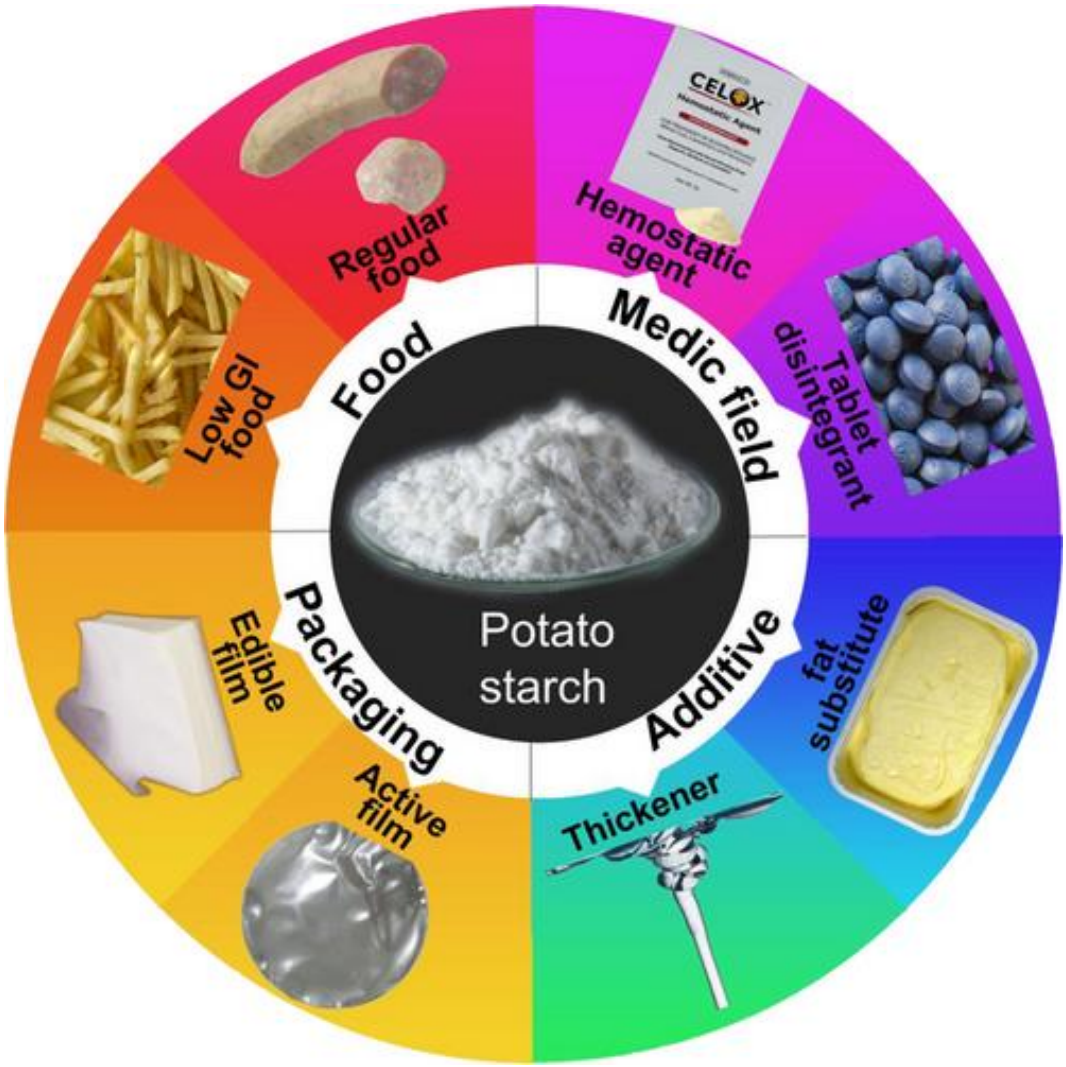

Figure 1. Starch from potatoes can be used in many different fields. Its characteristics are determined by the ratio of amylose to amylopectin as well as the structure of the branching, making it suitable for various applications (Tong et al. 2023).

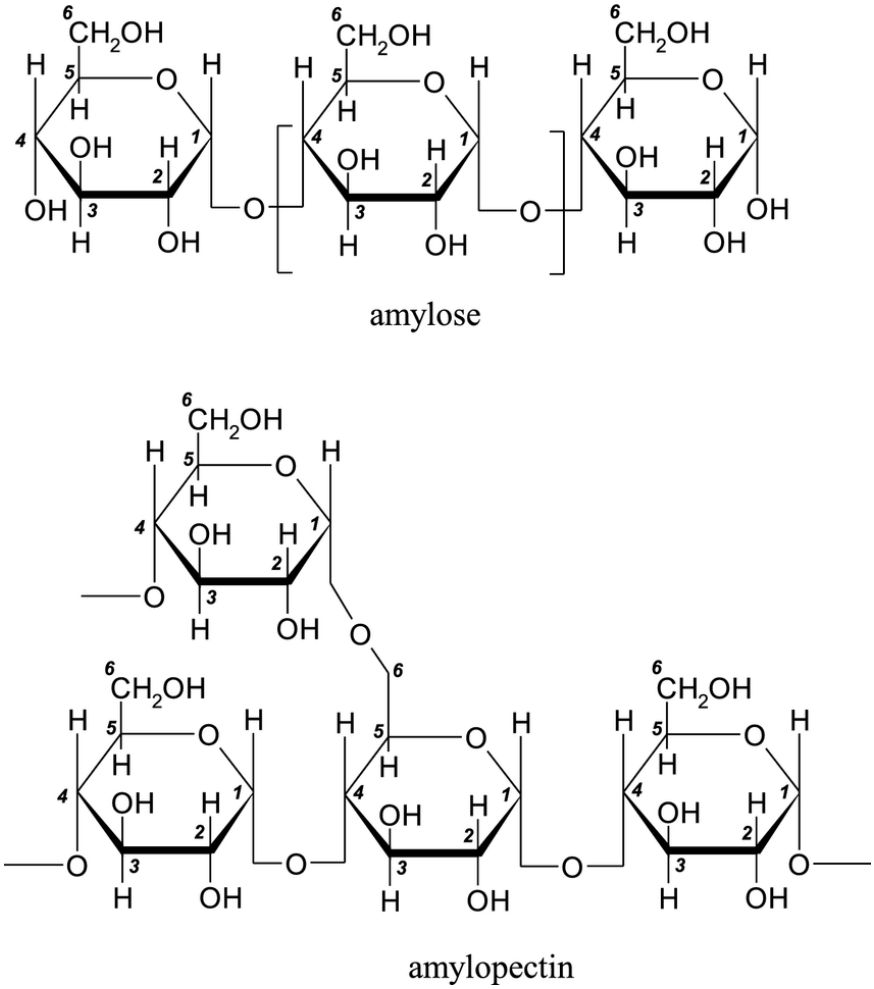

Figure 2. Starch is structured with 1,4-linked D-glucose. While amylose is considered linear, amylopectin branches through 1,6-linked D-glucose

Besides polysaccharides, potato starch also contains small amounts of non-carbohydrates, such as proteins, making up less than 0.5%, while lipids are mostly absent. Compared to many other starches, potato starch also contains phosphorus, which is believed to contribute to its unique characteristics (Bertoft, 2017).

1.2 Biosynthesis of starch

The biosynthesis of starch involves several enzymes, as shown in Figure 3, including SS (starch synthases), SBE (starch branching enzymes), DBE (starch debranching enzymes) and GBSS (Granule-bound starch synthase) (Nazarian-Firouzabadi & Visser, 2017). Important enzymes associated with starch production are SS1, SS2, SS3, and GBSS. SS1, SS2, and SS3 form

amylopectin, while GBSS is responsible for making long amylose chains. SS1 elongates short amylopectin chains (DP 6-7), SS2 extends intermediate chains (DP 6-14), and SS3 creates long amylopectin chains. The DP, or degree of polymerization, refers to the length of the polymer chain. Altering the expression levels of these enzymes influences starch properties (Ahmad et al., 2024). Therefore, by understanding the different roles of each enzyme involved in starch synthesis, it is possible to modify starches with specific characteristics for various applications. (Tong et al., 2023).

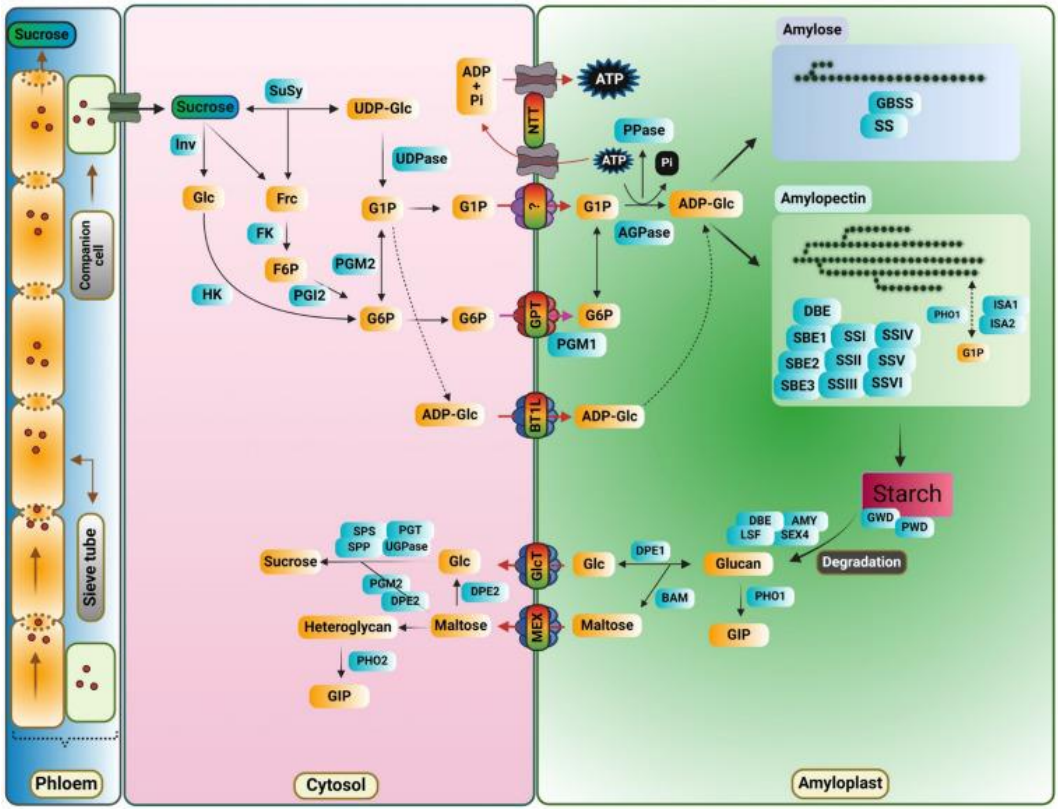

Figure 3 Sucrose imported through the phloem is metabolized through a network of enzymes, producing precursors for starch synthesis. Inside the amyloplast, multiple enzymatic reactions coordinate the formation of amylose and amylopectin. The figure highlights the complex nature of starch metabolism (Achmad, 2024)

Even though there has been much research conducted over the years focusing on understanding starch biosynthesis, much uncertainty remains.

Even though all starch is made up of amylose and amylopectin, there is much diversity in molecular structure between different starches, affecting the characteristics of starches from different plant species and even between different cultivars (Kossmann & Lloyd 2000). Not only is there a complex process with multiple enzymes involved, but the activity of the enzymes also varies depending on the life stage of the tubers. (Hawker, 1979).

1.3 Heterologous expression

One way to further study proteins and enzymes is to express them in various hosts such as mammalian cells, yeast, or bacteria. Among these, *E. coli* bacteria are a particularly suitable host and are currently a key organism used for both production and research. Since *E. coli* has many desirable qualities, such as a high growth rate, relatively simple nutritional requirements, genetic stability and ease of product purification, also *E.coli* can accumulate high levels of recombinant proteins, sometimes as much as 50 % of its total cellular proteins. (Francis & Page, 2010). This makes *E. coli* an attractive host for large-scale protein production (Inoye, 1999). When expressing a protein, the goal is often to maximize the yield of soluble protein. To achieve this, several parameters can be optimized, such as the growth media and growing conditions. By using optimal media along with testing various conditions such as inducing concentration, incubation time, and temperature, it is possible to achieve conditions with high protein yield and quality (Taylor, 2017). However, it also presents some significant challenges when expressing eukaryotic proteins in bacteria. When expressed in *E. coli* with its high growth rate, overexpression often leads to misfolded proteins and inclusion bodies since the folding machinery of *E. coli* is much simpler with more limitations compared to the native host (Gopal & Kumar, 2013), as well as problems with post-translational modifications that usually occur in eukaryotic cells (Francis & Page, 2010).

To control the expression of the gene of interest, it is possible to use vectors containing a “laclq-lac promoter-operator complex” (Khan et al., 2008), making it possible to repress the expression of the gene. In the complex, a repressor is binding to the operator downstream of the promoter. The expression can then be activated by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG), a structural analogue of lactose that binds to the repressor and allows for transcription, thus making it possible to control the expression of the gene (Khan et al.).

In addition to expressing the protein, the gene itself needs to be introduced into the bacteria, for example, via a vector. Gateway from Invitrogen is a widely used system for this. By conducting a BP reaction followed by an LR reaction, it is possible to generate a destination vector that can be inserted in a bacterial strain, followed by expression (Invitrogen, 2010). Figure 4 shows how the gene is inserted using flanked attachment (att-sites) sites to recombine it into donor and destination vectors, catalyzed by different enzymes.

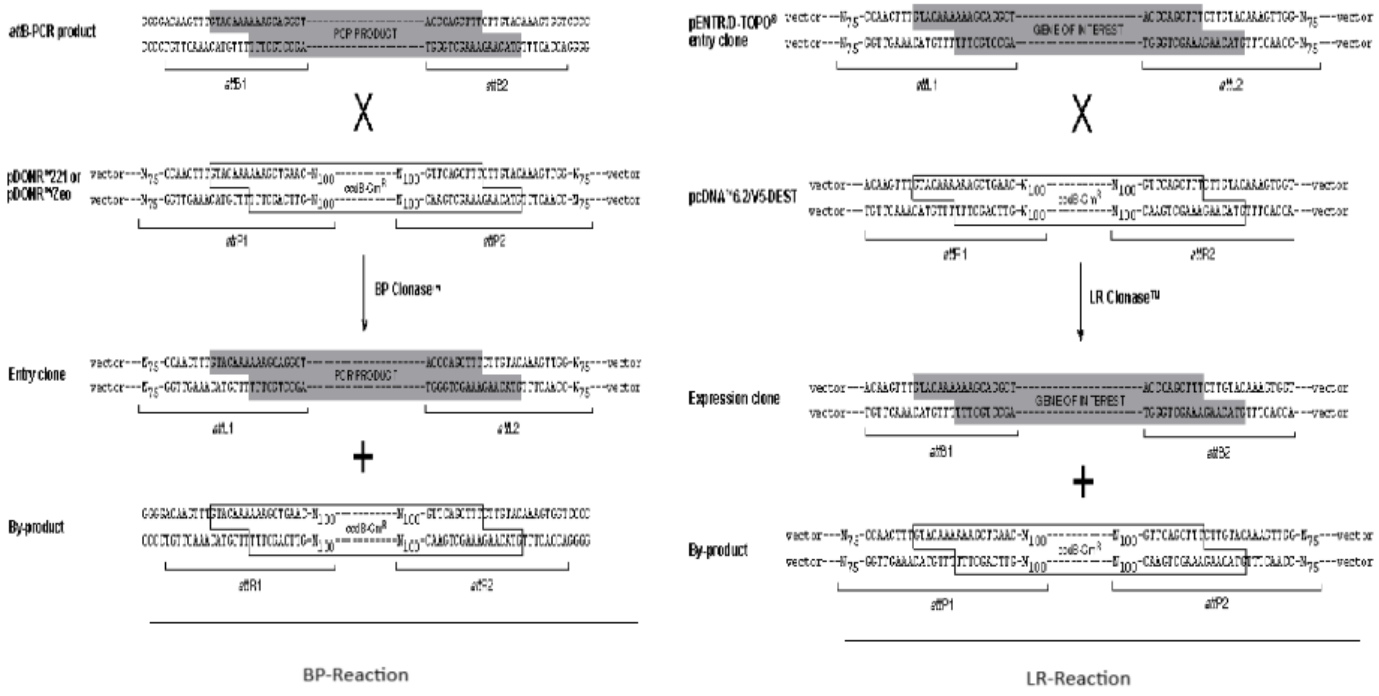

Figure 4. Figure illustration BP and LR reaction. (Invitrogen, 2010)

Achieving expression of soluble, functional proteins in *E. coli* allows for further research and various applications, such as pharmaceuticals and genetic engineering. After the BP and LR reaction is performed, it is important to evaluate the sample to ensure the insert is present. Since sequencing is costly, most researchers test their samples either through colony PCR or with restriction enzymes before sending them for sequencing (Miguel, 2013). When using PCR, one of the most critical steps is designing the primers correctly, as this is a common reason why the desired PCR product amplifies a fragment size different from what was expected. Usually, the longer the DNA sequence, the more issues arise when choosing primers, and the more DNA sequence available, the better the chance to find suitable candidates. By selecting suitable primers, it is possible to avoid issues such as primer-dimers and self-complementarity, as well as low melting temperatures and the correct ratio of GC base pairs (Rychlik, 1995). Another way to indicate the presence of the insert is to use

restriction enzymes. A restriction enzyme has specific cut sites within the sequence, and by selecting suitable restriction enzymes based on the sequence, it is possible to analyze the insert by examining the fragment sizes after digestion with the chosen restriction enzyme (D.C, 2011).

A widely used method for checking protein expression is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In this technique, proteins are denatured with the anionic detergent SDS, which also negatively charges the protein, allowing them to migrate through a polyacrylamide gel according to their molecular mass (Nowakowski et al., 2014). However, SDS-PAGE only indicates protein expression and does not provide information on protein activity. To overcome this limitation it is possible to use a Native-PAGE. In this approach, proteins are not denatured, and migration through the gel depends not only on size but also on folding and charge. After electrophoresis, the gel can be incubated under conditions that allow proteins to act on a substrate, making it possible to test whether they are functionally active aswell. (Nowakowski et al., 2014).

This project aimed to obtain soluble expression of SS1 protein derived from potato suitable for downstream applications. Due to the time limit of this project, it was not possible to perform a zymography to study potential activity. The result will be focused on the optimization of the protocol regarding heterologous expression of ss1 in *E. coli*. Potential further studies for activity will be presented during the discussion.

Materials

2.1 Bacterial Strains and Plasmids

- *E. coli* BL21 (OneShot, Invitrogen; Cat. No. 10543-015)
- pDONR221 – Gateway donor vector (Invitrogen; Cat. No. 12535017)
- pDEST17 – Gateway destination vector (Invitrogen; Cat. No. 11803012)
- *E. coli* TOP10 (OneShot, Invitrogen; Cat. No. C404010)

2.2 DNA Fragments and PCR Products

- TC + StSS1 fragment flanked with attB1 and attB2 sites

2.3 Primers

- R-SS1 (5′→3′): GAT TTG TGG GTG TTT GCA GAG
- F-DEST17 (5′→3′): GAA GGC TCT CAA GGG CAT
- F-DONR221 (5′→3′): GAT TTG TCC TAC TCA GGA GAG C

2.4 Reagents and Enzymes

- BP Clonase II Enzyme Mix – for BP reaction (Invitrogen; Cat. No. 11789020)
- LR Clonase II Enzyme mix – for LR reaction (Invitrogen; Cat. No. 11791020
- Proteinase K – for termination of BP reaction (Invitrogen; Cat. No. E00491)
- DreamTaq Green PCR Master Mix (2×) (Invitrogen; Cat. No. K1081)

- Coomassie Brilliant Blue G-250 staining reagent (prepared in-house)
- LR Cl

2.5 Chemicals

- Isopropyl β-D-1-thiogalactopyranoside (IPTG) – for induction (Invitrogen; Cat. No. 15529019)
- Ethanol

2.6 Buffers

- TE buffer, pH 8.0
- 5× LR Clonase buffer
- 1× Novex Tris-Glycine Native running buffer (prepared in-house)
- Lysis buffer (pH 7.3, prepared in-house, 1000 mL):
    - 0.945 g Monobasic $NaPO_4$
    - 6.1344 g Dibasic $NaPO_4$
    - 17.532 g NaCl
    - 100 mL Glycerol
    - 4 mM DTT
    - Lysozyme (150 µg/mL)
    - DNase (0.005 µg/mL)
    - $MgCl_2$ (0.005 µg/mL)
    - Complete EDTA-free protease inhibitor tablet (1 tablet/10 mL)

2.7 Media

- S.O.C Medium (Invitrogen; Cat. No. 15544034)
- LB agar + Kanamycin (50 µg/mL; prepared in-house)
- LB agar + Ampicillin (100 µg/mL; prepared in-house)
- LB broth + Kanamycin (50 µg/mL; prepared in-house)

- LB broth + Ampicillin (100 µg/mL; prepared in-house)
- LB broth (standard)

2.8 Gels

- Novex Tris-Glycine Mini Protein Gel, 8%
- 1% Agarose gel with GelRed (prepared in-house)

2.9 Kits

- GeneJet Plasmid Miniprep Kit – for plasmid isolation (Invitrogen; Cat. No. K0502)

2.10 Equipment and Instruments

- Shaking incubator
- Incubation chamber
- Refrigerated high-speed centrifuge
- Microcentrifuge
- Thermal cycler (PCR machine)
- Spectrophotometer
- UV transilluminator
- Gel documentation system
- Sonicator

Method

3.1 BP reaction

A BP recombination reaction was performed between the attB-flanked SS1 gene and the attP–containing pDONR221 to generate an entry clone, as shown in Figure 5.

0,5 µl of the attB-flanked SS1 gene (150 ng/µl) and 1 µl of pDONR221 (150 ng/µl) were added to 6,5 µl of TE buffer (pH 8.0) at room temperature. Then, 2 µl of BP Clonease enzyme mix was added to the reaction, which was vortexed and briefly spun in a microcentrifuge. The reaction was incubated for 1 hour. After incubation, 1 µl of proteinase K solution was added, vortexed, and incubated at 37°C for 10 minutes. One-shot TOP10 *E. coli* was thawed on ice, and 5 µl of the ligation reaction was added. The cells are very fragile, so they were mixed by gently tapping. The sample was incubated on ice for 30 minutes, followed by a heat shock at 42°C, and then returned to ice. 250 µL of recovery S.O.C. medium was added to the sample in a sterile environment, followed by incubation in a shaking incubator at 37°C and 225 rpm. Transformation was then spread on LB agar plates containing kanamycin at 50 µg/ml. Plates were incubated overnight at 37°C. The plates were visually checked the following day to examine the growth of colonies.

Figure 5. A BP reaction enables the recombination of an attB substrate with an attP substrate (donor vector) to generate an attL-containing entry clone, using the BP Clonase enzyme mix to catalyze the reaction.

To examine whether the transformation was successful, restriction enzymes were used to verify the potential positive sample before sequencing. Five colonies were selected for overnight culture and collected in 7 mL of liquid LB media with 50 µg/mL kanamycin at 37°C. Samples were taken after 18.5 hours and transferred to 2 mL microcentrifuge tubes for plasmid isolation. The samples were centrifuged at 12,000 rpm for 2 minutes, and the supernatant was discarded. Plasmid isolation solutions and buffers were obtained from the GeneJet miniprep kit by Invitrogen. The pellets were resuspended in 250 µL of resuspension solution. Then, 250 µL of lysis solution was added and mixed by inverting the tube five times. The sample became slightly transparent and viscous. Next, 350 µL of neutralization solution was added and mixed by inversion. The samples were centrifuged at 12.000 rpm, and the supernatant was transferred to GeneJET spin columns. The spin columns were centrifuged at 12.000 rpm, and the flow-through was discarded. 500 µl of wash solution was added, followed by 1 minute of centrifugation. The flow-through was discarded, and the wash procedure was repeated. After the last wash, all samples were centrifuged for 1 minute to ensure the removal of any ethanol residues. The collection pellet in the spin column was transferred to a new 1.5 mL microcentrifuge tube, and 50 µL of elution buffer was added. In new 1.5 mL microcentrifuge tubes, 2 µl of plasmid DNA from each sample was mixed with 2 µl of 10X Fast digest buffer, 12 µl of nuclease-free water, and 1 µl of each restriction

enzymes; Eco321 and BamHl which had one expected cut site in the insert at 4472 bp, while the control (empty pDONR) was expected to have two cut sites at 1165 bp and 3596 bp. All samples were incubated at 37°C for 12 minutes. Immediately after incubation, the sample was placed on ice and then loaded in a 1% agarose gel with GelRed and run at 100 V for 40 minutes. An empty donor vector was used as a control. The sample was derived from an isolated plasmid from a positively tested colony.

3.2 LR reaction

LR reaction was performed to recombine the entry clone from the BP reaction using an enzyme mix to create the expression clone, as seen in Figure 6. The entry clone derived from a positive-tested plasmid was used for the LR reaction. 1 µl of entry clone, 416 ng/µl, was mixed with 2 µl destination vector, 150 ng/µl, 4 µl 5X LR Clonase reaction buffer, and 9 µl TE buffer. 4 µl LR Clonase enzyme mix was added and briefly vortexed. The reaction was incubated for 1 hour at 25°C. 2 µl of proteinase K, two µg/µl, was added and incubated for 10 minutes at 37 °C. 5 µl of of the reaction was added to 50 µl BL21 cells and mixed by gently tapping the competent cells and immediately put on ice and incubated for 30 minutes followed by heat shocking the cells for 45 seconds at 37 °C, then incubated on ice for 2 minutes. 950 µl of recovery Medium was added. Samples were incubated at 37°C for 1 hour in a shaking incubator set to 210 rpm. After incubation, the samples were spread on LB plates containing ampicillin, 100 µg/mL, in a sterile environment to prevent contamination. Plates were incubated overnight at 37 °C.

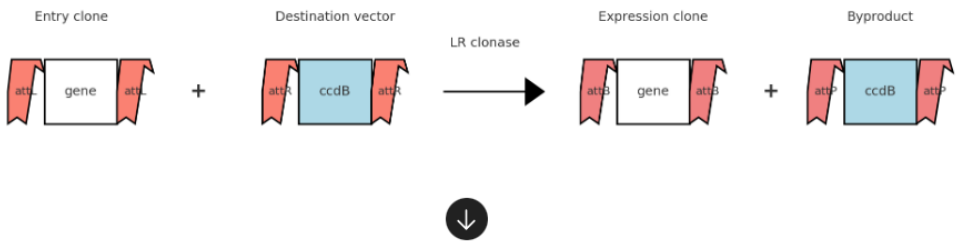

Figure 6 Enables recombination between an attL substrate (entry clone) and an attR site (destination vector) to produce an attB-containing expression clone. The reaction was catalyzed using LR Clonase enzyme mix.

The same procedure as in 3.2 was performed to confirm the LR reaction. A new restriction enzyme, Ppu21I, was used with three expected cut sites in the sample at 537 bp, 2510 bp, and 3580 bp. The control (empty pDEST17) had one expected cut site at 6354 bp. PCR was also used to confirm the LR reaction, the following protocol was used:

For the PCR assessment five samples was collected from the plates from the BP reaction with pipette tip and resuspended in 200 µl MQ . 1 µl of sample was mixed with 416 ng/µl of DNA and 225 µl of DreamTaq Green PCR Master Mix, 1 µl forward primer µM, 10 µand 1 µl reverse primer, 10 µM and 198 µl nuclease free water. Controls used; C1: Empty Destination vector, C2: MasterMix + Primers, C3: MasterMix – primers. The cycles for the PCR:

Table 1. Thermocycling of PCR

Step	Temp, °C	Time (Minutes)	No. Cycles
Initial denaturation	95 C	3:00	1
Denaturation	95 C	0:30	35
Annealing	58 C	0:30	
Extension	72 C	0:25	
Final extension	72 C	7:00	1

The primers were designed in CLC Workbench with a Tm value of 61.5 °C to -5 °C and between 18-25 bp. A suitable sample was selected and streaked on new ampicillin agar plates (100 µg/mL). These plates were used as the sample for expression.

3.3 Expression

To achieve expression, several parameters must be considered, including varying IPTG concentrations, incubation temperatures, and incubation durations. Therefore, the following procedure was performed multiple times. This outlines what was found to be potentially optimal. The different runs will be further discussed in the results and discussion sections.

Samples were collected from ampicillin agar plates dedicated to expression using an inoculation loop and then incubated in 10 mL of LB broth overnight. Empty BL21 cells were used as controls and were incubated overnight, then subjected to the same protocol. When collected after 16.5 hours, 4 mL of the culture was added to 400 mL of LB broth, divided into two sterile Erlenmeyer flasks (1000 mL), and incubated at 37°C on a shaking table at 210 rpm. Samples OD was measured from the starting point until the OD reached between 0.4 and 0.6. 1 mL of IPTG (100 mM) was added to induce expression, resulting in a final concentration of IPTG of 0.5 mM. Samples were incubated for 3 hours at 37°C, shaking at 210 RPM.

Samples were collected and OD was measured, followed by centrifugation at 4°C and 4000 rpm for 15 minutes. The supernatant was discarded, and the pellet was resuspended in 4 mL of lysis buffer (as specified in material 2.6), then incubated on ice for 30 minutes. All samples were then sonicated on ice for 10 seconds, followed by a 30-second rest, and the process was repeated six times. A 1 mL aliquot of whole cell extract was collected, and the remaining samples were centrifuged at 4°C for 45 minutes at 14,000 rpm in a high-speed centrifuge. The soluble fraction and pellet were separated. Samples and controls were loaded onto a cold Novex Tris

Glycine protein gel (8%) and run at 100 V in 1x tris-glycine running buffer. The entire process was conducted in a cold room at 4°C. The gel was collected after 2 hours and stained with Coomassie Blue. Finally, the gel was examined for results.

Figure 7 presents a schematic overview of the workflow used in this study, where it is possible to follow the gene insert from the BP reaction to the expression test. Some steps, such as SDS-PAGE, are not described in the Methods section since they were not performed due to time constraints. However, they are discussed in the Introduction and the Discussion sections.

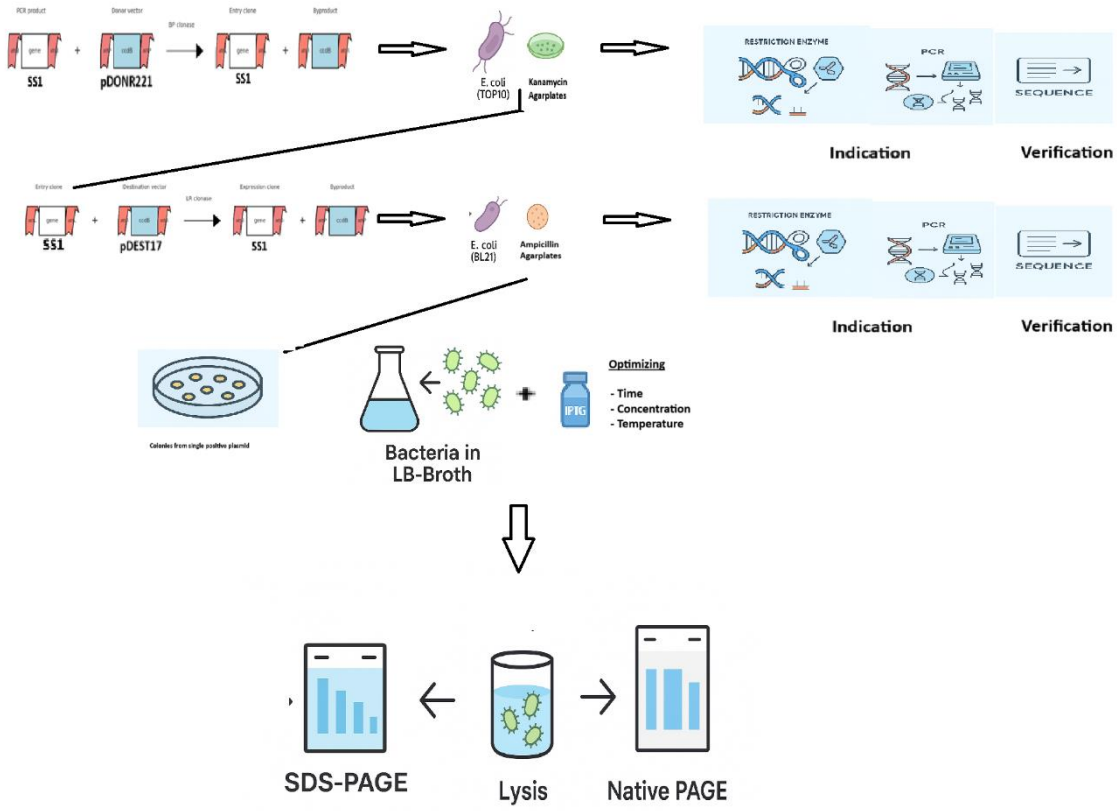

Figure 7. Illustration of workflow

Results

4.1 BP reaction

The BP reaction was conducted to generate an entry clone containing the SS1 insert; no issues were noted during the actual reaction. Samples collected from agar kanamycin plates (50 µl/mL) for plasmid isolation and electrophoresis gel run, no issues were encountered during plasmid isolation. Figure 8 presents a UV image of the electrophoresis gel run with samples from the BP reaction. Controls (empty pDONR) show two distinct bands around the 5000 bp and 1500 bp marks, while the samples have one band slightly above the control and around the 5000 bp mark.

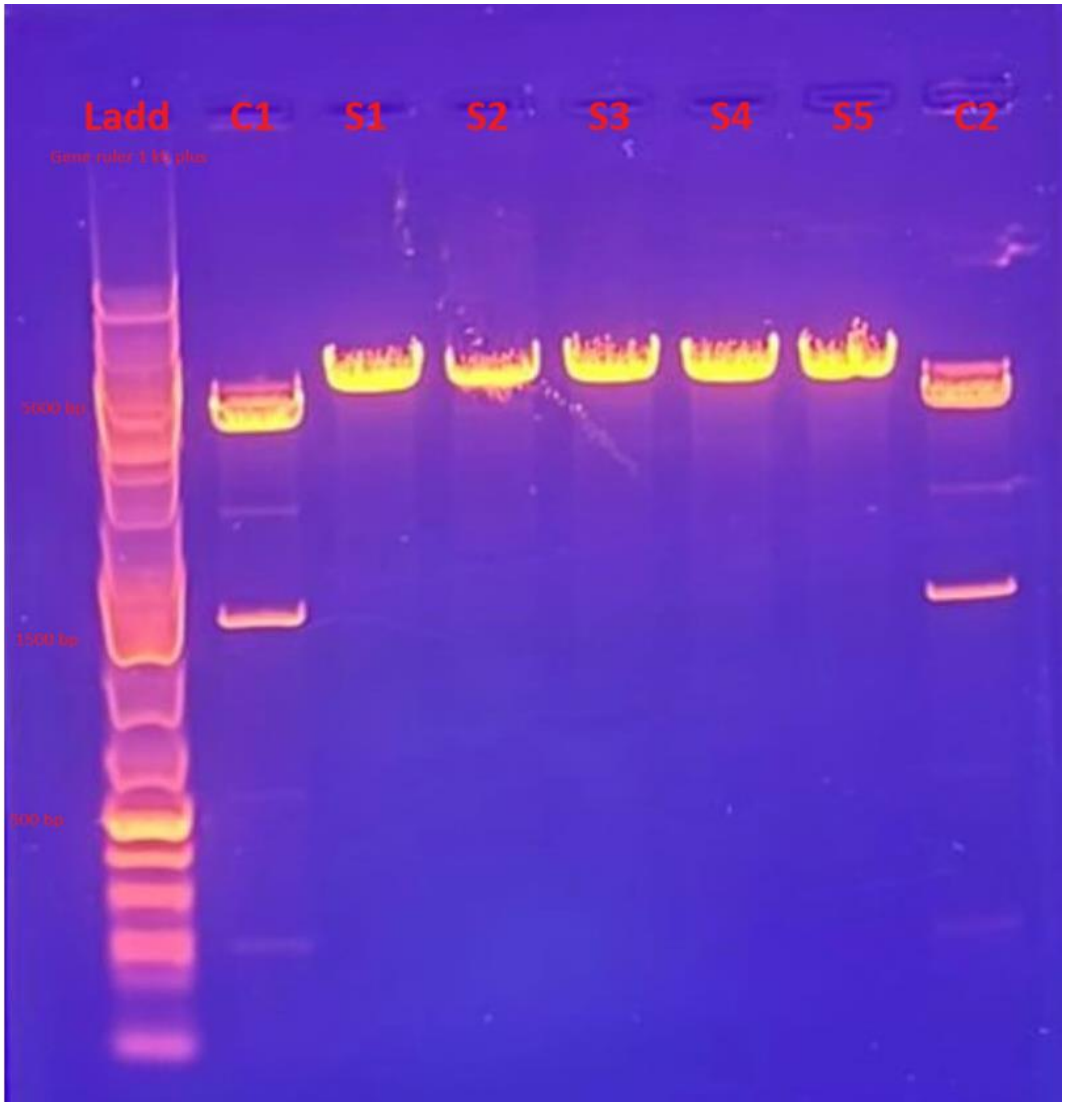

Figure 8 Agarose gel electrophoresis after digestion with restriction enzymes Eco321 and BamHI

Sample 1 was sent for sequencing and came back with positive results, confirming that the entry clone contained the SS1 insert.

4.2 LR reaction

An LR reaction was conducted to perform a recombination between the attL entry clone and the attR destination vector to generate an expression clone. Samples were collected for overnight culture and exhibited a milky color. Samples from the overnight culture were collected and diluted for the PCR run.

Figure 9 presents results from PCR with controls. C1 (Empty pDest) is showing very faint bands while C2 (MasterMix) and C3 (Mastermix + primers) show no bands. Samples 1, 2, and 3 show bands around 750 bp and 300 bp. Sample 4 shows no bands, while sample 5 exhibits bands at approximately 750 bp.

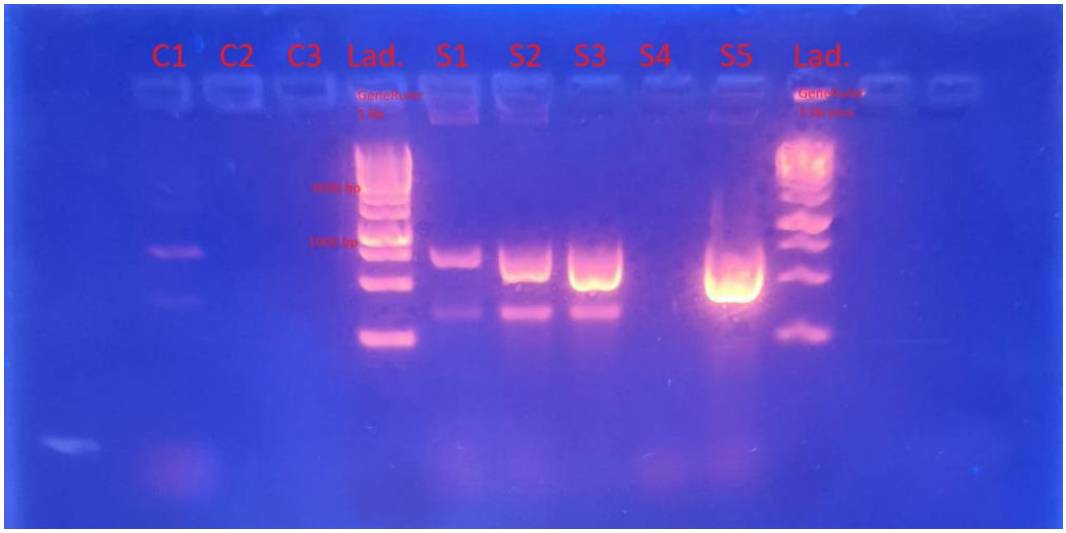

Figure 9 Agarose gel electrophoresis UV image after PCR on LR reaction samples.

Samples from overnight culture were also collected for plasmid isolation and digested with restriction enzyme Ppu21I, which has three expected cuts in the sample: 537 bp, 2510 bp, and 3580 bp. For the control (empty pDest17), there is one expected cut site at 6354 bp. Figure 10 shows a UV image of the electrophoresis gel after digestion with the restriction enzyme. Both controls display one band above 6000 bp; sample 1 shows three bands at approximately 500 bp, 3000 bp, and 4000 bp. Samples 2-5 exhibit the same bands as sample 1, with two additional bands appearing near 6000 bp and 8000 bp.

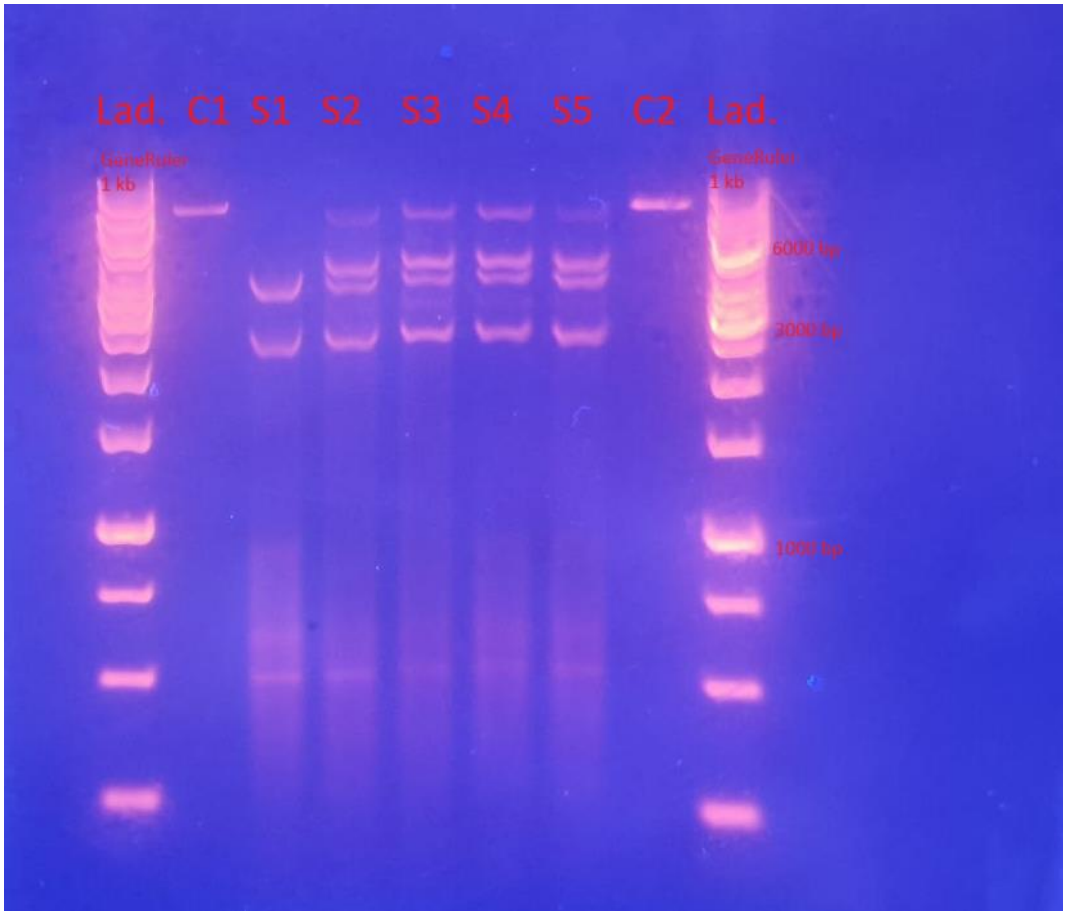

Figure 10 agarose gel electrophoresis UV-image after restriction enzyme

Sample 1 was selected for sequencing with positive confirmation of the insert. It was collected and streaked on new ampicillin agar plates (100 µl/mL).

4.3 Optimization for expression

Samples from an agar plate with colonies derived from sample 1 from the LR reaction were collected for an overnight culture. 3 mL of culture was collected from the overnight culture and added to 300 mL of LB broth. Samples were collected at OD 0.507. Samples were divided into three and induced with 0,2 mM IPTG. Samples 1 and 2 were incubated at 28 °C, and S1 was collected after 1 hour and S2 after 2 hours. Lysis and sonication were performed, and the samples were then frozen in liquid nitrogen. S3 was induced with 0.2 mM IPTG and incubated at 22 °C. Lysis and sonication

were performed on samples and control, samples from soluble, pellet and lysis fraction was collected from all samples and loaded on Native-PAGE with Novex Tris-glycine protein gel. Figure 11 presents the electrophoresis run with Novex gel. All samples and controls exhibit a smear, and there is no visible difference between the sample and the control.

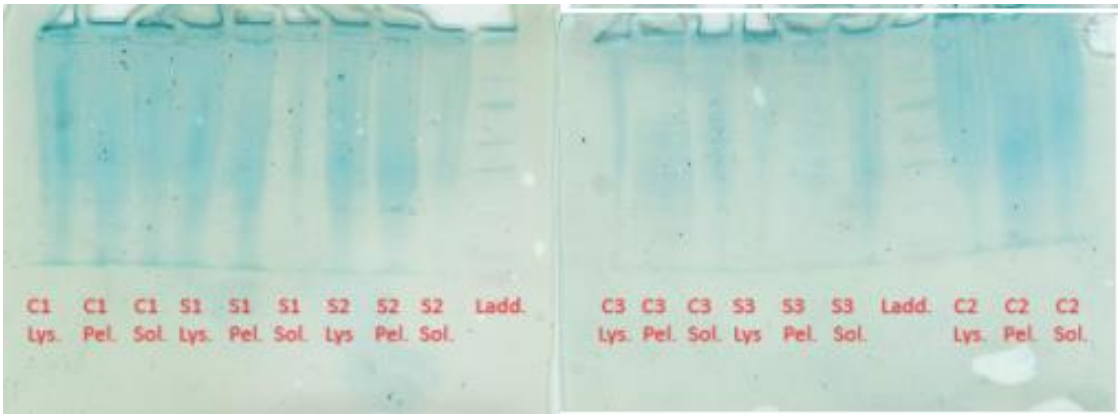

Figure 11. Electrophoresis was run on a Novex protein gel with three different inducing conditions.

Figure 12 shows the results from a gel run on a native PAGE with Novex Tris-Glycine protein gel. Two samples were prepared and collected when they reached two different OD levels: S1 with OD 0,57 and S2 with OD 0,50. C1 was collected with OD 0,71 and C2 with OD 0,58. All samples and controls were induced with 0.2 mM IPTG in 100 ml of LB broth and incubated at 37 °C for 5 hours. Samples and control are smeared without any distinct bands, and there is no noticeable difference between the control and the samples.

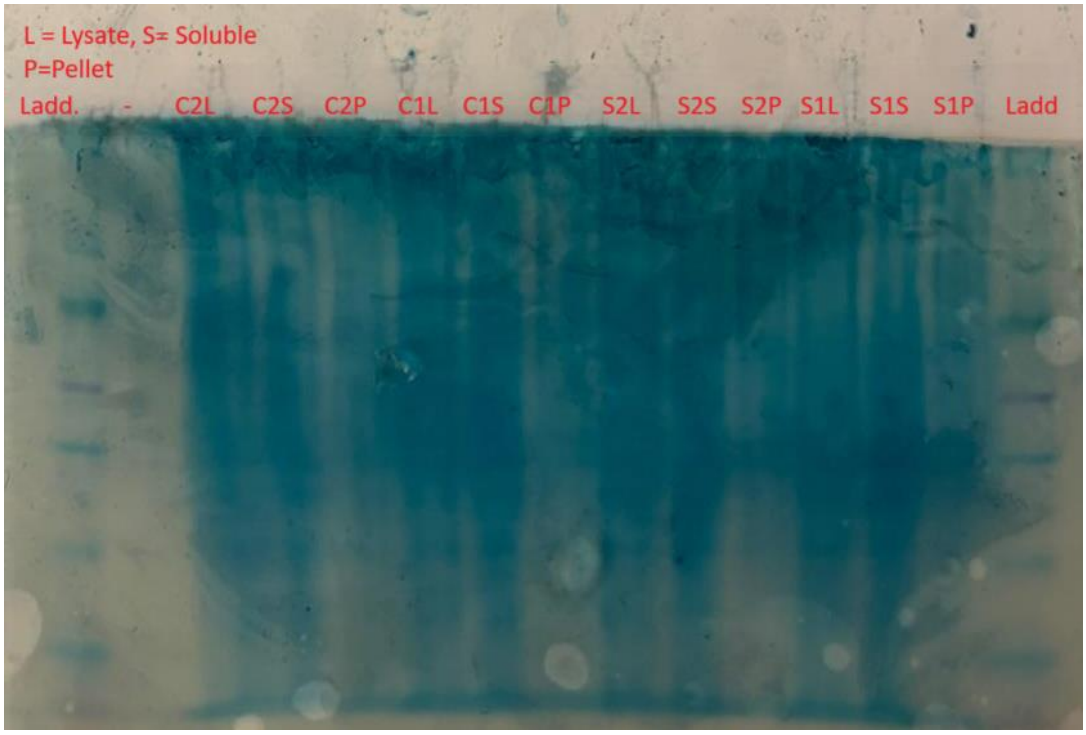

Figure 12 Electrophoresis on Novex protein gel, samples induced at different ODs

Figure 13 shows the results from the gel run on native-PAGE wth Novex Tric-Glycine protein gel. Samples were collected at OD 0,450, and the control at 0,659. Both the sample and control were IPTG-induced with 0.5 mM IPTG in 400 mL LB broth and incubated for 18.5 hours at 22°C. No distinct bands or difference between control and sample was noticed. Figure 14 shows a visual difference observed between the sample and control. The left picture shows collection after incubation and the right picture shows samples after centrifugation and supernatant discarded before Lysis. Sample is clear compared to a yellow tone in control

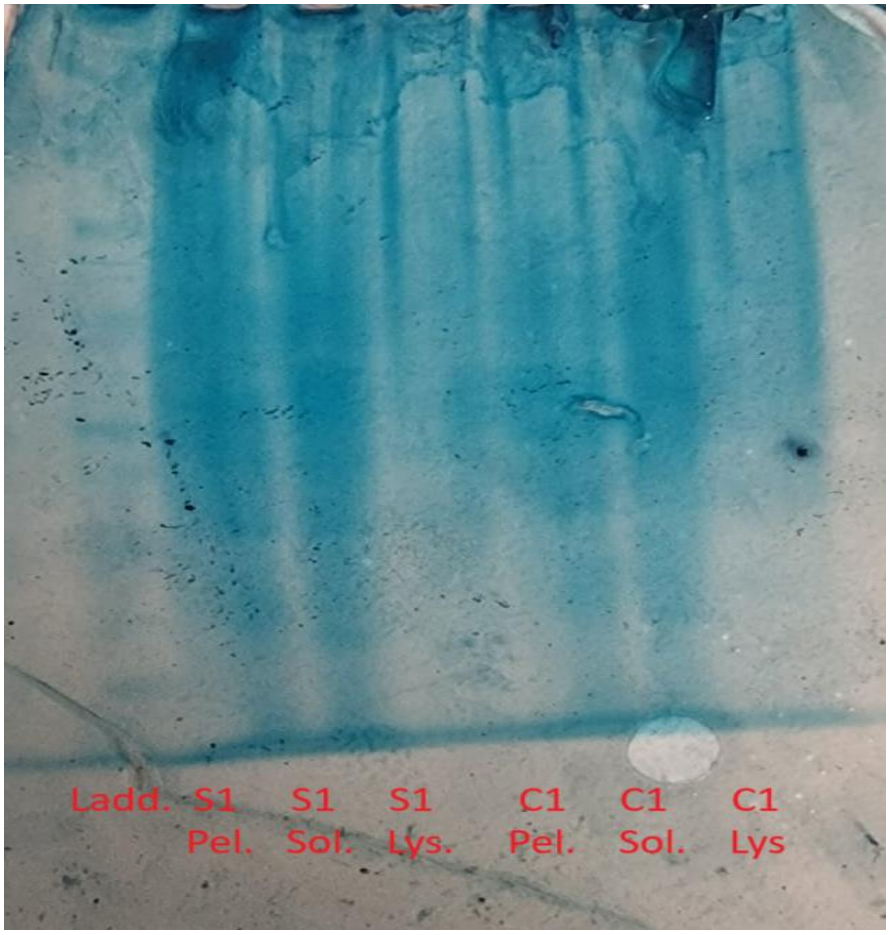

Figure 13 Electrophoresis on Novex protein gel with 0,5 mM IPTG concentration

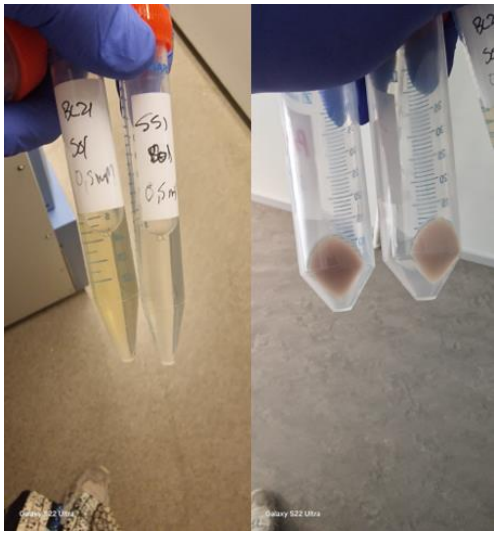

Figure 14 Visual difference between sample and control before lysis

Discussion

This project aimed to express SS1 in *E. coli* using the Gateway cloning protocol from Invitrogen. Much of the process when using IPTG to induce expression in *E. coli* involves optimizing induction conditions. Due to time constraints and some unexpected results, many experiments and procedures needed to be repeated to find favorable conditions for expression. Therefore, this discussion will focus on potential ways to express SS1 with IPTG and identify possible errors. Since there is no confirmation that SS1 was expressed, the discussion will also explore potential methods to further investigate both the expression and activity of SS1 in *E. coli*.

BP Reaction

The results from the BP reaction initially searched for indications of successfully inserted PCR products by colony PCR. This PCR run was not included in the results, as they were inconclusive.

Therefore, it was decided to proceed with restriction enzymes as an alternative to PCR. The restriction enzymes were expected to cut at control sites of 1165 bp and 3596 bp, and in the sample at 4472 bp. Compared to the ladder, the fragments appeared larger than expected cuts. However, samples showed cuts slightly larger than the control, while the control had two fragment sizes smaller than the sample, which was expected. Even though the cuts did not display fragments of the correct size compared to the ladder, it is important to consider that there can be many reasons for the differences in migration, especially since the ladder was based on linear DNA fragments while the samples were plasmid DNA. Therefore, the band placement was sufficient indication to send the plasmid for sequencing. The sequence confirmed that the insert into the entry clone from the BP reaction was successful.

LR reaction

After confirming the insertion in the entry clone, an LR reaction was initiated. To evaluate whether the entry clone had been recombined into the pDEST17 destination vector, PCR was initially used. Controls behaved as expected with only faint bands on the empty vector. Samples, on the other hand, showed two distinct bands, suggesting that more than one fragment was amplified during the PCR. After evaluating the initial primers, it became clear that they were optimized for plant DNA sequences rather than for *E. coli*, which can lead to non-specific bindings. This could be the reason for the multiple fragments seen in the PCR. There are also other factors to consider when troubleshooting faulty PCR results, such as annealing temperature, even though primers are regarded as the most critical component of a successful PCR run. (Bustin & Huggett 2017). It was therefore decided to proceed with restriction enzymes to investigate if LR reaction was successful. Restriction enzyme Ppuc21I was used to digest samples and controls to determine if the entry clone containing SS1 was recombined into pDEST17. For a successful insert, it was expected to see three fragment sizes in the sample and one larger fragment in the control. The controls displayed one larger cut as expected. Sample 1 shows three fragments at expected sizes, indicating the insert was successful. Samples 2-5 also display fragments of the correct size along with two larger fragments. This issue might be caused by the restriction enzyme not fully digesting the substrate, which leads to partially or undigested fragments migrating differently in the gel. This can result from a high substrate concentration or a short digestion time. Based on the results after using restriction enzymes, it was decided to send sample 1 for sequencing. The sequence confirmed the presence of SS1.

Protein expression

When assessing protein expression, the results were less definitive. Even with varying IPTG concentrations, incubation times, and temperatures, the

native gels did not display any clear bands compared to controls. Instead, there was smearing and faint bands.

One possible explanation is that SS1 was expressed but misfolded and accumulated into inclusion bodies. The absence of a clear band specific to SS1 also indicates that expression levels may have been too low to be detectable with Coomassie staining. When the IPTG concentration was increased to 0.5 mM along with a larger amount of the expression sample, a noticeable visual difference appeared between the sample and the control. This difference was not observed at lower IPTG concentrations used for induction. While it does not confirm expression, the visual difference might suggest altered metabolic activity between the control and expression cultures. Additionally, it could have been considered to primarily use an SDS page, as migration is based solely on size. Therefore, if the protein is expressed but misfolded, it would still be possible to detect expression and, in the next phase, adjust induction conditions to test for activity. Due to time constraints and the fact that SS proteins extracted from potatoes were successfully shown using Native-PAGE in a previous research conducted by Felix Gerlam in 2025, it was decided to proceed directly with Native-PAGE after inducing expression. Since that project was based on protein extracted from the native host, it might not be comparable. Therefore, for future studies, it is recommended that expression first is tested using SDS-PAGE. This is also suggested in a study by (Zerb, 2014).

Technical limitations and future directions

As further discussed, there may be other interfering parameters, so it could very well express SS1; the protocol just isn't optimized enough to detect that expression.

Since misfolding and inclusion bodies are often related to overexpression (*Miguel*, 2013), one approach is to induce expression at colder conditions for a longer period. Due to limited resources, it was not possible to induce expression below 22 Celsius.

These challenges are brought up in the report by (Miguel, 2013), where they optimized the conditions to express protein P5βR and P5βR2 in E.Coli. They tested a range of temperatures from 4 °C to 37 °C after induction with IPTG 0,3 mM, only samples grown at 15 °C or lower produced soluble active protein.

During the test, we also attempted to stain the gel with InVision His-Tag Gel Stain, but the outcome was the same as using Coomassie Blue. The His-Tag Gel Stain is an option because it only binds to the N-terminal His-tag. This was not reported in the methods or results, as the staining solution used had expired. Therefore, it was difficult to determine the reason for the inconclusive results.

Overall, there are several parameters to consider when aiming to express protein in *E. coli* using IPTG-induction, such as induction growing conditions, media composition, and gel staining, to mention a few. However, the benefit of successfully expressing a ss1 in *E. coli* can outweigh the effort. It allows for a deeper understanding of starch biosynthesis and the roles of SS proteins.

Conclusion and insights

This study emphasizes that many parameters must be considered when aiming to express soluble protein in *E. coli* heterologously. It is crucial to establish a workflow that allows easy testing of multiple parameters to optimize conditions for expression in *E. coli* as a host. However, studying specific proteins is challenging, and expressing them for purification with endogenous expression is even more difficult. Successfully expressing soluble, active SS1 in *E. coli* not only opens up opportunities for further studying SS1 but also provides a guide for expressing other homologous SS proteins from potato and exploring the mechanisms of these enzymes. This offers the potential to genetically engineer potatoes for starch production for various applications. Over time, this can lead to custom-made, purpose-specific starch potatoes for agriculture and industry without the need for postharvest modifications.

Although the original goal of obtaining soluble expression of SS1 in *E. coli* was not achieved, this study has been valuable. The successful insertion of SS1 into the entry clone was confirmed by sequencing, providing a solid starting point for future work. Expression after the LR reaction could not be verified, but by testing different induction conditions, this project helped identify potential challenges and narrowed down directions for further optimization. This study highlights both the complexity of heterologous protein expression and the importance of method development in future studies.

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Bilaga 1 TC + stSS1 (attb1, attb2)

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