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| Tips and Tools

Yeast recombinational cloning for heterologous biosynthesis of polyketides: a molecular microbiology laboratory module for undergraduate students

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ABSTRACT Recombinant plasmids are essential tools in molecular biotechnology, and reliable plasmid assembly methods have, therefore, become a prerequisite for the successful cloning and transfer of genes. Among the multitude of available plasmid assembly strategies, *in vivo* homologous recombinational cloning in yeast has emerged as a cost-effective and relatively simple method. Since we use this method routinely in our group for assembling large plasmids with secondary metabolite gene clusters and for direct heterologous production of polyketides in *Saccharomyces cerevisiae*, we developed an exercise module for undergraduate students where they would get hands-on experience with these molecular practices. The exercises target several molecular techniques, including PCR, restriction enzyme digestion, and yeast recombinational cloning. The students will learn about plasmid assembly and yeast transformation methods by performing these experiments while inherently acquiring new skills valuable for their subsequent laboratory work or projects.

KEYWORDS plasmids, yeast, *Saccharomyces cerevisiae*, cloning, transformation, laboratory exercise module, microbiology, biotechnology, problem-based learning

Plasmid assembly is one of the cornerstones of molecular biotechnology; therefore, it is not surprising that numerous *in vitro* methods are available, including restriction enzyme digestion- and ligation-based cloning (1), gateway cloning (2), Gibson assembly (3), and Golden Gate cloning (4). An alternative, more cost-effective method is *in vivo* homologous yeast recombinational cloning in *Saccharomyces cerevisiae*, where amplified DNA fragments are assembled with the help of overlapping sequences of as little as 15 bp (5, 6). We routinely use this method for constructing plasmids with large secondary metabolite gene clusters in a single step (>50 kbp) (7) and also for direct heterologous production of polyketides in *S. cerevisiae* (8).

Laboratory exercises offer students a hands-on learning experience and implement the knowledge obtained from the theoretical lectures. Here, we describe a 4-day setup of laboratory exercises where students learn how yeast recombinational cloning can be a fast tool for plasmid assembly and heterologous biosynthesis of secondary metabolites. The target molecule, to be heterologously expressed in *S. cerevisiae*, is the pigment 6-O-demethylfusarubinaldehyde, which is biosynthesized by the polyketide synthase FSR1 (=PKS3) in all members of the fungal genus *Fusarium* (9–12). Colonies with correctly assembled plasmids will emerge with a red coloration, significantly accelerating the verification process. Our exercises, which are based on the idea of active learning, aimed to help students develop higher-order thinking skills to reach the top of the "learning pyramid," where they can synthesize, analyze, and evaluate (13).

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Intended audience

This laboratory module is designed to supplement microbiology classroom courses for second-year undergraduate students in Chemical Engineering and Biotechnology at Aalborg University (https://www.en.aau.dk/education/bachelor/chemicalengineering-biotechnology). The exercise is part of the course Microbial Biotechnology (K-KT-B4-18), where the students are expected to gain knowledge of biochemical molecules, processes, and systems, for projects in technical microbiology, food technology, and biotechnology development or production. These steps are covered in this lab exercise, where the students learn basic molecular techniques in combination with advanced cloning procedures that allow for microbial production of a secondary metabolite. Therefore, the module is suitable for undergraduate students majoring in biotechnology who have been introduced to the basic concepts of cell biology and microbiology.

PROCEDURE

Materials

In this laboratory exercise, *S. cerevisiae* BY4743 (genotype: MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) is used for plasmid assembly and biosynthesis of 6-O-demethylfusarubinaldehyde. Cryostocks of the strain can be obtained from the American Type Culture Collection (ATCC), cat. number ATCC 201390. To enable optimal transcription of *fsr1*, the coding sequence was codon optimized and obtained in a pUC57 plasmid from Genscript. This plasmid designated pCOA-Leu-FvPPT1 also constitutively expresses the PKS helper enzyme *FvPPT1*, which activates the domain of the PKS responsible for movement of the growing polyketide chain. *FvPPT1* originates from *Fusarium verticillioides* and has been codon optimized for *S. cerevisiae*. The constitutive promoters P(TEF-1α) and P(EXP1) from *Yarrowia lipolytica* are used to express the target genes. All plasmids are available through Addgene (pUC57-fsr1—cat. number 179866, pCOA-Leu-FvPPT1—cat. number 179923, and the assembled plasmid pCOA-Leu-FvPPT1-fsr1—cat. number 179868). The instructor resources are provided in Supplemental Material S1, Appendices 1, 2, 3, and 4, while the student materials are provided in Appendices 5 and 6; all appendices are available online. A visual representation of the workflow is illustrated in Fig. 1.

Safety issues

In these exercises, *S. cerevisiae* will be genetically modified, and *E. coli* will be used for plasmid proliferation. These experiments are classified as Biosafety Level I in Denmark and many other countries, including the United States of America. In some countries, the laws may be different and should be followed. 6-O-demethylfusarubinaldehyde has not been reported to be toxic, but since it is a fungal-derived secondary metabolite, it should be treated cautiously.

Experimental design and DNA amplification (day 1)

Students begin the first day of the exercises with a PCR to amplify the plasmid backbone from pCOA-Leu-FvPPT1 and *fsr1* from pUC57-fsr1 and to introduce overlaps in the fragments which will aid the process of plasmid assembly through homologous recombination. While the PCRs are running, the objective and schedule are carefully introduced to the students. Students are given a short sequence of *fsr1* (first 21 amino acids) from *Fusarium solani*, which they should codon optimize for *S. cerevisiae* based on the provided codon usage table. Comparing their results with an automatically generated sequence from software, such as Snapgene, can spark a discussion on essential aspects of the transcription process and why introns are present in filamentous fungi but not in *S. cerevisiae*.

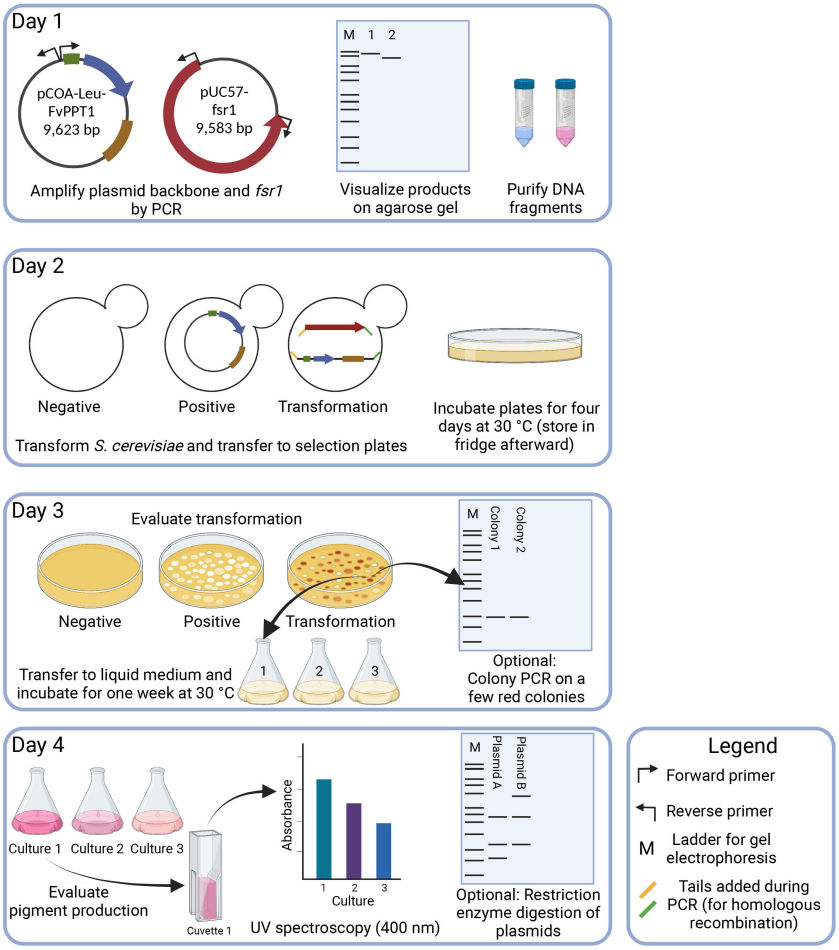

FIG 1 Flowchart of the experiments. The day notations do not reflect a chronological setup, i.e., the experiments do not run for four successive days but can be spread out upon necessity. Created with BioRender.com.

Transformation of *S. cerevisiae* (day 2)

On the second exercise day, students use competent *S. cerevisiae* cells to transform the purified PCR fragments, according to the protocol developed by Gietz and Schiestl (14). As a positive control, the cells are transformed with the intact pCOA-Leu-FvPPT1, while sterile water is used as a negative control. Discussing essential steps of the transformation method with the students allows for a comprehensive understanding of the process.

Evaluation of transformation efficiency and heterologous expression (day 3)

Students count the colonies on the plates containing the assembled pCOA-Leu-FvPPT1-fsr1 and the other two control plates (Fig. 2). Transformed *S. cerevisiae* should produce 6-O-demethylfusarubinaldehyde, conferring the emerging colonies a red pigmentation and thereby allowing immediate visual confirmation of linearized DNA fragments being assembled into the desired plasmid and, hence, successful transformation. Colonies with the correctly assembled pCOA-Leu-FvPPT1-fsr1 plasmid will appear in various red nuances, which can reflect the number of plasmids present in the cells, stage of cell growth, and cell fitness. The students then select three colonies for heterologous biosynthesis of 6-O-demethylfusarubinaldehyde in liquid media. Subsequently, they choose a (or a combination of) carbon source(s) from a selection of mono-, di-, and polysaccharides and add the amount they consider reasonable to a premade nitrogen-based medium lacking leucine. By letting the students choose the carbon source(s) for

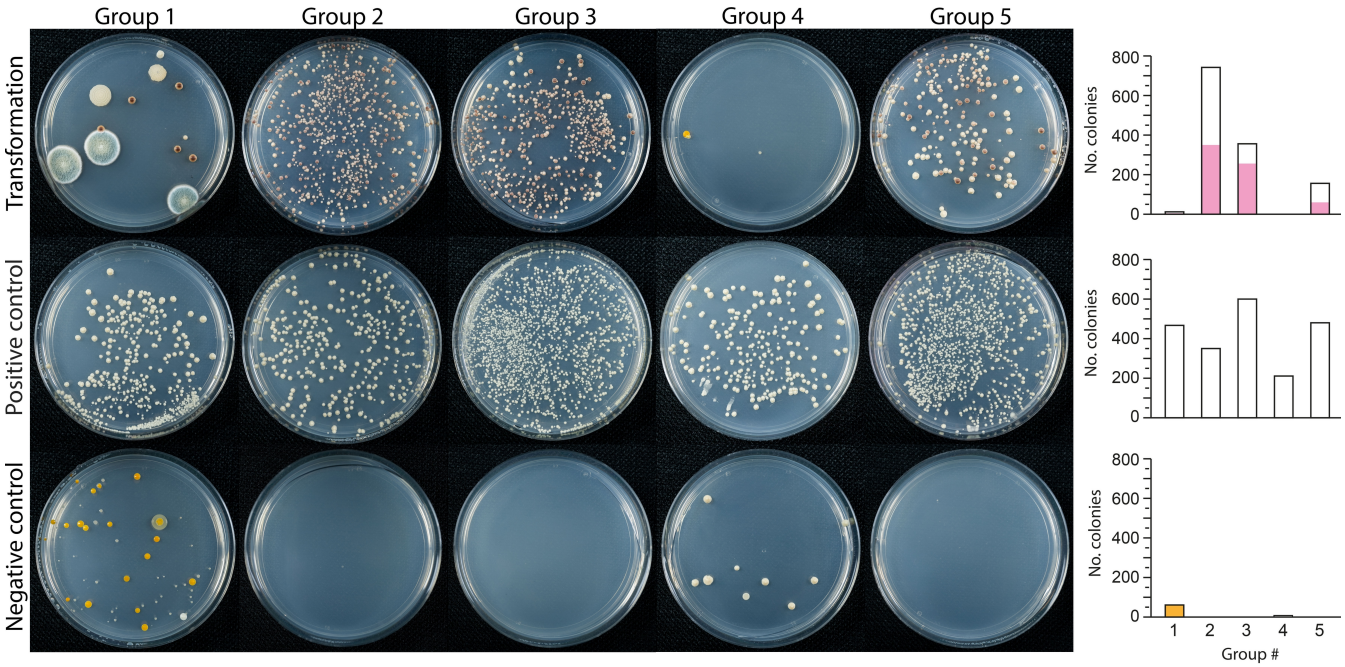

FIG 2 Results from exercises performed by five student groups. Transformation of *S. cerevisiae* with amplified pCOA-Leu-FvPPT1 backbone and fsr1 insert (transformation), preassembled pCOA-Leu-FvPPT1 (positive control), and water (negative control). The results look as expected for groups 2, 3, and 5, while for groups 1 and 4, only the positive control plates displayed the expected results. Conversely, the transformation plates of groups 1 and 4 displayed signs of contamination, potentially suggesting a lack of sterility during one of the steps of the transformation protocol. Additionally, the negative control plates for both groups 1 and 4 displayed unexpected growth, possibly because of cross-contamination for group 1 (due to the color of the colonies) and the addition of the positive control plasmid to the mix for group 4 (due to the colonies resembling the ones on the positive control plate). The bar chart on the right illustrates the number of colonies obtained by the groups. The chart for the transformation is differentiated in red and white to display the proportion of red vs white colonies.

heterologous expression, they are encouraged to study the primary metabolism in which the polyketide precursors, acetyl- and malonyl-CoA, are produced. The carbon source used by the yeast directly to produce acetyl- and malonyl-CoA will likely result in the highest pigment biosynthesis. Optionally, the transformed colonies can be validated directly from the transformation plates with colony PCR.

Quantification of pigment biosynthesis (day 4)

For the last exercise day, the students analyze the pigment biosynthesis of their 1-week-old cultures using UV spectroscopy at 400 nm. Optionally, the pCOA-Leu-FvPPT1 and pCOA-Leu-FvPPT1-fsr1 plasmids, isolated beforehand by the instructor, can be validated with restriction enzyme digestion.

Based on a survey organized at the end of the exercise module, the participating students unanimously agreed that the content was relevant to the course and education. Furthermore, the students answered that it allowed them to gain additional knowledge on top of the lectures (Supplemental Material S2).

CONCLUSION

This laboratory exercise module provides students with the experience of performing plasmid assembly and transformation using yeast recombinational cloning. In this process, the students learn essential molecular tools. Through this design, knowledge from classroom teaching can be transformed and used in a hands-on experience, which can promote collaboration between students and, thus, enhance their learning output. Moreover, if the students do not obtain the expected results due to, e.g., cross-contamination, such an outcome still provides learning opportunities through discussion and interpretation of potential reasons and mistakes that lead to these results.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Material S1 (jmbe00242-22-s0001.docx). Additional instructor resources: instructor preparation notes, complete materials list, student materials, and protocols.
Supplemental Material S2 (jmbe00242-22-s0002.docx). Student survey: results of the student answers in the survey conducted to evaluate the usefulness and appropriateness of the laboratory exercises.

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