Name: **Student number:**

Question 1 (max. 20 points)

Scientists have isolated a new yeast species that was found in various safe-to-consume foods such as dairy and cured meat products. The initial characterization of this yeast revealed that it can uptake and catabolize a wide range of substrates and when substrates are available in high concentrations it does not exhibit the Crabtree effect. Moreover, this yeast can grow in medium containing very high concentrations on salts. It was found that this yeast is efficiently secreting one type of polypeptide – a killer toxin capable to inhibit cell growth of most of the microbial organisms. In addition, no proteases are secreted by this yeast. Initial genetic analyses revealed that this yeast prefers non-homologous enjoying mechanism to repair DNA double strand breaks. Whole genome sequencing revealed that this yeast belongs to the CUG-clade yeasts. The CUG-clade yeasts prefer to translate the CUG codon into serine or alanine instead of leucine.

a) Describe which features of this yeast are advantageous and which features are disadvantageous in cell factory applications. Explain why. (max 5 points)

b) The thorough analysis shown that killer toxin polypeptide is constantly secreted out of the cells. Moreover, its production is not affected by changing environmental conditions. What can we learn about the gene expression of this toxin? How can we apply this knowledge to produce heterologous proteins in this yeast species? (max. 3 points)

c) Design a functional CRISPR-Cas9 tool for genetic engineering of the newly isolated yeast species. Assume that all required genetic components need to be assembled into one episomal shuttle vector. Describe in detail the functionality of each component. Besides a written description, you could include a schematic drawing of your design. (max. 12 points)

Question 2 (max. 10 points)

You are tasked to genetically engineer a new eukaryotic host. You need to express a heterologous pathway in the cytoplasm of the host. There is limited knowledge about cellular and genetic features of this host. However, few details are known; the strain is efficient in homologous recombination and has deficient *trp1* gene. In addition, you have successfully identified one suitable promoter that can be used to efficiently express a heterologous gene in this host.

a) Describe what strategies can be applied to express two or more genes using only one promoter? (max. 3 points)

b) You have managed to express a three-gene heterologous pathway in this host. However, your cell factory does not produce the desired product. Instead, it accumulates first intermediate of the pathway. Discuss possible reasons for this observation. (max. 2 points)

c) You need to investigate if, and where in the cell heterologous *gene-2* is expressed (see figure). This can be done by fusing fluorescent protein (FP) to the C-terminus of gene-2. Design a gene targeting construct using a counter-selectable marker strategy. Provide schematic drawing. Show all steps of the procedure, construct integration, marker loop-out and result. (max. 5 points)

Name: **Student number:**

Question 3 (max. 20 points)

The figure below shows a generalized metabolic pathway that converts substrate (S) to the final product (P_F). Metabolic reactions are depicted as black arrows. The reactions take place in the cytoplasm and mitochondria.

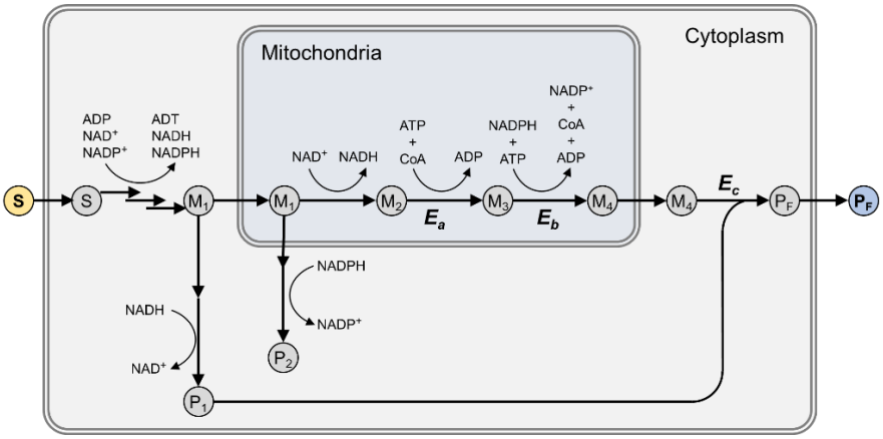
The conversion of metabolite M_2 into metabolite M_3 is carried out by enzyme $\mathbf{E_a}$. The activity of the enzyme $\mathbf{E_a}$ is controlled by the metabolite M_3 via negative allosteric regulation.

Enzyme $\mathbf{E_b}$ catalyzes conversion of metabolite M_3 to metabolite M_4. Reaction balance of this conversion is:

$$8\ M_3 + 7\ ATP + 14\ NADPH \rightarrow M_4 + 14\ NADP^+ + 8\ CoA + 6\ H_2O + 7\ ADP + 7\ P_i$$

Synthesis of the final product P_F is catalyzed by $\mathbf{E_c}$ enzyme and requires equimolar amounts of metabolite P_1 and M_4. Unfortunately, low catalytic activity of the endogenous enzyme $\mathbf{E_c}$ results in very low yields of the final product P_F.

Feeding experiments have been performed to further investigate the pathway. The cultivation media was supplemented with metabolites P_1, M_2 and M_4 and their effects on P_F production were measured. Metabolites were added individually or in pairs according to the table below.



Feeding experiment

	P_1	M_2	M_4
P_1	**N**	**+**	**++**
M_2	**+**	**+**	**++**
M_4	**++**	**++**	**++**

N: no effect, **+**: moderate improvement, **++**: strong improvement

a) Describe possible rate-limiting steps of this metabolic pathway? Consider the information provided in the text, the reaction balance, and the results from the feeding experiment to support your claims. (max. 7 points)

b) Propose cell factory engineering strategy to optimize the metabolic pathway and minimize the encountered bottlenecks. (max. 13 points)