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Iron-enriched Bread: Promoting Heme Production in *Saccharomyces cerevisiae* using HemQ

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Abstract. Several hundred million people around the world are facing iron deficiency problems, which can lead to a range of health problems, such as anemia and weakened immunity. Due to global climate change nowadays, the occurrence of extreme weather, the greenhouse effect, and the lack of nutrients in crops, particularly the lack of iron, have become more and more intense. In the case of limited nutrients in crop food, how to maximize the available iron content in food and absorption of iron by the human body has become an important problem. Considering that hemoglobin is the body's main conduit for iron absorption, we thought that by improving the synthesis of hemoglobin by yeast, we could allow enhanced iron intake. In the research, we introduced a bacterial *hemQ* gene into *Saccharomyces cerevisiae* and localized its expressed protein in mitochondria to enable a new metabolic pathway for promoting heme production.

Keywords: iron deficiency, iron absorption, hemoglobin, *Saccharomyces cerevisiae*

1. Introduction

For our project, we targeted the global food issue concerning the increasing iron deficiency rate due to population growth and climate change. The most problematic areas include Sub-Saharan Africa and South Asia, where stunted growth is most severe [1]. Smith et al. have reported that among the 1.4 billion people facing an increased burden of iron deficiency associated with CO_2 emissions, over 50% of the lost iron is derived from cereal grains and flour [2].

Additionally, meat as a main source of iron intake is scarce in many African households with less than 60% of African slum households consuming meat in an average week, and with vegetables being inefficient in energy provision, plain bread is the best option for most [3]. Bread has been the fundamental food source for thousands of years, the basic nutritional supply for most of the population. However, a study shows that replacing part of the diet with the recommended daily intake of fiber-rich wheat bread (300g) for 4 months may decrease iron levels in women who initially have enough iron stored in their bodies [4]. Thus, plain bread is not sufficient to satisfy our daily nutritional need for iron, bringing us the idea of enriching plain bread naturally with iron during fermentation.

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Reports show that the iron deficiency rate is increasing due to population growth and climate change globally. The high prevalence of iron-deficiency anemia it causes is now well established, largely due to the limited amount of dietary iron available for absorption. Even in developed countries like the US, Fe concentration in 62.1% of food products was reduced and Fe dietary intake declined during the 20 years from 1999 to 2018, and increases in prevalence of estimated anemia ranged from 10.5% to 103% depending on age and sex during the same period [5]. We sought to alleviate the issue by producing heme-iron-enriched bread manufactured with genetically modified yeast, allowing higher hemoglobin concentration in the bread, and resulting in enhanced iron absorption.

2. Possible strategies

2.1. Product

Our heme-iron-enriched bread aims to increase absorbable iron concentration to increase iron intake after consumption. In the human body, approximately 70% of iron is stored in hemoglobin and 25% in ferritin, hemoglobin was chosen as the iron vector instead of ferritin due to its significantly higher bioavailability for its heme iron than non-heme iron (which is present in ferritin), with its bioavailability approximately three times higher than the latter, thus being the ideal iron-fortification compound for its high absorption rate and that it's less affected by dietary factors [6,7].

The ideal application for our heme-iron-enriched bread is to serve as a refined food source that provides more iron aside from basic energy needs for populations with limited food diversity and scarce food sources, especially in Sub-Saharan Africa and South Asia, where malnutrition rate is top-ranking [8]. This could decrease the iron deficiency rate whilst ensuring that people still gain sufficient energy daily.

Advantages of heme-iron-enriched bread include more effective iron supplementation than previous iron-fortified bread (with manually added nutrient powders in the flour), high convenience in mass production, and easier introduction into the global market. Iron-fortified bread has been attempted by many in multiple countries, with results more or less showing that the iron supplements had very little effect on hemoglobin concentration [9,10].

Bread is a staple food widely consumed all over the world, with the average global consumption of bread ranging from 59 to 70 kg per year per capita [11]. Improving the nutritional quality of products consumed by a significant proportion of people is an essential strategy to meet the demands of consumers seeking better nutritional options and health benefits, all the while ensuring swift entry for this bread into the markets due to its regularity in daily life.

2.2. Approach and Pathway

To increase heme production in *Saccharomyces cerevisiae*, we would introduce the enzyme HemQ into the metabolic pathway to open an alternative pathway that utilises a former side-product, Fe-coproporphyrin III, to produce heme in an attempt to increase total heme content.

The classic pathway synthesises protoheme from coproporphyrinogen III through the use of HemF or HemN to produce protoporphyrinogen IX, then HemY, HemG, or HemJ to get protoporphyrin IX, and eventually HemH to acquire protoheme (heme), as shown in figure 1(A) as pathway on the right. The alternative pathway converts coproporphyrinogen III to coproporphyrin III using HemY, then to Fe-coproporphyrin III, and eventually to protoheme by HemQ, as the pathway on the left [12,13].

To achieve this, we plan to direct the target protein HemQ into the mitochondrial matrix by applying a suitable mitochondrial targeting sequence (MTS) from the gene of SOD2, and by attaching the MTS before HemQ at its N-terminus, the enzyme could be recognised and allowed through the double membrane of mitochondria, thus, HemQ could function in the correct place alongside the native enzymes in *S. cerevisiae* during the production of protoheme. The MTS of SOD2 is chosen for its advantage in growth complementation when compared to other genes, such as HSP10 [14].

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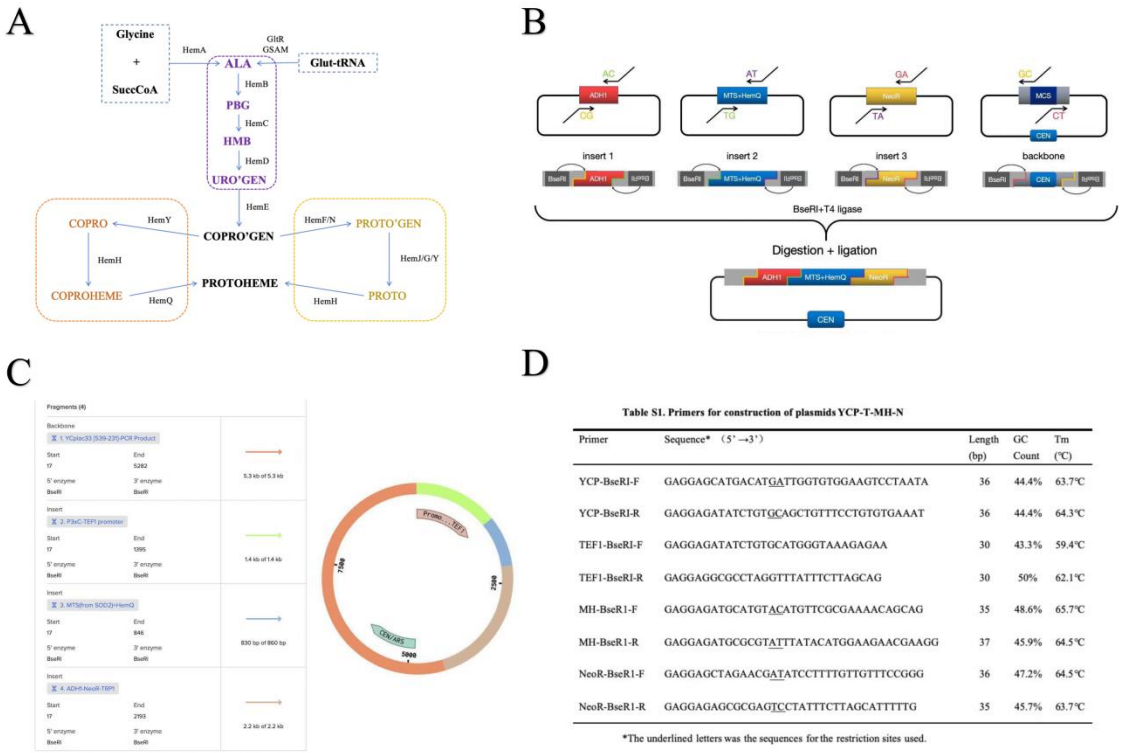


Figure 1. In (A) synthesis of heme in S. cerevisiae, the orange pathway is the new pathway. (B) Golden gate assembly of YCP-T-MH-N, (C)fragments and (D) primers shows details of construction of YCP-T-MH-N.

2.3. Host

Enhancing endogenous heme biosynthesis in yeasts has been proven as an efficient way to increase the content of recombinant hemoglobin. Unlike in bacteria, yeasts do not require the external addition of costly heme precursor, 5-ALA, for hemoglobin production, making yeasts more suitable than bacteria [15]. Yeasts, especially *S. cerevisiae*, are also the main used microorganisms in bread making.

Among the different yeasts (*S. cerevisiae*, *Y. lipolytica*, *K. phaffii*, and *K. lactis*), *S. cerevisiae* has advantages in many aspects. *S. cerevisiae* is a conventional microbial host with the advantage of a wider range of applicable genetic tools than other unconventional hosts. It is highly genetically tractable due to a well-annotated genome and the use of homologous recombination as the dominant DNA repair pathway, especially when compared to *K. phaffii* and *K. lactis* which use non-homologous end joining (NHEJ) making genetic manipulation more challenging. As we eventually need to consider the difficulty of production when using the modified yeast in real life, *S. cerevisiae* has high adaptability to harsh conditions, such as in households, or possible high ethanol concentrations and variable pH levels found in industrial-scale production sites.

S. cerevisiae strains can be divided into wild, commercial (industrial), and laboratory strains. Commercial strains have a better ability to utilise maltose than wild strains. In contrast, laboratory strains mainly derived from SC288C lack that ability, which indicates more steps and extra sugar needed in the bread-making process [16,17]. Thus, We plan to test several commercial bread-making *S. cerevisiae* strains from ATCC/CICC to find the best-fitting one with a high ability to utilise maltose and high hemoglobin productivity. Some problems may arise in the gene editing process due to common aneuploidy and polyploidy among commercial and wild strains, so specific effects and solutions must be tested [18].

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2.4. Feedstock & Process

As a food product, the flavor of the iron-enriched bread is also an important factor. Our feedstock choice is aimed at optimizing the efficiency of the yeast fermentation and the flavor of the final bread product.

Whilst adding ingredients to the dough, enzymes in the dough hydrolyze the starch into maltose, which regulates the flavor of the bread and provides nutrients to the yeast cells [19]. The addition of salt increases the rigidity of the gluten network in the dough, allowing it to hold more carbon dioxide from the fermentation, making the bread more elastic [20]. In addition, we add wheat bran, which increases the amount of fermentable sugars in the dough and improves the rate of pre-fermentation yeast fermentation. Apart from this, the alpha-amylase enzyme in the wheat bran promotes the release of maltose, which elongates the productive fermentation time [21].

The fermentation and kneading of the dough need to be alternated several times to ensure that the dough is moderately soft. The kneaded dough would be put to rise to promote yeast fermentation, with the temperature close to the optimum fermentation temperature for yeast (28-35℃). The first dough fermentation takes about 2-3 hours and the second about 40 minutes. Note that the dough should be covered with a damp cloth to ensure sufficient humidity during the fermentation process.

The dough will be baked in an oven at about 180 degrees centigrade for about 30 minutes. At this point, the Maillard reaction takes place involving amino acids and reducing sugars [22]. Whether this reaction is carried out properly determines the flavor and final colour of the bread. The exact baking time, temperature, and process will be adjusted in the experiment to ensure the hemoglobin content and the flavor of the final bread product.

2.5. Design Strategy

For the plasmid modification, we will use PCR to acquire the linear segment of vector YCplac33 with 2 primers to obtain 2 Type IIS Enzyme sites for an external enzyme BseRI. This foreign enzyme is chosen to evade possible unwanted digestions on YCplac33 due to the excessive amount of digestion sites of other preexisting Type IIS Enzymes. We would then attach the promoter (P3xC-TEF1), MTS, target protein (HemQ), resistance gene (NeoR), and terminator (TRp1) onto the plasmid using Golden Gate assembly to form the final plasmid YCP-T-MH-N.

2.5.1. Cassettes design and sequences

Generally, we select YCplac33 as the initial vector for it has a centromere sequence enabling it to replicate automatically. Research shows that YCp is suitable and has high transformation efficiency, so it would be useful for us to study the effects of a single hemQ gene [23]. According to comparative research in 2018, P3xC-TEF1, a constitutive promoter, has high stabilities and strengths higher than all other native promoters under 30°C, which can help us to maximize hemoglobin production [24]. The NeoR gene from Tn903 can be used for high-frequency transformation of wild-type strains of *S. cerevisiae* and can give them resistance to most aminoglycosides which makes selecting more effective [25,26]. Our target gene is hydrogen peroxide-dependent heme synthase ([*Geobacillus stearothermophilus*]. , GenBank:WP_033015420). To import the bacteria-origin gene hemQ into *S. cerevisiae*, we first optimised the codons of the target sequence to efficient mRNA translation and protein expression of HemQ in the yeast. Secondly, we plan to obtain the hemQ sequence by chemical synthesis after optimizing the codons. We choose Golden Gate Cloning to simultaneously assemble multiple gene elements into the final plasmid irreversibly. Primers are designed to amplify all of the elements and add unique overhangs to both ends of DNA fragments.

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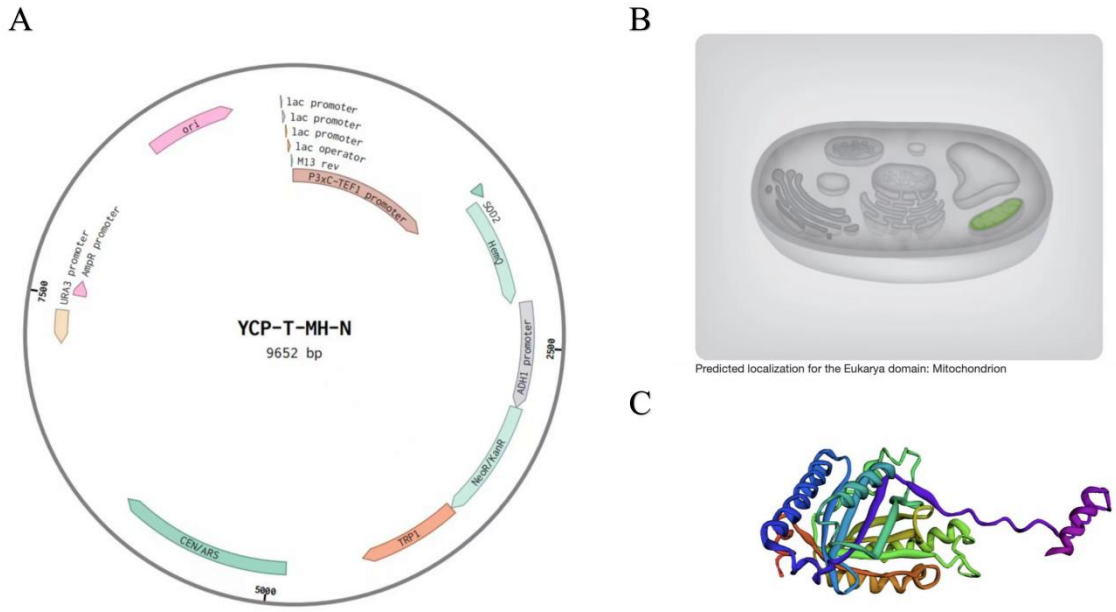


Figure 2. (A) Final assemble plasmid, YCP-T-MH-N; (B) shows that MTS-HemQ sub-cellular localisation is predicted to be in mitochondrion. MTS at the N-terminus of the protein shown purple in (C) MTS-HemQ Protein structure prediction using Robetta helps its location.

3. Conclusion

The use of genetically modified yeast to produce heme-iron-enriched bread presents a solution to the global issue of iron deficiency by increasing hemoglobin concentration in the bread, thus enhancing iron absorption. We import an enzyme (*hemQ*) into the mitochondrial matrix of *Saccharomyces cerevisiae* (our host) to enable a new metabolic pathway that produces protoheme (our target product). Prediction of protein structure using protein structure prediction software and sub-cellular localisation revealed that the target protein would have an extra signal peptide at the N-terminus, which was predicted to localise to the mitochondria, just as we had expected.

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Zimo Tang, Qianqian Zhou, and Jiayang Liu contributed equally to this work and should be considered co-first authors.

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