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OVEREXPRESSION OF PYRUVATE CARBOXYLASE IN *Saccharomyces cerevisiae* FOR CO_2 FIXATION

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ABSTRACT

Population growth has increased the demand for resources and consequently CO_2 emissions. Brazil is among the top four CO_2 emitters since 1850, bringing forward the need of global emission reductions. This study aims to enhance carbon fixation in *Saccharomyces cerevisiae* by replacing its native *PYC1* (pyruvate carboxylase) promoter (P_{PYC1}) with the strong *CCW12* promoter (P_{CCW12}). This is expected to boost *PYC1*/*PYC2* gene expression, thus increasing the anaplerotic reaction of pyruvate into oxaloacetate via atmospheric CO_2 fixation, which might enhance the conversion of substrate into bioproducts. Using *S. cerevisiae* CEN.PK113-5D as the host strain, the modified yeast can be a promise for efficient CO_2 capture, aiding in climate change mitigation.

Keywords: Carbon fixation. Climate change mitigation. Homologous recombination. Plasmid construction.

1 INTRODUCTION

In recent years, rapid population growth has resulted in a significant upsurge in the demand for feedstocks such as food, energy and consumer goods.[1] This increase is reflected in the growth of the emissions of greenhouse gases, for instance carbon dioxide (CO_2), whose effects are quite significant in terms of global warming and climate change.[2] Analyzing only CO_2 emission, between 1850 and 2021, Brazil was among the four largest emitters of this gas, which implies that measures to contain emissions must be implemented now, not only in Brazil, but worldwide. [3,4] Furthermore, in order to restrain the effects of the rise in world temperature, joint measures are needed in the fields of energy matrices, industrial processes, as well as the study and feasibility of new, more efficient CO_2 removal technologies. In the context of biorefineries, the use of CO_2 as a raw material has gained prominence, as it mitigates the effects of global warming and the use of CO_2 for the production of molecules of interest.[5,6]

One possibility is the use of genetically modified microorganisms incorporating atmospheric CO_2 in their metabolic pathways. In the case of industrial ethanol production, which uses the yeast *Saccharomyces cerevisiae*, implementing an engineered strain for this purpose will not only help to capture excess gas from the atmosphere, but also has the advantage of increasing the conversion factor of the substrate into products in fermentation processes.[7]

Therefore, the aim of this study was to genetically modify the *S. cerevisiae* CEN.PK113-5D strain by substituting its native PYC promoter with a more robust alternative, such as CCW12, via homologous recombination. This will enable the enhanced expression of *PYC1*/*PYC2* genes which play a crucial role in converting pyruvate into oxaloacetate, a key step in anaplerotic reactions for carbon fixation.

2 MATERIAL & METHODS

The strains used were *S. cerevisiae* strain CEN.PK 113-5D and the *Escherichia coli* DH5α strain. The yeast strain has a well characterized genome, which simplifies genetic analyses, and is an uracil auxotroph.[8]

S. cerevisiae CEN.PK 113-5D was cultured on solid YPD medium (yeast peptone dextrose), comprising 10 g/L yeast extract, 20 g/L bacteriological peptone, 20 g/L glucose, and 20 g/L agar (only for solid medium). Colonies were transferred to liquid YPD media with identical nutrients. The culture was grown overnight in a shaker at 30°C, at 200 rpm. *E. coli* DH5α was cultivated in LB medium, with growth on both solid media (LB medium supplemented with 15 g/L) at 37°C and in liquid media in a shaker at 37°C and 180 rpm.

Both the promoter gene and the uracil gene sequences were amplified from the pDX8.4 plasmid[9] using the primers DPO 002 F, DPOR 003 R and DPO 004 F, DPO 005 R, respectively. The pEERM1 plasmid (Addgene #64024) was amplified with the primers CVS 430 F and DPO 001 R, selecting the kanamycin marker for the construction of the target plasmid. Subsequently, new primers for the *CCW12* and *URA3* genes were synthesized with homology to the pEERM1 plasmid. All sequences were amplified according to the protocol of Phusion® High-Fidelity DNA Polymerase (NEB). The resulting plasmid, pEERM1, promoter, and uracil were constructed via Gibson assembly according to the GeneArt Gibson Assembly® Cloning protocol. All primers in this work are summarized in Table 1.

Table 1 Primers used in this work.

Name	Primer	Sequence (5' - 3')
CVS 430 F	pEERM1	CCAGGCATCAAATAAAACGAAAG
DPO 001 R	pEERM1	CCCCGAAGACACTATAGGTCT
DPO 002 F	CCW12	GGAGCTCGACCATTTCATATGCAAAGC
DPO 003 R	CCW12	GGCGGTGTACATTATTGATATAGTGTTTAAGC
DPO 004 F	URA3	CATGTCGAAAGCTACATATAAGGAACG
DPO 005 R	URA3	GAAGCTCTAATTTGTGAGTTTAGTATACATG
DPO 006 F	URA3	CGTATAAGAGACGTATAGGAGACCTATAGTGTCTTCGGGGCATGTCGAAAGCTACATATAAGGAACG
DPO 007R	URA3	TAAGTTTCTTTTATTTTGCTTTGCATATGAAATGGTCGAGCTCCGAAGCTCTAATTTGTGAGTTTAGTATACATG
DPO 008 F	CCW12	ATAAGTAAATGCATGTATACTAAACTCACAAATTAGAGCTTCGGAGCTCGACCATTTCATATGCAAAGC
DPO 009 R	CCW12	CCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGGCGGTGTACATTATTGATATAGTGTTTAAGC
Primer 1 F	Lev	TTCGATAGATACAAGGAGGTCTTGAGTATGTAGATAAACGAAAAGAAGATAACAAAAGGAAAATCTCAGCCTCTCCCCTTCCTCTTAGACCATGT CGAAAGCTACATATAAGGAACG
Primer 2 R	Lev	TCCTCTATTAGCCACCAATATTTTGTTCTTTTCACCCAAGAGATTGAAGTTATCTCTCAAGCCGGCGAATTTTCTTTGCGACATTATTTCTTGGTATG TTAGGAAAGAATAAA

Following the Gibson assembly protocol, colony PCR was carried out according to Master Mix Taq Pol (2X) Cellco and the restriction enzyme single digestion with EcoRI was used to confirm the construction of the plasmid of interest pD.I.1 (Figure 1).

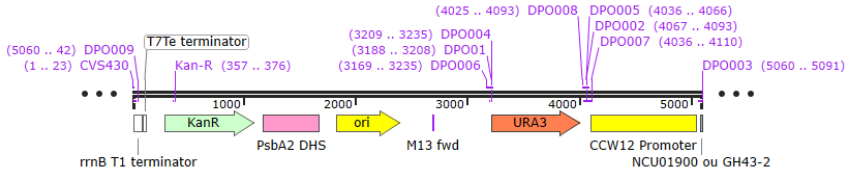

Figure 1 Plasmid pD.I.1 map – showing the construction of the primers.

The transformation of the strain CEN.PK113-5D with the modified plasmid was conducted using the polyethylene glycol (PEG)-LiAc method.[10,11] Following the transformation, the yeast cells were cultured under selective conditions to promote integration of the plasmid into the genome. Subsequent analyzes focused on assessing the major physiological parameters of the modified yeast strain, including its ability to capture CO_2 and increase product conversion yield.

3 RESULTS & DISCUSSION

The agarose gels with the sequences amplified using primers 2-5 are shown in Figure 2. **A** represents the amplification of the *CCW12* promoter gene, **B** represents the *URA3* gene, and **C** represents the amplification of the pEERM1 plasmid, along with the promoter genes and selection marker amplified for homology to the pEERM1 plasmid, all of which were successful.

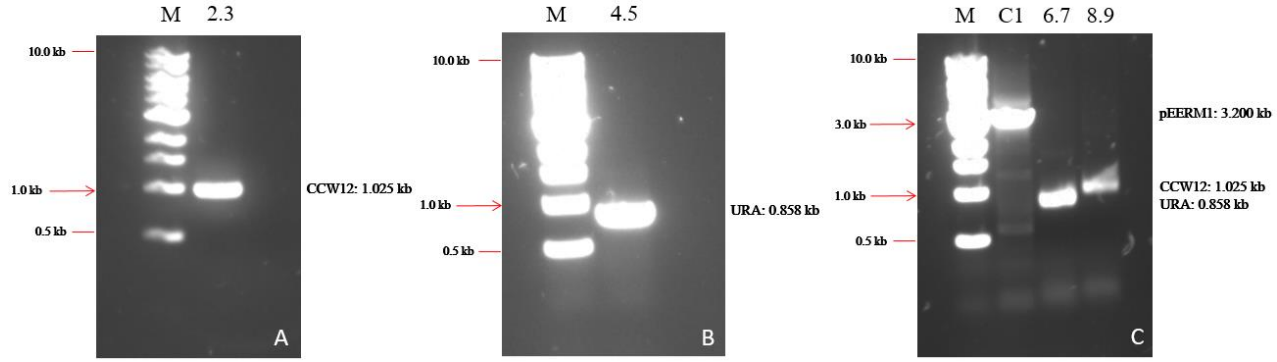

Figure 2 PCR amplifications of the genes *CCW12* (A), *URA3* (B) and plasmid pEERM1 (C)
Legend: M (1 kb plus molecular marker), *CCW12* promoter gene (2.3 and 6.7), and *URA3* selection marker gene (4.5 and 8.9).

Figure 3 displays the results of the Gibson Reaction Protocol. **A** shows the growth of transformed colonies on a Petri dish. Panels **B** and **C** present the subsequent colony PCR and single restriction enzyme digestion performed to confirm the correct assembly of the plasmids.

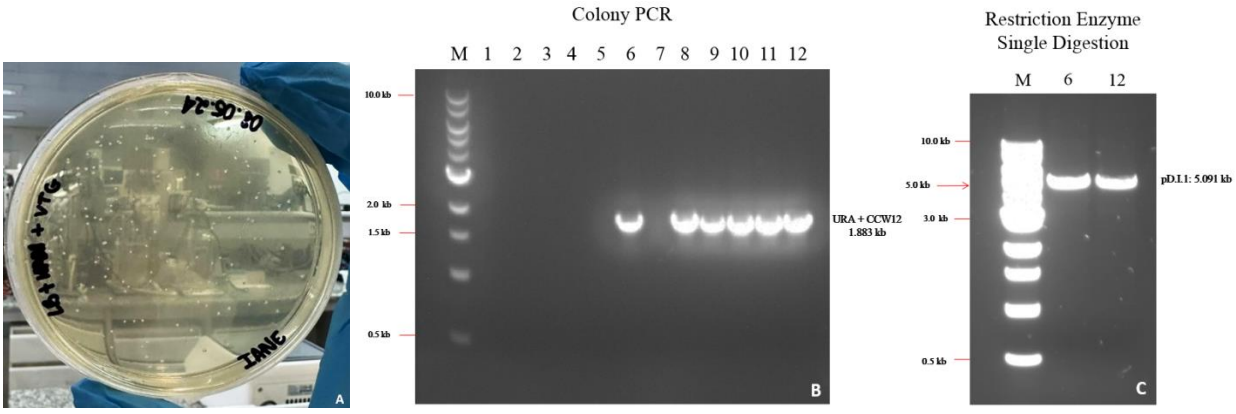

Figure 3 Plate of transformants from the Gibson assembly reaction (A), colony PCR from the Gibson assembly reaction (B), and restriction analysis with the EcoRI enzyme (C). Label: 2.3 (CCW12), 4.5 (URA), C1 (pEERM1) M (molecular marker 1 kb plus).

With the confirmation of the constructed plasmid, primers “1 and 2 Lev” were designed to create homology between the yeast locus and the plasmid. Immediately after the yeast transformation and confirmation, the strain will be evaluated and will provide insights into the effectiveness of this strategy in enhancing carbon fixation and its potential applications in bioprocess engineering.

4 CONCLUSION

This study offers a potential for harnessing the metabolism of *S. cerevisiae* CEN.PK 113-5D for CO_2 capture. The amplification of the *URA3* and *CCW12* promoter genes in the transformed colonies compared to the control highlights the successful insertion and expression of these genes in the new plasmid, pD.I.1. This suggests the potential of the plasmid as an efficient vector, creating a yeast capable of fixating CO_2. This approach could be a way of mitigating greenhouse gas emissions, representing a step forward in the development of biological solutions to address issues related to climate change and environmental sustainability challenges.

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