Funded by the European Union

ACTPAC	
Project number:	101135289
Project name:	A Complete Transformation PAth for C-C backboned plastic wastes to high-value Chemicals and materials
Topic:	HORIZON-CL6-2023-ZEROPOLLUTION-01-5
Type of action:	HORIZON-IA
Starting date of action:	01.01.2024
Project duration:	48 months
Project end date:	31.12.2027
Deliverable number:	D4.1
Deliverable title:	Successful genetic engineering of ATCC 20962 towards efficient diacid production
Document version:	Ver3 (revised after 1RP review)
WP number:	WP4
Lead beneficiary:	03-UM
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Nature of deliverable:	R
Dissemination level:	PU
Delivery date from Annex 1:	01.01.2025
Actual delivery date:	11.07.2025 (finalised as forseen on 20.12.2024, it was submitted late due to on-going amendment to the project’s formal description)

Document history

Version	Date	Beneficiary	Description
0.1	19.12.2024	03-UM	For internal review
0.2	19.12.2024	03-UM	Edited after internal review
1.0	20.12.2024	03-UM	Final version and submitted version

Executive Summary

ACTPAC consortium aims at the bio-chemical conversion of polyethylene (PE) (petroleum-based plastic) into bio-based polyesters (bio-based plastics). Shortly, PE waste streams are transformed into a well-defined range of C_6-C_{18} alkanes which are amenable for further conversion towards diols and diacids monomers that are finally used for polyester production. The conversion of the long-chain alkanes (C_{12}-C_{18}) into functionalized monomers is a crucial step that relies on the metabolic capabilities of yeast and bacteria to achieve efficient results.

The yeast *Saccharomyces cerevisiae* has initially been considered a possible strain for converting long-chain alkanes into diacids due to its non-pathogenicity. *S. cerevisiae* does not normally utilize alkanes but is a well-known chassis organism that can easily be genetically modified. In contrast, the yeast *Candida tropicalis* (now *Candida viswanathii*) is known to be an excellent diacid producer from alkanes or fatty acids but is categorized as a human pathogen and thus a risk-class organism. This will make industrial utilization of such a strain impossible in Europe. For this, in the first 6 months of the work package, all necessary literature research and setting up of the engineering strategies for *S. cerevisae* were performed. In June it was observed that *Candida viswanathii* ATCC 20962 (formerly named *Candida tropicalis*), which is a powerful diacid producer, was reclassified to risk class 1, based on good experience with safe handling under large-scale production conditions. For this, and to finally reach higher product titers it was decided that the strategy with *S. cerevisiae* should not be followed up. UM and the project coordinator AU agreed with the EU project responsible for adapting the work package towards *C. viswanathii* as the production strain.

C. viswanathii already possesses the α,ω-oxidation pathway required for the efficient conversion of alkanes to diacids and is described to be genetically accessible for metabolic engineering. For this, different aspects were considered to engineer and cultivate the strain successfully: 1) which plasmid and genetic toolkit to use, 2) which transformation method to use, and 3) which process parameters and equipment to use to enable efficient biotransformation.

For 1) an already described plasmid for the engineering of *Candida* sp. based on the CRISPR-Cas9 technology was chosen and tested. But even though different attempts have been made, genomic engineering has not achieved yet and optimization is still ongoing.

For 2) different transformation methods were tested to identify the most efficient one which were mainly, the lithium-acetate method and electroporation. Since no colonies were obtained on selective plates after transformation using the lithium acetate method by different attempts, this method was evaluated to be not effective for this strain. Instead, electroporation was successfully established.

For 3) the aggressive nature of alkane substrates causes challenges in terms of equipment compatibility. During initial tests and fermentations, it became apparent that some tubings in the bioreactor required replacement to withstand the aggressive substrates. New and compatible components were procured and installed. In addition the GC-MS device which was applied for was delivered, but final installation is delayed up to now. For this, initial measurements on an external device were established to guarantee initial analysis of the conversion of alkanes towards diacids. By addressing these challenges, the project is well-positioned to optimize the bioprocess and achieve high diacid production efficiencies.

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Acronyms & Abbreviations

Term	Description
cPCR	Colony PCR
CYP	Cytochrome P450 enzyme
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectroscopy
gDNA	Genomic DNA
GDPR	General Data Protection Regulation
HRF	Homologous Repair Fragment
NRS	Nourseothricin
ON	Overnight
PCR	Polymerase chain reaction
WP	Workpackage
WT	Wild type

1 Introduction

1.1 Overview

The main objective of WP4 is to provide highly efficient microbial strains that perform the conversion of the long-chain alkanes (C_{12}-C_{18}), supplied by WP1 and WP2, into long-chain α,ω-diacid and diol monomers for PE-like polyesters for the following polymerization in WP6-WP9. This will be mainly done by genetic and metabolic engineering of bacterial and yeast strains to perform the conversion of the long-chain alkanes. This deliverable report focuses only on the progress of the generation of optimized yeast strain for the production of long chain diacids. The parallel progress on the bacterial strain development for the mid- to long-chain diols will be presented in the next deliverable from UM (D4.2).

C. viswanathii, formerly known as *C. tropicalis*, is a suitable chassis for α,ω-diacids due to its innate capacity to assimilate alkanes into α,ω-diacids through the α-oxidation and the ω-oxidation pathways involving the P450 hydroxylase system [1], [2]. Alkanes are first oxidized to fatty acids through a three-step α-oxidation and following the fatty acids are oxidized to α,ω-diacids via another three-step ω-oxidation (Figure 1). To avoid the hydrophobic substrate consumption for biomass formation and to redirect the substrate flux towards the product of interest, the first gene involved in β-oxidation of *C. tropicalis* was deleted [1].

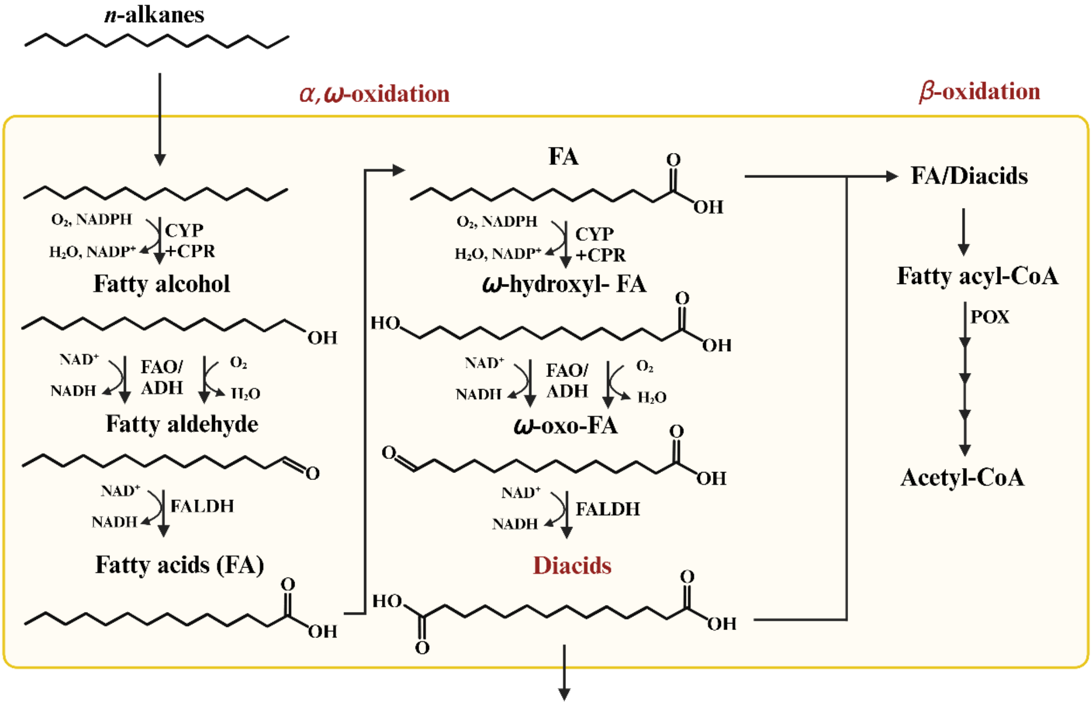

Figure 1. *n*-alkanes are transported into the cell membrane and then α,ω-oxidation occurs leading to the production of diacids. Subsequently, fatty acids and diacids undergo β-oxidation producing acetyl-CoA. CYP+CPR: cythocrome P450 monooxygenase/cytochrome P450 oxidoreductase complex, FAO/ADH: fatty alcohol oxydase/alcohol dehydrogenase, FALDH: fatty aldehyde dehydrogenase, POX: acyl-CoA oxidase.

For this, many optimization strategies have been described in literature which include overexpression of the P450 hydroxylase system and various alkane-specific promoters. These approaches were recently reported to result in an improved production of 1,14-tetradecanedioic acid of up to 210 g/L. But, *C. tropicalis* was classified for a long time as a risk class 2 organism which renders its industrial application in Europe impossible. By that, the efficient oxidation system was planned to be transferred to the non-pathogenic yeast model strain *S. cerevisiae*.

S. cerevisiae is an attractive non-pathogenic strain with broad industrial use for the production of various chemicals and metabolites. This is mainly due to its relatively easy genetic manipulation and the established collection of genetic tools [3]. *S. cerevisiae* does not naturally grow on alkanes, nor produces α,ω-diacids. Thus, engineering of the metabolism is necessary to enable the utilization of the alkanes towards the desired products. *S. cerevisiae* has been already engineered to express the cytochrome P450 hydroxylase system from *C. tropicalis* or other plant-sourced genes and showed α,ω-diacids production but only to a maximum of 5.8 mg/L [4], [5].

The initial plan of WP4 was to transfer the whole gene set for efficient alkane conversion from the pathogenic *C. tropicalis* (*viswanathii*) to the non-pathogenic *S. cerevisiae*. For this, all necessary strains, genetic tools, and primers were designed and ordered for that approach (data not shown).

By the new finding that *C. viswanathii* ATCC 20962 is classified as non-pathogenic, the plan was changed towards the engineering of *C. viswanathii* to further optimize the alkane conversion specifically towards long-chain diacids. An extensive literature research was conducted to guide further engineering of *C. viswanathii* towards optimization of alkane conversion. Additionally to the overexpression of the P450 hydroxylase system, also the *POS5* gene and the *FAO2* gene, NADH kinase and the long-chain alcohol oxidase respectively (Figure 1), were reported to be overexpressed and resulted in higher titers [6],[7]. These approaches were selected as a first engineering strategy. Together with the targeted deletion of various CYP encoding genes, which will massively influence the substrate and product spectrum of the optimized strain and the deletion of PAH1, phosphatidic acid phosphatase gene, to possibly enlarge endoplasmic reticulum and hence higher diacid production.

Table 1. List of targeted strategies for *C. viswanathii* derived from literature and beyond.

Targets	Description	Titers diacid (g/L)	Reference
ATCC 20962	Blocked β-oxidation	111	[6]
CYP52A with *CPR*	Overexpression of different *CYP52A* genes and *CPR*	156	[6]
FAO2	Overexpression	170	[6]
POS5	Overexpression	224	[6]
ADH	Overexpression	-	-
FALDH	Overexpression	-	-
Δ*CYP52A*s	Deletion of different *CYP52A*s for alkane substrate specificities studies	-	-

PAH1	Deletion of *PAH1* for enlarged endoplasmic reticulum and hence higher product production	-	[8]

To enhance the strain capabilities for the efficient and specific production of long-chain diacids, a proper transformation and genetic engineering method has to be established in the laboratory. Here, it is important to mention, that *C. viswanathii* has a diploid genome and does not undergo meiosis, which makes the generation of deletion strains more difficult. Each allele needs to be targeted independently. Thus, using a recyclable system such as CRISPR-mediated genome editing is highly advantageous. Lombardi *et al*. developed a CRISPR-Cas9-based system for different *Candida* strains, including *C. tropicalis*, and also Pham *et al*. described a CRISPR system specifically for *C. viswanathii*.

Within WP4, the main scope of this first deliverable was to identify the most appropriate yeast strain and, to establish the genetic engineering technique, including transformation method and gene editing system. In addition, the establishment of suitable extraction and analytical methods should be performed to guarantee a robust and reliable measurement of the substrates and products as well as intermediates.

For the transformation of yeast strains, the lithium acetate method is an efficient method usually employed for *S. cerevisiae* [9]. However, electroporation was also employed successfully in different *Candida* species, including *C. viswanathii* [6],[9]. In this study, both methods were applied to *C. viswanathii* and multiple efforts were made to optimize transformation protocols to reach high efficiencies. While the lithium acetate method was classified towards non-efficient category, an optimized electroporation protocol was successfully established. Genomic engineering was attempted by using the CRISPR-Cas9 based pCT-tRNA system by targeting the *POX2* gene and the CYP450 encoding *CYP52A19* gene as proof-of-concept and towards specific substrate and product range, respectively [6]. Within the first 3 months, no successful deletion variants could be obtained, likely linked to a non-active Cas9 expression in ATCC 20962. Consequently, a new CRISPR-Cas9-based plasmid with different features (promoters and terminators) was decided to be cloned to improve expression in this strain. This cloning is currently ongoing and is expected to enable efficient genetic modification of *C. viswanathii*. These advancements will support further strain engineering strategies to optimize the production of specific long-chain α,ω-diacids.

Furthermore, addressing the aggressive nature of alkanes, which causes the degradation/disintegration of certain parts and tubings of the bioreactors, these parts had to be replaced by more robust and compatible materials (silicon tubings are not suitable and must be replaced by e.g. viton tubings of the correct inner diameter). Additionally, a GC-FID system as inquired by the proposal had to been set-up to monitor the biotransformation process. With these improvements in place, bioprocess engineering efforts are currently ongoing.

1.2 Relation to other tasks and deliverables

D4.1 receives no input from other tasks or deliverables. D4.1 provides output to the following ACTPAC tasks and deliverables (Table 2): The UM part for diols will be addressed independently.

Table 2. D4.1 Output for other tasks and deliverables

Deliverable	Due Date	Output from D4.1
D4.2	M18	Successfully designed alkane-converting *P. polymyxa* strain for long-chain diol production
D5.1	M32	Optimized process variants for the yeast and/or the *P. polymyxa* strain with feeding strategies for maximal substrate conversion
D5.2	M36	Engineering strategies for further strain improvement identified via process analytics and strain characteristics

1.3 Structure of the deliverable

In this deliverable report, our results are described in section 2, divided in 4 subsections. Our conclusion from the results are summarized in section 3.

2 Successful metabolic engineering of ATCC 20962 towards specific diacid production

2.1 Strain selection to use for conversion of alkanes into diacids

As a suitable *S. cerevisiae* strain , the CEN.PK21C strain was selected based on its well described genetic manipulation and good growth behaviour [10] (Table 3). For the initially intended amplification of the alkane converting genes *C. viswanathii* ATCC 20962 was selected, and finally chosen as the strain for conversion and metabolic engineering towards higer product titers and targeted specificity of the chain length of substrates and products.

Table 3. List of strains

Strain	Genotype	Source
Saccharomyces cerevisiae CEN.PK2-1C (3000A)	*MAT*a; *his3D1*; *leu2-3_112*; *ura3-52*; *trp1-289*; *MAL2-8c*; *SUC2*	EUROSCARF
Candida viswanathii ATCC 20962	*pox5:ura3A; pox5:ura3A pox4A:ura3A; pox4B:URA3A*	ATCC

2.2 Transformation and genetic engineering of *C. viswanathii*

2.2.1 Selection and optimization of the transformation method

Two transformation methods were tested for *C. viswanathii*: lithium acetate method and electroporation [9],[11]. To engineer this strain the pCT-tRNA plasmid, containing the CRISPR-Cas9 gene, the sgRNA cassette and all the necessary parts for selection and replication in both *Escherichia coli* and *C. viswanathii* was selected [11]. Nourseothricin (Nrs) was chosen as the selection marker. Additionally, the pCTO-empty vector, an overexpression plasmid based on the pCT-tRNA that does not contain the CRISPR-related parts, was cloned and now it can be used to overexpress genes of interest in the future. Two different plasmids for the expression of two different CYP52, specifically CYP52A17 and CYP52A19, as shown in Table 4 were aleady cloned. The two genes were combined with different promoters from the CYP52A genes. Both plasmids (pCT-tRNA and pCTO-empty) were used to analyse the transformation efficiency of the two methods.

Table 4. List of plasmids used

Plasmid	Description	Reference
pCT-tRNA	Plasmid for engineering *Candida* sp.	[6] (Plasmid #133813 in AddGene)
pCTO-empty	Plasmid without Cas9 and sgRNA cassette	This study
pCTO-CYP52A17	Overexpression plasmid for *CYP52A17*	This study
pCTO-PCYP52A13-CYP52A17	Overexpression plasmid for *CYP52A17* with promoter of *CYP52A13*	This study
pCTO-PCYP52A13-CYP52A19	Overexpression plasmid for *CYP52A19* with promoter of *CYP52A13*	This study
pCT-POX2	Plasmid targeting *POX2*	This study
pCT-POX2-II	Plasmid targeting *POX2* with another sgRNA for deletion	This study
pCT-CYP52A19	Plasmid targeting *CYP52A19*	This study
pCTCv-POX2-Q28	Plasmid with promoters and terminators from *C. viswanathii,* targeting *POX2*	This study – cloning in progress
pCTCv-POX2-Q28-Nrs	Plasmid with promoters and terminators from *C. viswanathii,* targeting *POX2*, including *Nrs* in between the homologies for integration	This study – cloning in progress

The lithium acetate method is based on the concept that lithium cations neutralize charges on the cell membrane and the DNA added to the transformation mixture binds to the cell wall of the yeast to make it available for uptake into the cell [9]. A heat shock applied to the cells should facilitate the uptake. It is known that different strains require different durations of the heat-shock. Thus, 25 min, 40 min, 60 min and 80 min at 42 °C as well as an overnight incubation at RT in the presence of ~ 1000 ng of the pCTO-empty plasmid were tested. Only the 40 min and the overnight experiments showed colonies, which accounted for seven and one respectively (Figure 2A).

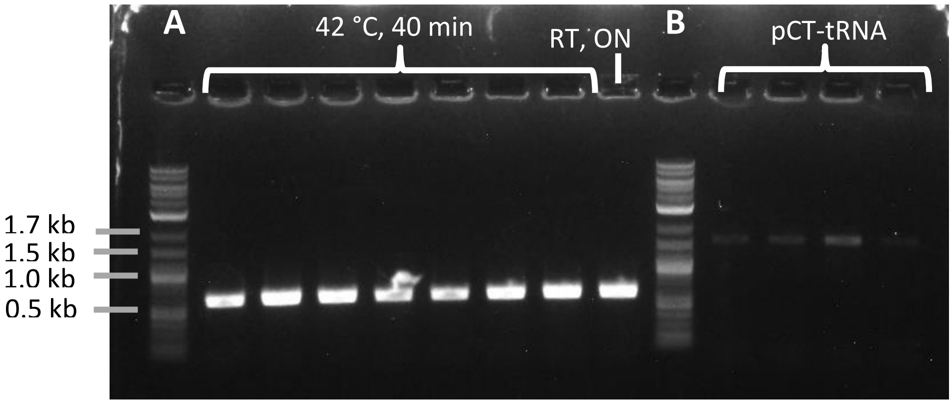

Figure 2. (A) Lithium acetate transformation of *C. viswanathii*: First seven colonies with 40 min heat shock at 42 °C, last colony with an overnight incubation at RT. Band of 0.6 kb confirms the presence of the plasmid pCTO-empty in the cell; **(B)** Electroporation of pCTO-empty in *C. viswanathii* with four colonies checked for the presence of pCT-tRNA (1.6 kb expected) and all four confirmed.

In parallel, the electroporation method based on Pham *et al*. was tested, using ~ 1000 ng of the plasmid DNA [6]. As given in Table 5, the transformation of the pCT-tRNA, which was not targeting any gene, resulted in just five colonies, whereas the transformation by use of pCTO-empty resulted in more than 200 colonies (Figure 2B). To improve transformation efficiency for the CRISPR containing plasmid, the pCT-tRNA was linearized using the restriction enzyme SpeI. Since the linearization enabled much higher transformation efficiencies, utilization of linearized plasmids was chosen as a standard for all following experiments.

Table 5. Electroporation of *C. viswanathii* assessing different conditions.

Plasmid	Number of transformants obtained
pCT-tRNA	5
pCTO-empty	217
pCT-tRNA, linearized with SpeI	232

2.2.2 Genomic engineering of *C. viswanathii*

After the successful establishment of the transformation method via electroporation, the targeted deletion of one allele of *POX2* (acyl-coenzyme A oxidase 2 gene involved in β-oxidation), and the *CYP52A19* encoding gene (one allele of a *CYP52* gene), were selected as proof-of-concept for succesfull gene deletion. For this, the plasmids pCT-POX2 and pCT-CYP52A19 were cloned and homologous repair fragments (HRF) were created with a total size of 2 kb including 1 kb for the upstream homologous sequence and one for the downstream homologous sequence. However, no clones showing the successful deletion PCR fragment were encountered with the pCT-POX2 plasmid with the repair fragment (Figure 3) (Table 6). To analyze if the non-homologous end joining pathway was more active than the homology recombination, for six transformants the PCR amplicons were sent for sequencing to identify putative single point mutations, insertions, or deletions. However, only wild-type gene sequences were identified. By that, the correct targeting of the gRNA was questioned and consequently, another gRNA was designed and cloned into the plasmid pCT-POX2-II. However, transformations with this plasmid and the corresponding repair templates also failed to produce any positive deletion clones. In the protocol used, a standard 4 h of recovery period in 2 × YPD medium following transformation was extended by an additional 20 h in 2 × YPD with supplementation of 25 (µg/mL) of Nrs.

This approach resulted in an increased number of transformants, but still no successful deletion. Thus, it was decided to clone new plasmids (pCTCv-POX2-Q28 and pCTCv-POX2-Q28-Nrs) according to Pham *et al* [3]. This group used a plasmid that mainly contains native promoters and terminators from *C. viswanathii* to express the Cas9 gene and sgRNA cassette. In contrast, the pCT-tRNA uses sequences from different fungi and yeast species.

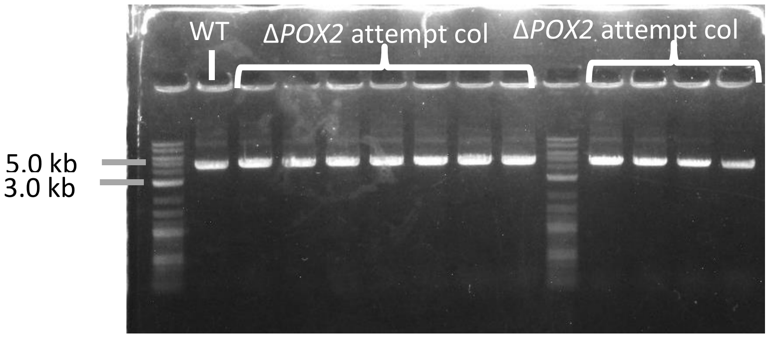

Figure 3. Δ*POX2* colony PCR (cPCR) for confirmation of POX2 locus editing: WT gDNA as a control for non-edited phenotype (4375 bp) and eleven colonies from editing attempt showing WT amplicons (amplicon of edited locus: 2453 bp expected).

Table 6. Transformation efficiencies for genomic engineering of *C. viswanathii*.

Plasmid	**4 h of recovery**		**24 h: 4 standard recovery + 20 h with Nrs**	
	Number of transformants obtained	**Number of edited transformants obtained**	**Number of transformants obtained**	**Number of edited transformants obtained**
pCT-POX2 linearized with SpeI + HRF	648	No deletion	Not attempted	Not attempted
pCT-POX2-II linearized	64	No deletion	234	No deletion

with SpeI + HRF				
pCT-CYP52A19 linearized with SpeI + HRF	62	No deletion	278	No deletion

2.2.3 Establishment of an efficient colony PCR protocol for *C. viswanathii*

Once *C. viswanathii* is transformed, colony PCR (cPCR) must be performed to check if the targeted modification occurred. The typical approach is picking one colony from the selection plate as DNA template and performing PCR using GoTaq® G2 polymerase did not result in any PCR amplicon (data not shown). Thus, boiling the picked colony for 10 min with 20 mM of NaOH prior to the PCR was tested. Yet, this method was successful only if the template of the cPCR was a plasmid, while no PCR amplicon was detected when the genomic DNA served as the template. However, extracted genomic DNA (gDNA) could successfully be used as a template. Indeed, this is a time-consuming method for a usually fast screening method. In the next step, the picked colony was cultivated overnight in 3 mL of YPD medium. Cells of 0.5 mL and 1.0 mL of that culture were boiled in 50 µL of NaOH (20 mM) and used as a template for the cPCR. Various adjustments were tested, including the use of different template concentrations (Figure 4). This method resulted in positive amplicons but is inefficient due to the tedious and time-consuming procedure, which is unsuitable for a fast engineering approach. The treatment of single colonies with CHELEX is decribed for certain types of microorganisms to increase efficiency of cPCR [12]. By that, treatment of the picked colonies of *C. viswanathii* with 5 % of CHELEX at 40 °C for 20 min was performed before the PCR This method proved to be very succesfull and was from then on used for all the cPCRs (Figure 5). By that an efficient and quick cPCR protocol was established the first time for *C. viswanathii*.

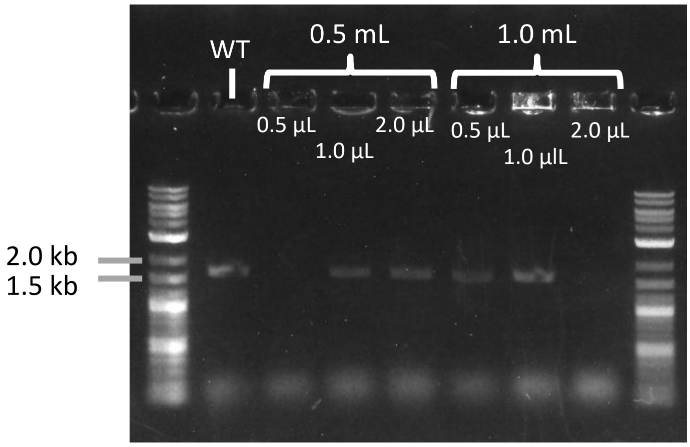

Figure 4. Test of using cells of 0.5 mL or 1.0 mL overnight culture boiled in 50 µL NaOH (20 mM) as template for cPCR. (WT: extracted gDNA as control, amount of volume used as template shown in µL; 1508 bp expected for all PCRs).

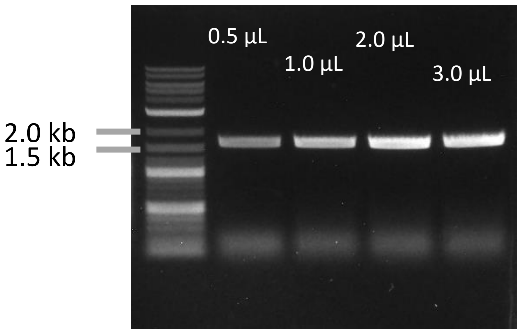

Figure 5. Testing of the CHELEX method with different volumes as template shown in µL (1508 bp expected for all PCRs).

2.3 Bioprocess engineering towards diacid production

To enable the efficient production of diacids, it is essential to define a precise bioprocess. Indeed, the use of living microorganisms as whole-cell biocatalysts requires specific attention to growth conditions and process parameters. The diacid production process consists of two phases: a growth phase, which provides an appropriate number of biocatalysts (cell density), and a biotransformation phase, where diacid production occurs with the alkanes fed to the cells. A pH shift is required between the two phases and the design of the accurate feeding strategy represent essential steps. Currently, first fermentations and tests have been running. However, due to the aggressive nature of the alkane substrates, it became necessary to order and install new parts and tubings for the bioreactors to ensure compatibility and maintain process integrity. These initial efforts underscore the importance of tailoring the bioprocess set-up to the challenges of diacid production from alkanes. With the equipment compatibility issues now addressed, the focus can shift towards refining and optimizing the process.

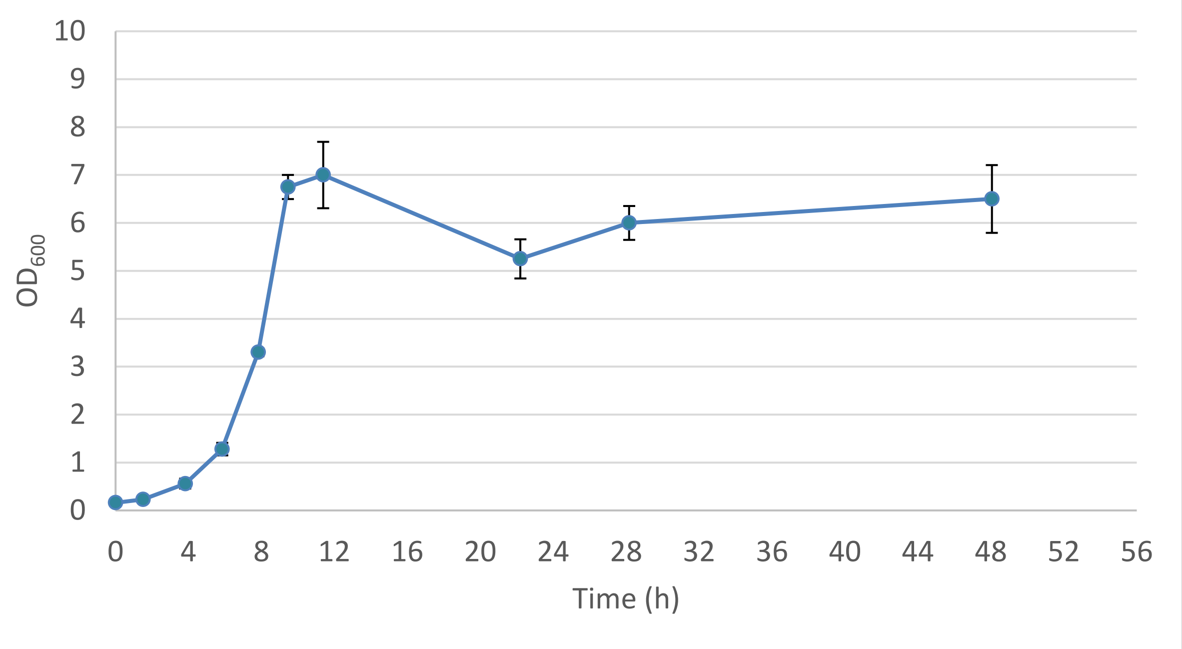

Figure 6. Growth curve of *C. viswanathii* in OPT-1 medium in shake flasks at 30 °C and 200 rpm shaking over the course of 48 h. Duplicates are shown.

2.4 GC-FID analysis of substrates and products

A proper and robust analytical method for the identification and quantification of the substrates and products is essential for this project. Due to shipping and technical delays in setting up our GC-MS, we have used a GC-FID available in our department. As a starting point, we mainly focused on preparing standards of one substrate and one diacid, dodecane, and dodecanedioic acid with which we plan to start fermentations. Standards were prepared and derivatized by using BSTFA-TMCS *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide) to introduce trimethysilyl groups instead of the hydroxy groups. The samples were analyzed using a Shimadzu GC-FID hosting the column Rtx-35ms (30 m x 0.53 mm x 1.00 µm), using the following conditions: the initial column temperature was 100 °C and held for 2 min, increased to 240 °C at 20 °C/min for 1 min, finally increased to 280 °C at 15 °C/min and kept for 2 min. Dodecane eluted at 3.6 min and dodecanedioic acid at 9.7 min (Figure 7).

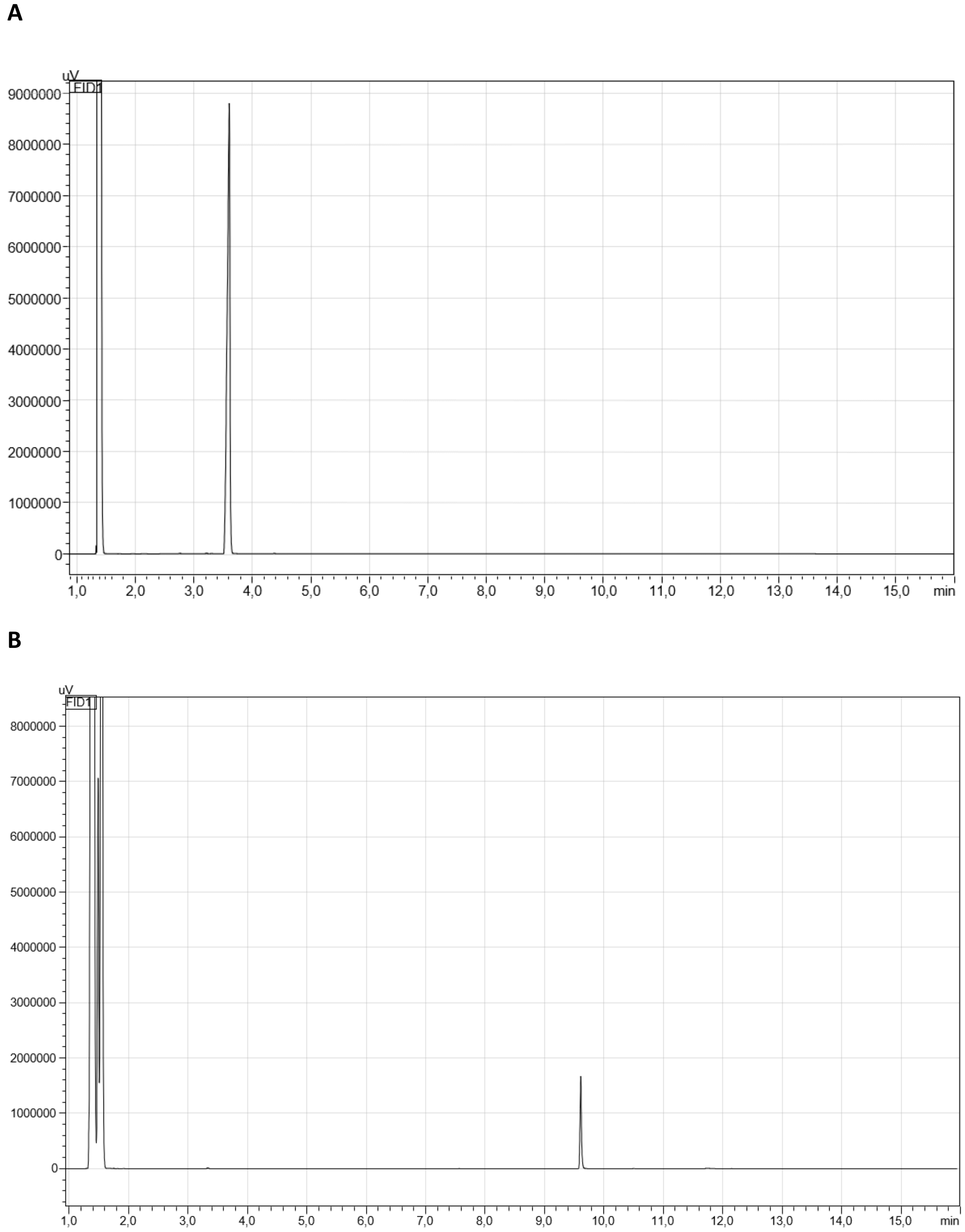

Figure 7. (A) Chromatogram of the standards of dodecane and **(B)** dodecanedioic acid using GC-FID.

3 Conclusions

In this task of our WP4 of the ACTPAC project, the non-pathogenic yeast *C. viswanathii* ATCC 20962 was first identified as the strain to genetically engineer for the production of diacids and we have subsequently defined which strategy to take once the genetic engineering method will be established. Furthermore, in this task, we have focused on establishing a proper transformation and genetic engineering method to use for this specific strain.

Two different transformation methods were tested: the lithium acetate method and electroporation. Yet, only the electroporation method enabled high numbers of transformants with pCTO-empty plasmid, 31-fold higher compared to the lithium acetate method. Consequently, this method was further used and optimized by linearization of the pCT-tRNA plasmid prior to transformation yielding 46-fold higher transformants compared to the use of non-linearized DNA fragments. Different strategies were also employed to enable successful deletions, including different target loci, different gRNAs and different recovery times. Up to now, no successful deletion strain has been obtained. Due to a most probable non-effective Cas9 expression using the pCT-tRNA plasmid, a new plasmid carrying native promoters and terminators from *C. viswanathii* is currently under preparation (cloning). To also complete the genetic manipulation of this strain, a proper and fast cPCR is a must-have. Colony PCRs for *Candida* sp. cells are usually time-consuming processes which first require an overnight culture of the single colonies, subsequent genomic DNA extraction and formation of spheroplasts. In this task, we have developed an efficient CHELEX-dependent cPCR, which will enable much faster progress in the genetic and metabolic engineering in the next steps.

Finally, we have addressed bioreactor equipment issues and set-up a GC-FID system that will both help in refining and optimizing the process for enhanced production of diacids and diols.

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