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ABSTRACT BOOK

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LACTIC ACID PRODUCTION BY ENGINEERED *OGATAEA POLYMORPHA* YEAST STRAINS EXPRESSING PROTISTAN LACTATE DEHYDROGENASE

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The rise in the global demand for lactic acid requires more intensive research on microbial lactic acid fermentation. The ability to biosynthesise lactic acid is rare among yeast, however metabolic engineering approach allows to use these microorganisms as efficient lactic acid producers due to heterologous expression of lactate dehydrogenase genes. *Ogataea polymorpha* being a thermotolerant yeast species with the ability to grow on renewable and low-cost substrates offers a great opportunity for engineering applicable lactic acid producing yeast strains.

The lactate dehydrogenase (*LDH*) gene from the protist *Plasmodium falciparum* was introduced into the wild type strain of *O. polymorpha* under the control of the strong constitutive promotor of glyceraldehyde-3-phosphate dehydrogenase gene. *LDH* gene codes an enzyme that catalyses the conversion of pyruvate into lactic acid. The initial selection of transformant yeast strains was performed on a YPD medium with the addition of colourful pH indicator bromophenol blue. 9 selected strains were studied for their ability to produce lactic acid and ethanol during high temperature flask fermentation on a minimal YNB medium with 10% glucose. The transformant yeast strain that produced the highest amount of lactic acid was selected for further investigation of its lactic acid producing properties. The next step would be to study the lactic acid and ethanol production on different carbon sources (including xylose, methanol, and glycerol) and agitation rates as well as in pH buffering conditions.

CONSTRUCTION OF THE YEAST RIBOFLAVIN OVERPRODUCERS BY INTRODUCTION OF GENES *RFE1*, *RIB6*, AND *GND1* INTO WILD STRAINS OF *CANDIDA FAMATA* VKM Y-9

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Riboflavin (vitamin B2) is a yellow compound, which can be produced by plants and microorganisms. It is a precursor of coenzymes, flavin mononucleotide and flavin adenine dinucleotide, which are involved in numerous enzymatic reactions in all organisms. *Candida famata* is a natural riboflavin-producing strain, belonging to the flavinogenic yeast, which overproduces riboflavin in iron-deficient media.

In previous studies, it was demonstrated that increasing the expression of the *RFE1* gene, which encodes riboflavin excretase, in the *C. famata* riboflavin overproducer BRP resulted in a 1.4-1.8-fold enhancement of riboflavin production (*Dmytruk et al., 2011, 2014; Tsyrulnyk et al., 2020*). Furthermore, it was observed that overexpression of the *GND1* gene, coding 6-phosphogluconate dehydrogenase, led to 1.3 fold overprodution of riboflavin in yeast (*Ruchala et al., 2022*) Recently, the riboflavin structural gene *RIB6* was overexpressed (coding 3,4-dihydroxy-2-butanone-4-phosphate synthase) in the best riboflavin producer BRPI *C. famata*. The obtained strain BRPI/RIB6 accumulated 13% more riboflavin than the parental strain (*Petrovska. et al., 2022*).

The aim of this work was to obtain recombinant strains with increased synthesis of riboflavin due to simultaneous expression of the mentioned regulatory and structural genes *RFE1*, *GND1*, and *RIB6* in *C. famata*. As a result, the plasmids with different combination of these genes were successfully constructed based on the vector pTTb, which harbors a strong constitutive promoter *Dh*_TEF1. The fidelity of these plasmids was verified by restriction digestions and PCR detections. Subsequently, the linearized plasmids were introduced into the wild-type strain *C. famata* VKM Y-9 by electroporation. The presence of a gene expression cassettes in the yeast genome were confirmed by PCR. In the best obtained recombinant strain, riboflavin production increased by 1.8 times compared to the initial strain.

Our results suggest that overexpression of genes *RFE1*, *GND1*, and *RIB6* led to significant riboflavin oversynthesis in the yeast. Simultaneous overexpresiion of these genes in the best available riboflavin overproducer BRPI *C. famata* strain is planned.

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METABOLIC ENGINEERING OF THE YEAST *CANDIDA FAMATA* FOR OVERSYNTHESIS OF RIBOFLAVIN FROM XYLOSE.

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Flavinogenic yeast *Candida famata* are characterized by the ability to grow on unconventional substrates, in particular on xylose. The metabolism of xylose in yeast starts with its reduction by xylose reductase (XR, EC 1.1.1.21) to xylitol, which is further oxidized to xylulose, this reaction is catalyzed by xylitol dehydrogenase (XDH, EC 1.1.1.9). Xylulose, at the next stage, is phosphorylated by xylulokinase (XK, EC2.7.1.17) to xylulose-5-phosphate. This sugar in the non-oxidizing phase of PPP is converted by phosphopentoepimerase to ribulose-5-phosphate which is used as the riboflavin precursor. To increase riboflavin production from xylose genes *XYL1* and *XYL2* were overexpressed.

The *XYL1* was cloned between TEF1 promoter and terminator into the corresponding sites of pT-SAT to create pT-SAT-X1. The *XYL2* gene with TEF1 promoter and terminator was amplified and cloned into pT-SAT-X1 to create pT-SAT-X1/X2. Obtained expression cassettes were introduced into *C. famata* BRPI strain, and the selection of *NTC* transformants was provided.

The ability of resulted strains to biomass accumulation and riboflavin production in xylose medium was studied. *XYL1* overexpression in recombinant BRPI strain improved growth kinetics up to 1.1-1.2 times, the production of riboflavin was about 1,36 times higher as compared to the initial strain. The BRPI/XYL1/XYL2 strain demonstrated 1.44-fold increase in riboflavin production.

Overexpression of *XYL1* gene elevated riboflavin production in synthetic medium with xylose, whereas additional overexpression of *XYL2* gene did not lead to further increase in riboflavin synthesis.

EFFECT OF *ATG1, ATG6, ATG15* GENE DELETIONS ON FORMALDEHYDE DEHYDROGENASE DEGRADATION IN THE METHYLOTROPHIC YEAST *KOMAGATAELLA PHAFFII*

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Exploring the mechanisms of cytosolic protein degradation holds significant importance both in terms of fundamental understanding and in terms of practical applications. The study investigated the ways of degradation of cytosolic enzyme formaldehyde dehydrogenase (*Fld1*) in different strains of methylotrophic yeast *Komagataella phaffii*. It was shown that *Fld1* degrade through the vacuolar pathway irrespective of the duration of methanol induction. This conclusion was verified through assessing the change in specific enzymatic activity, Western blot analysis and fluorescence microscopy studies. The disruption of *Atg1* and *Atg6*, which are involved in autophagy initiation and autophagosome formation, does not affect the degradation of *Fld1*. In contrast, the *Atg15* lipase, responsible for breaking down the membranes of autophagic bodies in the vacuole, is essential for the degradation of this enzyme in *K. phaffii.* The absence of *Fld1* degradation in *atg15Δ* after the shift from methanol on glucose suggests the protection of the enzymes as autophagic cargos in autophagic bodies, retaining their membrane integrity.

SEARCHING THE GENE INVOLVED IN THE AUTOPHAGY OF CYTOSOLIC AND PEROXISOMAL PROTEINS IN METHYLOTROPHIC YEAST *KOMAGATELLA PHAFFII*

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When the methylotrophic yeasts are transferred from the methanol medium to the glucose-containing medium, the formation of most of the enzymes involved in methanol utilization is repressed at the transcriptional level, and the enzymes already present in the cell undergo rapid degradation and proteolysis, and the cell's carbon metabolism switches to a glycolytic pathway. Until now, the mechanisms of degradation of cytosolic proteins of their own, as well as of recombinant foreign proteins of biotechnological significance with cytosolic localization in methylotrophic yeast, remain unclear. however, previously study suggest that they are partially degraded in the autophagic pathway. (Dmytruk et al., 2021)

To identify factors that influence this process, a developed system for the selection of recombinant strains of *K. phaffii* with impaired autophagic degradation of the heterologous model cytosolic protein (yeast β-galactosidase) was used for insertional tagging of the genes involved in cytosolic proteins degradation. Obtained *K. phaffii* transformants were analyzed to identify the strains with increased residual β-galactosidase activity aimed to identify genes specifically involved in the process of autophagic degradation of cytosolic proteins. In this study, the two autophagic degradation disrupted *K. phaffii* mutant strains were isolated. After then the four plasmids encompassing sequences of the insertion cassette pPICZ-B together with the sequences of the genomic DNA of *K. phaffii* which were flanking the insertion cassette in the genome of the study *K. phaffii* mutant were obtained.

It is important to mention that β-galactosidase of methanol-grown *K. phaffii* transformants can be assayed directly on plates using X-Gal staining, which opens opportunity to isolate the mutants defective in degradative inactivation of cytosolic proteins in *K. phaffii*. Therefore, the UV mutagenesis and chemical mutagenesis with N-Methyl-N′-nitro-N-nitrosoguanidine were carried out to screening the mutant *K. phaffii* strain with impaired autophagic degradation in this study.

DEVELOPING A NOVEL SELECTION METHOD FOR YEAST *OGATAEA POLYMORPHA*

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Methylotrophic yeast *Ogataea polymorpha* is the model organism for studying the mechanisms of thermotolerance, peroxisome homeostasis, methanol metabolism, production of heterologous proteins on industrial scale and of high temperature alcoholic fermentation. Still, the new tools for continue molecular research are needed, namely additional selective markers for the selection of recombinant strains with the desired physiological characteristics. The aim of this study was to develop the new selective approach for *O. polymorpha* usable in metabolic engineering experiments.

As a potential selective agent, metformin was used - a substance that suppresses gluconeogenesis and, as shown in our previous studies, increases the level of ethanol production from xylose, but not from glucose during high-temperature alcoholic fermentation. The minimal toxic concentration for *O. polymorpha* NCYC495 strain was found to be 50 mg/ml of metformin. The introduction of a vector for the overexpression of the *CAT8* gene restores the growth of yeast *O. polymorpha* on a YNB medium with glucerol and metforimin. The presence of the vector for overexpression in the transformants was confirmed by the PCR method. In summary, the introduction of pUC19/pGAP/CAT8/NTC (Ruchala et. al., 2017) into genome of *O. polymorpha* wild-type strain was sufficient to obtain transformants with restored expression of genes involved in gluconeogenesis and can be used as a new method of selecting recombinant strains.

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THE TRANSCRIPTION FACTOR SEF1 ACTIVATES EXPRESSION OF RIBOFLAVIN BIOSYNTHESIS STRUCTURAL GENES IN THE FLAVINOGENIC YEAST *CANDIDA FAMATA*

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Riboflavin (RF, also known as vitamin B_2) is a yellow water-soluble compound, one of the most important vitamins for human and animals. It is a precursor for flavin coenzymes FAD and FMN, which participate in multiple redox reactions in living cells. For a long time, it has been produced in large-scale industry exclusively with the use of microorganisms, such as bacteria *Bacillus subtilis*, fungi *Ashbya sp.*, and yeast *Candida famata.*

Although the metabolic pathway for RF biosynthesis in different yeast species is well established, little is known about its regulation. Sef1, the transcriptional factor of the zinc cluster family $Zn(II)_2Cys_6$, plays a central role in the regulatory circuit of RF biosynthesis in *Candida famata*. It positively regulates RF biosynthesis and is induced under iron deficiency in the growth medium. However, the mechanism by which Sef1 activates RF synthesis remains unclear. We hypothesized that Sef1 activates the transcription of RF synthesis structural genes by interacting with their corresponding promoters (*RIB*-promoters). We developed a Yeast-One-Hybrid system based on the yeast *Saccharomyces cerevisiae* to confirm this hypothesis. The yeast was transformed with two expression constructs: the first contained the reporter gene *LAC4* from *Kluyveromyces lactis*, encoding β-galactosidase, under the control of *RIB*-promoters; the second carried *SEF1* ORF under the galactose-inducible *GAL1* promoter. *S. cerevisiae* strains containing both plasmids were grown on media supplemented with galactose to induce Sef1, or with glucose as a control. Activity of β-galactosidase indicated the interaction of Sef1 with *RIB*-promoters.

The obtained results demonstrate that Sef1 interacts with and activates the promoter of the *RIB1* gene. Furthermore, a Sef1 binding sequence (TAAAAATCCGAACCCCGG) has been identified in the *RIB1* gene promoter, as evidenced by modified versions of this promoter. Substitution or deletion of this site leads to the loss of β-galactosidase activity. Sef1 also activates the promoters of *RIB3*, *RIB6*, and *RIB7* genes; however, the β-galactosidase activity observed under their activation is 50%, 73%, and 22% of that observed under *RIB1* promoter activation, respectively. Sef1 does not interact with *RIB2* and *RIB5* promoters.

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TIME AND DOSE-DEPENDENT INDUCTION OF IMMUNOGENIC CELL DEATH IN MURINE MELANOMA BY A NOVEL THIOSEMICARBAZONE DERIVATIVE

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During the last two decades, it has been demonstrated that multiple anticancer drugs can induce a specific pathway of programmed cell death in cancer cells that activates the anticancer immune response in the host. The underlying mechanism was named immunogenic cell death (ICD). It is crucial for the long-term success of anticancer therapies. Apart from doxorubicin (Dox), oxaliplatin and multiple other ICD-inducing chemotherapeutics, recent evidence suggests the ICD-inducing capabilities of novel thiosemicarbazone derivatives, such as COTI-NMe_2, synthesized by the group of Prof. C. Kowol from the University of Vienna. The current study aimed to compare the ICD-inducing properties of COTI-NMe_2 in two lines of murine melanoma and investigate the possibility of cross-immunization using these two lines.

Methods: cell culture studies *in vitro*, trypan blue assay, tumor cell inoculation *in vivo* , morphophysiological analysis of tumor-bearing animals, haemocytometry. Murine melanoma cells of the B16F10 line (wild type) and the B16F10/ADR line (Dox-resistant) were treated with COTI-NMe_2 for 24 and 48 hours to determine cytotoxicity and LC_{50} using trypan blue assay. For animal immunization studies, C57BL/6 mice were inoculated with the COTI-NMe_2-treated cells. As a negative control, we used necrotic tumor cells subjected to multiple freeze-thaw cycles (-196 to +20°C, 3x) and thus killed without ICD induction. Dox-treated cells were used as a positive control. After 14 days, the mice were rechallenged with alive B16F10 and B16F10/ADR cells. Blood samples were collected from surviving animals 30 days after the immunization and analyzed on an automatic blood analyzer Dymind DF51 Vet.

Despite being more resistant to Dox compared to B16F10 cells, B16F10/ADR melanoma was not more resistant to COTI-NMe_2 when treated for 24h and tended to be more susceptible to it when treated for 48h. Comparison of different time and dose combinations of COTI-NMe_2 treatment showed that the cell vaccine prepared from B16F10 cells treated with 500 nM of COTI-NMe_2 for 48 hours was the most effective in immunization against alive B16F10 cells in comparison with other treatment combinations (1μM/48h, 5μM/24h, 10μM/24h). However, the rechallenge with alive B16F10/ADR cells has not demonstrated any signs of immunization under the current experimental setup. Interestingly, the tumor growth dynamics after the rechallenge in mice previously inoculated with treated B16F10 cells were similar to those animals that were initially inoculated with treated B16F10/ADR cells, demonstrating the generally more aggressive phenotype of B16F10/ADR melanoma.

Overall, we demonstrated that treatment with lover doses of COTI-NMe_2 for longer time periods is more effective in preparing cell vaccines from B16F10 cells. Although no signs of cross-immunisation between B16F10 and B16F10/ADR have been detected so far, it cannot be excluded entirely yet, considering the similarities between these two lines of melanoma. More studies are needed to investigate the possibility of cross-immunization and the conditions under which it can or cannot occur.

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CYTOTOXIC AND PRO-APOPTOTIC ACTIVITY OF NEW THIOPYRANO[2,3-D]THIAZOLES DERIVATIVES TOWARDS COLORECTAL CARCINOMA CELLS

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Colorectal cancer (CRC) is the 3rd most common cancer worldwide and 2nd leading cause of death after lung cancer. Patients with advanced disease have few alternatives for treatment, which contributes significantly to colorectal cancer mortality. So, development novel, safe, and effective anticancer compounds is one of the main goal of modern medicine.

The aim of our work was to study cytotoxic and pro-apoptotic activity of thiopyrano[2,3-d]thiazoles (Les-6547, Les-6557) towards human colorectal adenocarcinoma cells.

The MTT and clonogenic assays were used for measure the cell viability, proliferation and ability of a single cell to grow into a colony which indicate the treatment cytotoxicity. Flow cytometry analysis of apoptosis induction, changes in mitochondrial membrane potential, and caspases 3/7, 8, and 9 activities were performed.

Human colorectal adenocarcinoma cells (HT-29, DLD-1) are more sensitive to the action of the Les-6547 and Les-6557 compared to pseudo-normal cells (human keratinocytes, mouse fibroblasts) suggested the selectivity of their action. Les-6547 and Les-6557 at 1 μM dose completely inhibit the viability, proliferation and colony formation ability of HT-29 colorectal cancer cells. Les-6547 and Les-6557 increased number of apoptotic cells, cells with reduced mitochondrial membrane potential, and cells with active caspases-8, -9, -3/7. This indicates that the studied compounds induce apoptotic cell death by intrinsic and extrinsic pathways.

Our team thanks the UKRAINIAN MILITARY - the HEROES of Ukraine who defend us and our country from russian aggression. It is thanks to them that we have the opportunity to work.

FOURIER TRANSFORM INFRARED SPECTROSCOPY PROFILING OF CULTURE MEDIUM CONDITIONED BY HUMAN HEPATOMA HEPG2 CELLS

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Human hepatoma HepG2 cells possess an epithelial-like morphology, perform many differentiated hepatic functions, and are convenient experimental models in hepatotoxicity studies. Here we utilized ATR-FTIR spectroscopy in order to track nutrient utilization, a release of waste products and signaling molecule by HepG2 cells into culture medium for 48 h. Prior to measurement, culture medium samples and the same medium devoid of cells were dried at room temperature to minimize background interference from water. Reflection spectra in the mid-infrared region (400–4000 cm−1) were then recorded using an FTIR spectrometer Nicolet iS50 equipped with a single-bounce diamond attenuated total reflection (ATR) accessory. Spectra were acquired with a spectral resolution of 4 cm−1, averaging 32 scans per spectrum.

Key peaks observed in the spectra were identified at 1648-1651 cm−1, 1574-1579 cm−1, 1400-1404 cm−1, 1300-1305 cm−1, 1075-1077 cm−1, and 1026-1030 cm−1. Notably, alterations in the conditioned culture medium were discerned through shifts in peak position, changes in peak intensity, and alterations in peak ratios. Specifically, a decrease in intensity was observed at the 1030 cm−1 and 1075 cm−1 peaks, corresponding to C–O stretching vibration indicative of carbohydrate fingerprinting, particularly glycogen absorption [1, 2]. Furthermore, a diminished intensity was observed around the 1404 cm−1 band, representative C-H banding vibrations of multiple amino acid side chains and lipids [3]. Of particular interest was the increased prominence of the peak at approximately 1303 cm−1 following exposure to HepG2 cells, correlating with amide III vibrations, specifically collagen [4].

These findings underscore specific changes induced by HepG2 cell interactions within the microenvironment. Further studies utilizing analysis of principle compounds is required to obtain reliable data on the hepatoma cells and its microenvironment interplay under various treatments.

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