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Ph.D. thesis

**REGULATION OF β -GALACTOSIDASE DEGRADATION AND
METABOLIC ENGINEERING OF XYLOSE FERMENTATION IN THE
METHYLOTROPHIC YEASTS *KOMAGATAELLA PHAFFII* AND *OGATAEA
POLYMORPHA***

09 – Biology

091 – Biology

Applying for the Doctor of Philosophy degree

The dissertation contains the results of my research. The use of ideas, results, and
texts of other authors are linked to the appropriate source

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Lviv – 2025

ANNOTATION

Mingxing ZUO. Regulation of β -galactosidase degradation and metabolic engineering of xylose fermentation in the methylotrophic yeasts *Komagataella phaffii* and *Ogataea polymorpha* – Qualifying scientific work on the rights of the manuscript.

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This dissertation primarily focuses on three topics: the first involves isolating mutants with defects in cytosolic β -galactosidase degradation in the methylotrophic yeast *Komagataella phaffii* through the use of the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); The second is to develop new dominant selectable markers for future applications in metabolic engineering of the yeast *O. polymorpha*; the third addresses the development and implementation of metabolic engineering strategies to produce *Ogataea polymorpha* strains with enhanced efficiency of ethanol production from xylose.

Komagataella phaffii (previously known as *Pichia pastoris*) is an obligate aerobic, methylotrophic yeast capable of using methanol as sole carbon and energy source (Mastropietro et al., 2021). Its traits – such as respiratory growth to very high cell densities, efficient protein secretion, and robust expression of recombinant proteins driven by strong inducible promoters – make *K. phaffii* highly valuable in the pharmaceutical and biotechnology sectors.

However, when *K. phaffii* is transferred from methanol to glucose medium, most of the enzymes involved in methanol utilization and already present in the cell undergo rapid degradation and proteolysis (Kirkin et al., 2009). Although *K. phaffii* is a robust industrial species known for high-level protein overproduction, effective minimizing cytosolic degradation of recombinant proteins remains challenging. This rapid degradation significantly limits potential of this yeast for large-scale industrial applications. Currently, two primary pathways of protein degradation are recognized.

The first is proteasomal degradation, a cytoplasmic process that predominantly targets ubiquitinated, short-lived proteins for breakdown (Pohl & Dikic, 2019). The second pathway, known as autophagy, is lysosome- (or vacuole-) dependent and involves not only the degradation of proteins but also the breakdown of intracellular organelles and other macromolecular complexes within lysosomes (vacuoles), where the resulting small molecules can be recycled for cellular use (Mizushima & Komatsu, 2011; Sibirny, 2016).

Previous research has shown that formaldehyde dehydrogenase, formate dehydrogenase, and fructose-1,6-bisphosphatase in *K. phaffii* are selectively degraded through the autophagy pathway (Dmytruk et al., 2021; Dmytruk et al., 2020). Therefore, modifying and optimizing the autophagy pathway has the potential to enhance the production of heterologous proteins in this yeast. Moreover, methylotrophic yeasts are regarded as exemplary model organisms for elucidating the mechanisms underlying autophagy, with a number of genes implicated in autophagy and pexophagy, such as *ATG26*, *ATG28*, *ATG35*, *TRS85*, *GCR1*, *HXS1* and *GSS1*, identified in *O. polymorpha* and *K. phaffii* by the group of A. Sibirny at Institute of Cell Biology NAS of Ukraine. However, despite extensive knowledge of the degradation mechanisms for proteins and cellular organelles, the processes underlying the degradation of intrinsic cytosolic proteins and recombinant heterologous proteins in the cytosol of methylotrophic yeast remain poorly characterized. To investigate the autophagic degradation mechanisms of cytosolic proteins in *K. phaffii*, it is essential to isolate mutants that are specifically involved in this process.

In our previous work, we developed a vector for expressing the β -galactosidase gene *LAC4* from *Kluyveromyces lactis*, fused with the fluorescent tag green fluorescent protein (GFP) and regulated by the methanol-inducible promoter of the *FLD1* gene. This vector was transformed into *K. phaffii* and subsequently employed to directly assay β -galactosidase activity on YPD plates using X-gal staining, thereby providing an opportunity to isolate mutants that are defective in the degradation of cytosolic proteins in *K. phaffii* (Dmytruk et al., 2020). Besides, using this transformant, the insertion mutant with defects in β -galactosidase inactivation due to a mutation in a

novel gene, designated *ACG1*, which is involved in the autophagy of cytosolic and peroxisomal proteins was isolated (Zazulya et al., 2023).

In this dissertation, the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was utilized to select new mutant strains exhibiting impaired degradation of β -galactosidase. The impairment of this enzyme's degradation in the selected mutants was assessed based on their blue color on YPD with X-gal plates following the shift from methanol to glucose. Four mutants displaying elevated β -galactosidase activity on glucose compared to the parental strain were identified. Viability assays and phloxine B assays conducted under nitrogen starvation conditions revealed growth defects in these mutants. Furthermore, the biomass of the mutant strains was significantly diminished relative to that of the parental strain, indicating an impairment in autophagy. These findings suggest that the selected mutants possess defects in autophagy. Additionally, to investigate pexophagy, residual activity of the key peroxisomal matrix protein alcohol oxidase (AOX) was measured in the selected mutants after shifting cells from methanol to glucose. The results demonstrated that mutants MNNG-1 and MNNG-3 exhibited defects in both selective autophagy and pexophagy, while mutants MNNG-2 and MNNG-4 were characterized exclusively by defects in selective autophagy of cytosolic β -galactosidase. In the future, sequencing of the mutant strains is anticipated to uncover specific genes associated with autophagy defects, which could provide critical insights into the molecular mechanisms governing autophagy. Identifying these genes may reveal novel regulatory pathways and targets for modulating autophagy in methylotrophic yeast, with potential applications in enhancing protein production, engineering metabolic pathways, and understanding cellular homeostasis. This study thus establishes a foundational basis for further investigations into autophagy and protein degradation, paving the way for advances in biotechnology and pharmaceutical applications where precise control of autophagic processes is essential.

In another topic of this dissertation, novel dominant selectable markers were developed for the nonconventional yeast *Ogataea polymorpha*. *O. polymorpha* is a methylotrophic, thermotolerant yeast capable of xylose alcoholic fermentation, and

serves as both an important model eukaryotic microorganism for studying molecular mechanisms of life processes and a promising cell factory. Genetic engineering techniques are now commonly employed to create novel strains with altered metabolic pathways and investigate fundamental aspects of eukaryotic cellular biology. The expanding applications of these techniques necessitate new selection markers to identify recombinant strains exhibiting desired physiological traits. For commercial applications, yeast vectors containing only native genes (self-cloning vectors) are particularly advantageous (Akada et al., 2002). These self-marker systems are particularly effective for transforming prototrophic industrial yeast strains (Hashida-Okado et al., 1996; Hashida-Okado, Ogawa, et al., 1998). However, despite significant advancements in molecular tools for *O. polymorpha*, self-cloning selection markers for this organism are still unavailable. Therefore, in this study, we evaluated two potential dominant selective markers for *O. polymorpha*: the mutated *AUR1* gene and the native *IMH3* gene, which confer resistance to aureobasidin and mycophenolic acid, respectively. These markers were tested for their potential application in metabolic engineering of *O. polymorpha*.

Two mutant versions of the *AUR1* gene were created to develop aureobasidin-resistant *O. polymorpha* strains. The first variant contained two amino acid substitutions: leucine to phenylalanine at position 53 (L53 CTT→F53 TTT) and histidine to tyrosine at position 72 (H72 CAT→Y72 TAT). The second variant carried a single substitution of alanine to cysteine at position 156 (A156 GCA → C156 TGT). The two *AUR1* variants were separately transformed into wild-type *O. polymorpha* and selected on YPD medium containing aureobasidin (0.3 mg/L). Only transformants carrying the A156C mutation formed colonies, while no growth was observed for strains containing the L53F/H72Y mutations. These results demonstrate that the *AUR1* gene variant with the A156C substitution is effective as a selection marker for aureobasidin-resistant *O. polymorpha* transformants.

Additionally, we constructed a plasmid containing the *O. polymorpha IMH3* gene, which confers resistance to mycophenolic acid. The NdeI-linearized plasmid was transformed into wild-type *O. polymorpha*. Transformants appeared on YNB medium

containing mycophenolic acid (40 mg/L) after five days of cultivation, with a transformation frequency of 20 transformants per microgram of DNA. The results demonstrated that introducing an additional copy of the *IMH3* gene into the wild-type *O. polymorpha* genome was sufficient to generate mycophenolic acid-resistant transformants, establishing *IMH3* as an effective selection marker.

To demonstrate the utility of the *IMH3* selection marker, a plasmid was constructed for simultaneous overexpression of three genes under the GAP promoter: *TAL1* (cytosolic transaldolase), *TKL1* (cytosolic transketolase), and *AOX1* (peroxisomal alcohol oxidase). This plasmid was transformed into the advanced ethanol producer strain *O. polymorpha* BEP/ Δ cat8. Transformants were selected on YNB medium containing mycophenolic acid (40 mg/L) after five days of cultivation. We evaluated ethanol production during xylose fermentation in the resulting strain BEP/ Δ cat8/*TAL1*/*TKL1*/*AOX1* compared to its parental strain. The engineered strain showed enhanced ethanol production during xylose fermentation, accumulating 39% more ethanol than the parental BEP/ Δ cat8 strain at 43 hours, with continued fermentation observed up to 67 hours. These results demonstrated that both the mutated *AURI* gene (conferring aureobasidin resistance) and the native *IMH3* gene (conferring mycophenolic acid resistance) as effective dominant selection markers for *O. polymorpha* strain engineering.

In the third topic in this dissertation, we primarily focus on elevated bioethanol production from xylose in *O. polymorpha*. To tackle the escalating challenge of diminishing oil resources, there is an urgent global demand for green and clean energy solutions. In this context, bioethanol has emerged as an ideal renewable clean energy source, attracting significant attention and interest from global researchers.

Currently, commercial bioethanol production primarily relies on first-generation ethanol, which is derived from raw materials such as corn in the United States, sugar cane and sweet potatoes in Brazil, and wheat and sugar beets in Europe. However, this production method, which relies on food crops, has sparked widespread discussions about the competition between food and fuel in the world. This competition can not only destabilize the food supply but also drive up food prices, potentially impacting

vulnerable groups in society. Therefore, the production of second-generation ethanol using agricultural waste, such as lignocellulose biomass, has gradually garnered increasing attention. This production method has significant advantages as it does not compete with food resources for human consumption, effectively utilizes existing agricultural by-products, reduces dependence on food crops, and promotes sustainable development. With advancements in technology and supportive policies, second-generation ethanol is expected to become an important direction for future bioenergy development.

The hydrolysis of lignocellulosic biomass mainly produces hexoses and pentoses, with glucose as the predominant hexose and xylose as the principal pentose, accounting for approximately one-third of the total sugar content, second only to glucose. Currently, the main microorganism used for ethanol production is *Saccharomyces cerevisiae*; however, this yeast cannot naturally utilize xylose to produce ethanol. The lack of robust microbial strains capable of efficiently fermenting both pentose and hexose sugars to ethanol has created a significant technical barrier to achieving high yields and productivities (Lu, 2021). Therefore, the microorganism with efficient xylose fermentation is a critical prerequisite for the development of a viable process for converting lignocellulose into fuel ethanol.

The natural xylose-metabolizing yeast *O. polymorpha* can tolerate high temperatures (up to 50°C) during fermentation, making it suitable for simultaneous saccharification and fermentation (SSF) of lignocellulosic hydrolysates (Zaldivar et al., 2001). However, wild-type *O. polymorpha* produces only minimal ethanol, with production levels around 0.5 g/L in xylose medium (Ruchala et al., 2020). In our previous study, various metabolic engineering and classical random selection techniques were applied to develop recombinant *O. polymorpha* strains. These strains achieved up to a 40-fold increase in ethanol production from xylose at high temperature. (Ruchala et al., 2017; O. Kurylenko et al., 2018). Nonetheless, the rate of xylose uptake remains notably lower than that of glucose, especially during mixed-sugar fermentations, where xylose utilization typically initiates only after glucose is completely consumed. This sequential consumption leads to a prolonged fermentation

cycle, as the slower uptake of xylose delays the overall process. To further enhance the efficiency of ethanol production from xylose, additional strategies should be investigated. Previous studies have demonstrated that transcription factors and sugar transporters play key roles in regulating ethanol fermentation from xylose (Semkiv et al., 2022). However, the role of sugar sensors in xylose fermentation remains unexplored. Investigating sugar sensors could provide crucial insights into how cells detect and respond to xylose, potentially leading to strategies that improve xylose uptake and utilization.

Therefore, this dissertation focuses on investigating the roles of the hexose sensor gene *HXS1* and the *AZF1* gene, which encodes the *O. polymorpha* homolog of the *S. cerevisiae* transcription factor with sensing properties, in the alcoholic fermentation of xylose and glucose by *O. polymorpha*.

First, the gene *HXS1*, which encodes a hexose transporter-like sensor and is a close homolog of the *S. cerevisiae* sensors *SNF3* and *RGT2*, was overexpressed in the advanced ethanol producer *O. polymorpha* BEP/ Δ cat8 and wild-type strains. The role of this gene in xylose utilization and fermentation had not been previously elucidated. The recombinant strain obtained showed a 10% increase in ethanol production from glucose and 40% increase in ethanol production from xylose compared to the BEP/ Δ cat8 strain. When *HXS1* was overexpressed in the WT background, both glucose and xylose fermentation were activated, and ethanol production from xylose increased relative to the WT (0.83 g/L of ethanol against 0.49 g/L by WT strain at 72 h of fermentation). At the same time, strains with overexpression of the gene *AZF1*, which encodes a transcription activator involved in carbohydrate sensing, were also constructed on the background of the WT and the advance ethanol producer strains, respectively. These transformants demonstrated 10% more ethanol in glucose medium and 2.4 times more ethanol in xylose medium than wild-type strain. Additionally, it resulted in a nearly 10% increase in ethanol accumulation on glucose and in the xylose medium, there was an above 30% increase than the advanced ethanol producer *O. polymorpha* BEP/ Δ cat8. The data suggest that overexpressing the *AZF1* and *HXS1* genes has a positive impact on ethanol production from xylose in *O. polymorpha* strains.

Given that efficient xylose fermentation is essential for optimizing bioethanol production from lignocellulosic biomass, understanding these mechanisms is necessary for advancing industrial applications and enhancing the economic viability of biofuels. Besides, the strains developed in this study can be further utilized to establish stable ethanol superproducers.

Keywords: β -galactosidase, MNNG, Autophagy deficient, *Komagataella phaffii*, Selective-marker; Lignocellulose, Xylose, Alcoholic fermentation, Sensors, *Ogataea polymorpha*.

АНОТАЦІЯ

Мінсін ЗУО. Регуляція деградації β -галактозидази та метаболічна інженерія ферментації ксилози у метилотрофних дріжджів *Komagataella phaffii* та *Ogataea polymorpha*. – Кваліфікаційна наукова праця на правах рукопису.

Дисертація на здобуття наукового ступеня доктора філософії за спеціальністю 091 – Біологія. – Інститут біології клітини НАН України, Львів, 2025.

Дисертація головним чином зосереджена на трьох розділах: перший передбачає виділення мутантів з дефектами цитозольної деградації β -галактозидази у метилотрофних дріжджів *Komagataella phaffii* за допомогою хімічного мутагену N-метил-N'-нітро-N-нітрозогуанідину (MNNG); метою другого є розробка нових домінантних селективних маркерів для застосування у майбутньому в метаболічній інженерії дріжджів *O. polymorpha*; третій стосується розробки та впровадження стратегій метаболічної інженерії для отримання штамів *Ogataea polymorpha* з підвищеною ефективністю продукції етанолу з ксилози.

Komagataella phaffii (раніше відомі як *Pichia pastoris*) є облигатними аеробними метилотрофними дріжджами, здатними використовувати метанол як єдине джерело вуглецю та енергії (Mastropietro et al., 2021). Їх властивості, такі як здатність до респіраторного росту до дуже високої концентрації клітин, ефективна секреція білка та стабільна експресія рекомбінантних білків, контрольована сильними індукцибельними промоторами, роблять *K. phaffii* дуже цінними у фармацевтичному та біотехнологічному секторах.

Однак, якщо *K. phaffii* перенести з метанолу у середовище з глюкозою, більшість ферментів, які беруть участь в утилізації метанолу і вже присутні в клітині, зазнають швидкої деградації та протеолізу (Kirkin et al., 2009). Незважаючи на те, що *K. phaffii* є надійним промисловим видом, для якого характерний високий рівень синтезу білка, мінімізація цитозольної деградації

рекомбінантних протеїнів залишається проблематичною. Така швидка деградація значно обмежує потенціал цих дріжджів для великомасштабного промислового застосування. На сьогодні відомі два основних шляхи деградації протеїнів. Перший – це протеасомна деградація, цитоплазматичний процес, який переважно стосується убіквітинованих, короткоживучих білків (Pohl & Dikic, 2019). Другий шлях, відомий як автофагія, відбувається у лізосомах та вакуолях, за якого деградують не лише окремі протеїни, але також і органели та інші макромолекулярні комплекси, завдяки чому продукти розщеплення - невеликі молекули можуть бути повторно використані клітиною для власних потреб (Mizushima & Komatsu, 2011; Sibirny, 2016).

Попередні дослідження показали, що формальдегіддегідрогеназа, форміатдегідрогеназа та фруктозо-1,6-бісфосфатаза в *K. phaffii* вибірково розщеплюються шляхом автофагії (Dmytruk et al., 2021; Dmytruk et al., 2020). Таким чином, модифікація та оптимізація шляху автофагії має потенціал для посилення синтезу гетерологічних білків у клітинах цього виду дріжджів. Крім того, метилотрофні дріжджі вважаються зразковими модельними організмами для з'ясування механізмів, що лежать в основі автофагії, з низкою генів, залучених до автофагії та пексофагії, таких як *ATG26*, *ATG28*, *ATG35*, *TRS85*, *GCR1*, *HXS1* та *GSS1*, ідентифікованих у *O. polymorpha* та *K. phaffii* групою А. Сибірного в Інституті біології клітини НАН України. Однак, незважаючи на знання про механізми деградації білків і клітинних органел, процеси, що лежать в основі деградації власних цитозольних білків і рекомбінантних гетерологічних білків у цитозолі метилотрофних дріжджів, є на сьогодні погано охарактеризованими. Щоб дослідити механізми автофагічної деградації цитозольних білків у *K. phaffii*, важливо виділити мутанти, у яких цей процес відбувається специфічно.

У нашій попередній роботі ми розробили вектор для експресії гена β -галактозидази *LAC4* з *Kluyveromyces lactis*, злитого з флуоресцентною міткою зеленого флуоресцентного білка (GFP), під контролем метанол-індукованого промотора гена *FLD1*. Цей вектор був трансформований у *K. phaffii* та відповідно

використаний для безпосереднього аналізу активності β -галактозидази на чашках із середовищем YPD з барвником X-gal, таким чином дозволивши виділити мутанти з дефектною в деградацією цитозольних білків у *K. phaffii* (Dmytruk та ін., 2020). Крім того, на основі цього трансформанта отримано інсерційного мутанта з дефектами інактивації β -галактозидази внаслідок мутації в новому гені, позначеному як *ACGI*, який бере участь в аутофагії цитозольних і пероксисомних білків (Zazulya et al., 2023).

У цій дисертації хімічний мутаген N-метил-N'-нітро-N-нітрозогуанідин (MNNG) був використаний для відбору нових мутантних штамів, що демонструють порушення деградації β -галактозидази. Порушення деградації цього ферменту у вибраних мутантів оцінювали на основі їх синього кольору на чашках із YPD з X-gal після зміни метанолу на глюкозу у якості ростового субстрату. Було ідентифіковано чотири мутанти, що демонструють підвищену активність β -галактозидази на глюкозі порівняно з батьківським штамом. Крапельний тест та визначення концентрації флоксину В, проведені в умовах голодування за Нітрогеном, виявили дефекти росту цих мутантів. Крім того, біомаса мутантних штамів була значно меншою порівняно з батьківським штамом. Ці дані свідчать про те, що вибрані мутанти мають дефекти аутофагії. Також, щоб дослідити пексофагію, у відібраних мутантах після переведення клітин з метанолу на глюкозу вимірювали залишкову активність ключового протеїну матриксу пероксисоми – алкогольоксидази (AOX). Результати продемонстрували, що мутанти MNNG-1 і MNNG-3 мають дефекти як селективної аутофагії, так і пексофагії, тоді як мутанти MNNG-2 і MNNG-4 характеризувалися виключно дефектами селективної аутофагії цитозольної β -галактозидази. Очікується, що в майбутньому секвенування мутантних штамів дозволить встановити конкретні гени, пов'язані з дефектами аутофагії. Це дозволить досягти критичного розуміння молекулярних механізмів, що є в основі регуляції аутофагії. Ідентифікація цих генів може виявити нові регуляторні шляхи та мішені для модуляції аутофагії в клітинах метилотрофних дріжджів з потенційним високристанням для покращення продукції білка, розробки

метаболічних шляхів і розуміння клітинного гомеостазу. Таким чином, це дослідження створює основу для подальшого вивчення автофагії та деградації білків, прокладаючи шлях до прогресу в біотехнології та фармації, де точний контроль автофагічних процесів є важливим.

Також у цій дисертації було розроблено нові домінантні селективні маркери для неконвенційних дріжджів *Ogataea polymorpha*. *O. polymorpha* – це метилотрофні термотолерантні дріжджі, здатні до алкогольної ферментації ксилози, і є важливим модельним еукаріотичним мікроорганізмом для вивчення молекулярних механізмів клітинних процесів та в той же час перспективним продуцентом білків та інших корисних сполук. Методи генетичної інженерії сьогодні широко застосовуються для створення нових штамів із видозміненими метаболічними шляхами та для дослідження фундаментальних аспектів еукаріотичної клітинної біології. Розширення меж використання цих методів потребує нових селективних маркерів для ідентифікації рекомбінантних штамів, що виявляють бажані фізіологічні риси. Особливо вигідними для комерційного застосування є дріжджові вектори, що містять тільки нативні гени (самоклоновані вектори) (Akada et al., 2002). Ці автомаркерні системи є особливо ефективними для трансформації прототрофних промислових штамів дріжджів (Hashida-Okado et al., 1996; Hashida-Okado, Ogawa, et al., 1998). Однак, незважаючи на суттєві досягнення у розробці молекулярних методів для *O. polymorpha*, маркери для самокловування є досі недоступними. Таким чином, у цій роботі, ми оцінили два потенційні домінантні маркери для *O. polymorpha*: мутований ген *AUR1* та нативний ген *IMH3*, що забезпечують стійкість до авребазидину та мікофенолової кислоти, відповідно. Ці маркери були протестовані для їх потенційного використання у метаболическій інженерії *O. polymorpha*.

Дві мутантні версії гену *AUR1* було створено для розробки штамів *O. polymorpha*, стійких до авребазидину. Перший варіант містить два заміщення амінокислотних залишків: лейцин на фенілаланін у позиції 53 (L53 CTT → F53 TTT) та гістидин на тирозин у позиції 72 (H72 CAT → Y72 TAT). У другому варіанті проводилася одноразова заміна аланіну на цистеїн у позиції 156 (A156

GCA → C156 TGT). Два варіанти *AUR1* були окремо трансформовані в *O. polymorpha* дикого типу та відібрані на середовищі YPD, що містить авребазидин (0,3 мг/л). Лише трансформанти, що несуть мутацію A156C, утворювали колонії, тоді як для штамів, що містять мутації L53F/H72Y, ріст не спостерігався. Ці результати демонструють, що варіант гена *AUR1* із заміною A156C ефективний як маркер селекції для трансформантів *O. polymorpha*, стійких до ауреобазидину. Крім того, ми сконструювали плазмиду, що містить ген *IMH3* *O. polymorpha*, який надає стійкість до мікофенолової кислоти. NdeI-лінеаризовану плазмиду трансформували в *O. polymorpha* дикого типу. Трансформанти відбирали на середовищі YNB, що містить мікофенолову кислоту (40 мг/л), після п'яти днів культивування з частотою трансформації 20 трансформантів на мікрограм ДНК. Результати продемонстрували, що введення додаткової копії гена *IMH3* в геном *O. polymorpha* дикого типу було достатнім для отримання трансформантів, стійких до мікофенолової кислоти, що свідчить про ефективність *IMH3* як маркера селекції. Щоб продемонструвати ефективність маркера селекції *IMH3*, ми сконструювали плазмиду для одночасної надекспресії трьох генів під контролем промотора *GAP*: *TAL1* (цитозольна трансальдолаза), *TKL1* (цитозольна транскетолаза) і *AOX1* (пероксисомна алкогольоксидаза). Ця плазміда була трансформована в найкращий продуцент етанолу штам *O. polymorpha* ВЕР/Δcat8. Трансформанти відбирали на середовищі YNB, що містить мікофенолову кислоту (40 мг/л), після п'яти днів культивування. Ми оцінили виробництво етанолу під час ферментації ксилози в отриманому штамі ВЕР/Δcat8/*TAL1*/*TKL1*/*AOX1* порівняно з його вихідним штамом. Сконструйований штам продемонстрував посилену продукцію етанолу під час ферментації ксилози, акумулюючи на 39% більше етанолу, ніж батьківський штам ВЕР/Δcat8 через 43 години, із продовженням ферментації до 67 годин. Ці результати продемонстрували, що як мутований ген *AUR1* (надає стійкість до авребазидину), так і нативний ген *IMH3* (надає стійкість до мікофенолової кислоти) є ефективними домінантними маркерами селекції для розробки штаму *O. polymorpha*.

В третьому розділі цієї дисертації ми в основному зосереджуємося на підвищеній продукції біоетанолу з ксилози в *O. polymorpha*. Щоб подолати зростаючу проблему зменшення нафтових ресурсів, існує нагальний глобальний попит на екологічні та чисті енергетичні рішення. У цьому контексті біоетанол став ідеальним відновлюваним чистим джерелом енергії, привертаючи значну увагу та зацікавленість світових дослідників.

В даний час комерційне виробництво біоетанолу в основному покладається на етанол першого покоління, який отримують з такої сировини, як кукурудза в Сполучених Штатах, цукрова тростина і солодка картопля в Бразилії, а також пшениця і цукрові буряки в Європі. Однак цей метод виробництва, який базується на продовольчих культурах, викликав широкі дискусії про конкуренцію між продовольством і паливом у світі. Ця конкуренція може не тільки дестабілізувати постачання продовольства, але й підвищити ціни на продукти харчування, потенційно вплинувши на вразливі групи суспільства. Тому виробництво етанолу другого покоління з використанням сільськогосподарських відходів, таких як лігноцелюозна біомаса, поступово привертає все більшу увагу. Цей метод виробництва має значні переваги, оскільки він не конкурує з харчовими ресурсами для споживання людиною, ефективно використовує наявні побічні сільськогосподарські продукти, зменшує залежність від продовольчих культур і сприяє сталому розвитку. Завдяки прогресу в технологіях і підтримці політики, очікується, що етанол другого покоління стане важливим напрямком майбутнього розвитку біоенергетики.

Під час гідролізу лігноцелюозної біомаси в основному утворюються гексози та пентози, причому глюкоза є переважною гексозою, а ксилоза – основною пентозою, що становить приблизно одну третину загального вмісту цукрів, поступаючись лише глюкозі. В даний час основним мікроорганізмом, який використовується для виробництва етанолу, є *Saccharomyces cerevisiae*; однак ці дріжджі не можуть природно використовувати ксилозу для продукції етанолу. Відсутність стійких мікробних штамів, здатних ефективно ферментувати як пентозу, так і гексозу до етанолу, створила значну технічну

перешкоду для досягнення високої продуктивності (Lu, 2021). Таким чином, мікроорганізми з ефективною ферментацією ксилози є критичною передумовою для розвитку життєздатного процесу перетворення лігноцелюлози в паливний етанол.

Природні дріжджі *O. polymorpha*, що метаболізують ксилозу, можуть витримувати високі температури (до 50°C) під час бродіння, що робить їх придатними для одночасної сахарифікації та ферментації (SSF) лігноцелюлозних гідролізатів (Zaldivar et al., 2001). Однак *O. polymorpha* дикого типу виробляє лише мінімальну кількість етанолу з рівнем близько 0,5 г/л у ксилозному середовищі (Ruchala та ін., 2020). Щоб підвищити ефективність продукції етанолу з ксилози, різні методи метаболічної інженерії та класичні методи селекції були застосовані для розробки рекомбінантних штамів *O. polymorpha*, які демонструють 40-кратне збільшення продукції етанолу з ксилози при високій температурі в нашому попередньому дослідженні (Ruchala та ін. ., 2017; Куриленко та ін., 2018). Тим не менш, швидкість поглинання ксилози залишається помітно нижчою ніж швидкість поглинання глюкози, особливо під час бродіння змішаного цукру, де утилізація ксилози зазвичай починається лише після повного споживання глюкози. Таке послідовне споживання призводить до подовження циклу ферментації, оскільки повільніше поглинання ксилози затримує загальний процес. Для подальшого підвищення ефективності продукції етанолу з ксилози слід вивчити додаткові стратегії. Попередні дослідження показали, що фактори транскрипції та транспортери моносахаридів відіграють ключову роль у регулюванні ферментації етанолу з ксилози (Semkiv et al., 2022). Однак роль білків-сенсорів у ферментації ксилози залишається невивченою. Дослідження білків-сенсорів може надати важливу інформацію про те, як клітини розпізнають ксилозу і реагують на неї, що потенційно приведе до стратегій, які покращують поглинання та використання ксилози.

Таким чином, ця дисертація зосереджена на дослідженні ролі гена гексозного сенсора *HXS1* та гена *AZF1*, який кодує гомолог транскрипційного

фактора з *S. cerevisiae* із сенсорними властивостями, у спиртовому бродінні ксилози та глюкози *O. polymorpha*.

По-перше, ген *HXS1*, який кодує сенсор, подібний до транспортера гексози, і є близьким гомологом сенсорів *S. cerevisiae* *SNF3* і *RGT2*, був надекспресований у покращеному продуценті етанолу *O. polymorpha* ВЕР/ Δ cat8 і штамів дикого типу (WT). Роль цього гена в утилізації ксилози та ферментації раніше не була з'ясована. Отриманий рекомбінантний штам продемонстрував збільшення продукції етанолу з глюкози на 10% і збільшення продукції етанолу з ксилози на 40% порівняно зі штамом ВЕР/ Δ cat8. Коли *HXS1* був надекспресований на фоні WT, ферментація як глюкози, так і ксилози були активовані, і продукція етанолу з ксилози збільшилася відносно тієї у WT (0,83 г/л етанолу проти 0,49 г/л з штамом WT за 72 години бродіння). У той же час штам з надекспресією гена *AZF1*, який кодує активатор транскрипції, що бере участь у сенсингу вуглеводів, також були сконструйовані на основі WT і покращеного продуцента етанолу (*O. polymorpha* ВЕР/ Δ cat8), відповідно. Одержані трансформанти продукували на 10% більше етанолу на середовищі з глюкозою та в 2,4 рази більше етанолу на середовищі з ксилозою, ніж штам дикого типу. Крім того, це призвело до збільшення акумуляції етанолу майже на 10% на глюкозі та на 30% більше в ксилозному середовищі, ніж у покращеного продуцента етанолу *O. polymorpha* ВЕР/ Δ cat8. Дані свідчать про те, що надекспресія генів *AZF1* і *HXS1* позитивно впливає на виробництво етанолу з ксилози в штамів *O. polymorpha*. Враховуючи, що ефективне бродіння ксилози має важливе значення для оптимізації виробництва біоетанолу з лігноцелюлозної біомаси, розуміння цих механізмів є необхідним для вдосконалення промислового застосування та підвищення економічної ефективності біопалива. Крім того, штамів, розроблені в цьому дослідженні, можуть бути використані для створення стабільних надпродуцентів етанолу.

Ключові слова: β -галактозидаза, N-метил-N'-нітро-N-нітрозогуанідин (MNNG), дефіцит автофагії, *Komagataella phaffii*, лігноцелюлоза, ксилоза, спиртове бродіння, сенсори, *Ogataea polymorpha*.

The list of publications of the acquirer:

Seven scientific works have been published on the dissertation topic, including **three** manuscripts in international publications and **four** abstracts of reports in the materials of conferences, scientific congresses, and congresses.

Articles in periodical scientific publications indexed in databases Web of Science Core Collection and/or Scopus:

1. **Zuo M**, Dmytruk OV, Dmytruk KV, Kang YQ, Sibirny AA. (2025). Isolation of mutants defective in cytosolic β -galactosidase degradation in the methylotrophic yeast *Komagataella phaffii*. *Cytology and Genetics*, Vol. 59, No. 1, pp. 71–78. doi: <https://doi.org/10.3103/S0095452725010104>. **Q4**, Scopus and WoS. (*The acquirer, together with co-authors, conducted research, analyzed and summarized the obtained data, participated in the writing and design of the publication*).

2. Bratiichuk D, Kurylenko O, Vasylyshyn RV, **Zuo M**, Kang YQ, Dmytruk K, Sibirny AA. (2020). Development of new dominant selectable markers for the nonconventional yeasts *Ogataea polymorpha* and *Candida famata*. *Yeast*, 37(9-10):505-513. doi: <https://doi.org/10.1002/yea.3467>. **Q2**, Scopus and WoS. (*The acquirer, together with co-authors, conducted research, analyzed and summarized the obtained data, participated in the writing and design of the publication*).

3. Semkiv MV, Ruchala J, Tsaruk AY, Zazulya AZ, Vasylyshyn RV, Dmytruk OV, **Zuo M**, Kang YQ, Dmytruk KV, Sibirny AA. (2022). The role of hexose transporter-like sensor HXS1 and transcription activator involved in carbohydrate sensing AZF1 in xylose and glucose fermentation in the thermotolerant yeast *Ogataea polymorpha*. *Microbial Cell Factories*, 21, 162. doi: <https://doi.org/10.1186/s12934-022-01889-z>. **Q1**, Scopus and WoS. (*The acquirer, together with co-authors, conducted research, analyzed and summarized the obtained data, participated in the writing and design of the publication*).

Abstracts of reports at domestic and international conferences, scientific congresses and congresses:

4. **Zuo M**, Vasylyshyn RV, Sibirny AA. Improvement of alcoholic fermentation in yeast by protoplast fusant of *Saccharomyces cerevisiae* and *Ogataea polymorpha* strains. // 1st International conference of young scientists of the Institute of Cell Biology and the University of Rzeszów “Current Issues in Cell Biology and Biotechnology”, June 6, Lviv, Ukraine. – 2021. – P. 24.

5. **Zuo M**, Vasylyshyn RV, Sibirny AA. The role of transcription activator involved in carbohydrate sensing *azf1* in xylose and glucose fermentation in the thermotolerant yeast *Ogataea polymorpha*. // Conference of Young Scientists of Institute of Cell Biology, June 8, Lviv, Ukraine. – 2022. – P.19.

6. **Zuo M**, Dmytruk OV, Dmytruk KV, Sibirny AA. Searching the gene involved in the autophagy of cytosolic and peroxisomal proteins in methylotrophic yeast *Komagatella phaffii*. // Conference of Young Scientists. May 20, Lviv, Ukraine. – 2024. – P.3.

7. **Zuo M**, Dmytruk OV, Dmytruk KV, Sibirny AA. Screening for mutant strains with autophagy defects of cytosolic protein β -galactosidase in the methylotrophic yeast *Komagatella phaffii*. // 7th Congress of the All-Ukrainian public organization “Ukrainian Society of Cell Biology” with international representation. 11-13 September, Lviv, Ukraine. – 2024. – P.29.

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LIST OF CONDITIONAL DESIGNATIONS

AOX – Alcohol oxidase

AUR1-inositol phosphorylceramide synthetase

ADH– Alcohol dehydrogenase

BSA – Bovine serum albumin

bp – Base pair

CTAB – Hexadecyltrimethylammonium bromide

DTT – DL-Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

ER– Endoplasmic reticulum

GFP – Green fluorescent protein

GRAS – Generally Recognized As Safe

IMH3-IMP dehydrogenase

K-P buffer– Potassium Phosphate Buffer

LAC4 – β -galactosidase gene

MNNG – N-methyl-N'-nitro-N-nitrosoguanidine

OD – Optical density

ONPG – O-nitrophenylgalactoside

PCR – Polymerase Chain Reaction

PDC– Pyruvate decarboxylase

PMSF – Phenylmethylsulfonyl fluorid

SSF – Simultaneous Saccharification and Fermentation

WT – Wild-Type

X-GAL – 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

XDH– Xylitol dehydrogenase

XR– Xylose reductase

YNB –Yeast Nitrogen Base

INTRODUCTION

Relevance of the topic

Methylotrophic yeasts (organisms which can metabolize methanol as the sole carbon and energy source) are regarded as one of the most efficient producers of intrinsic and recombinant proteins with industrial significance, such as insulin, hepatitis B virus surface antigen, interferons, alcohol oxidase enzymes, nitrilases and others (Krasovska et al., 2007; Grabek-Lejko et al., 2013). As a representative of methylotrophic yeasts, *Komagataella phaffii* (formerly known as *Pichia pastoris*) has the following characteristics: respiratory growth to extremely high cell densities, effective protein secretion and high-level expression of recombinant proteins from strong inducible promoters. These characteristics make it widely applied in the pharmaceutical and biotechnological industries. Therefore, it is also called the “biotech yeast” (Heisteringer et al., 2020).

As a robust industrial species, it not only overproduces proteins at high levels but also requires minimizing the degradation of recombinant proteins in the cytosol. However, the stability of heterologous proteins during over-synthesis in methylotrophic yeasts, especially when it transferred from methanol to 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 degradation and proteolysis, and the cell’s carbon metabolism switches to a glycolytic pathway. This process is called catabolic degradation (Dmytruk et al., 2021). There are two types pathways of protein degradation: one is proteasomal degradation occurring in the cytoplasm. This system typically recognizes and degrades ubiquitinated individual proteins, predominantly short-lived ones (Pohl & Dikic, 2019). The second pathway is lysosome-(vacuole) dependent and is known as autophagy. This process involves not only proteins but also intracellular organelles or other macromolecular compounds. Subsequently, these compounds are degraded and recycled within lysosomes (vacuoles) (Mizushima & Komatsu, 2011; Sibirny, 2016).

Previous research has demonstrated that the secretion of heterologous proteins in yeast are closely associated with the level of autophagy. Modifying and transforming the autophagy pathway can enhance the production of heterologous proteins. Y.Liu et al utilized *Kluyveromyces marxianus* to express the heterologous protein feruloyl esterase. Through radiation mutagenesis, a novel mutant named T1 was obtained. The protein yield of this mutant is approximately ten-fold higher than that of the parent strain. Transcriptomic analysis revealed enhanced intracellular vesicle trafficking and diminished autophagy in the T1 mutant strain. Further investigation identified a single cytosine deletion in the Mtc6p protein, which contributed to the decrease in autophagy. Additional experiments demonstrated that attenuating or fully inhibiting autophagy led to increased yields of heterologous proteins. These results suggest inhibiting autophagy significantly enhances the production of secreted proteins in *K. marxianus* (Y. Liu et al., 2018). This study is currently the only research that links autophagy with heterologous protein expression in yeast, suggesting a potential connection between autophagy deficiency and improved efficiency in heterologous protein production. Although the precise mechanism of how attenuated autophagy regulates yeast protein secretion remains unelucidated, this study offers a novel perspective for promoting the secretion of heterologous proteins in yeast by modulating the autophagy pathway. It triggers in-depth investigations into the relationship between autophagy and protein secretion, and lays a theoretical foundation for the production of industrialized proteins using yeast.

Furthermore, it has also been reported that the expression of recombinant proteins in *K. phaffii* can be increased by inhibiting proteolytic degradation. Mutants with damaged proteases in *K. phaffii* such as SMD1163 (*his4 Pep4 Prb1*) and SMD1168 (*his4 pep4*), have been developed to reduce protein degradation. However, the disadvantages of such strains were reduced growth rate and low transformation efficiency, which will be unfavorable for large-scale industrial applications (Dmytruk et al., 2020). Consequently, these limitations highlight the need for alternative

approaches to address protein degradation without compromising growth and productivity, ensuring strains are viable for efficient, large-scale production processes.

Up to date, despite a large array of information on the mechanisms of degradation of proteins and cellular organelles, the mechanisms of selective degradation of intrinsic cytosolic proteins, as well as recombinant heterologous proteins with cytosolic localization in methylotrophic yeast, remain poorly understood (Mizushima & Komatsu, 2011). That is why the study into the mechanisms of degradation of cytosolic proteins in methylotrophic yeast is an extremely important task. To explore the relationship and mechanisms between cytosolic proteins and autophagic defects in *K. phaffii*, isolating mutants that are specifically defective in autophagic degradation processes will be vital for enhancing our understanding of how cytosolic proteins are degraded in *K. phaffii*, thereby shedding light on the underlying mechanisms involved and facilitating future advancements in biotechnological applications.

In this study, the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used to select mutant strains with impaired degradation of heterologous β -galactosidase expressed under control of methanol-induced *FLDI* promoter. It was observed that the increased activity of β -galactosidase, relative to the parental strain, when the cells were transferred from methanol to glucose medium was due to defects in selective autophagy pathways in the mutant strains. These obtained mutants are conducive to exploring the relationship between autophagy and protein secretion. By utilizing the regulation of autophagy to optimize the yeast expression system and enhance the production of target proteins, it will favorably drive forward the process of industrialized protein production by yeast in the future.

Additionally, another research topic in this thesis is to develop the new dominant selective markers for *O. polymorpha* usable in metabolic engineering experiments. Methylotrophic yeast *O. polymorpha* is the model organism for studying the mechanisms of thermotolerance, peroxisome homeostasis, methanol metabolism and of high temperature alcoholic fermentation (O. O. Kurylenko et al., 2018; Manfrão-Netto et al., 2019; Ryabova et al., 2003). Currently, genetically engineered approaches

are widely used for construction of new strains with modified metabolic pathways and for studying basic aspects of eukaryotic cell biology. The growing potential of such approaches requires additional suitable markers for selection of recombinant strains with desired physiological characteristics. The *ADE11*, *LEU2*, *MET6* and *URA3* genes have been developed as selective markers in combination with the corresponding auxotrophic *O. polymorpha* recipient strains. Various counter-selection systems have been succeeded for elimination of drug-resistance markers from genome of different yeast species. However, the most desired for commercial application appeared to be the self-cloning yeast vectors carrying no heterologous genes (Akada et al., 2002). Such markers have not been developed yet for *O. polymorpha*. Therefore, in this study, the mutated *AUR1* and native *IMH3* genes from *O. polymorpha* conferring resistance to aureobasidin and mycophenolic acid were tested as potential selectable markers. Aureobasidin inhibits Aur1, an enzyme catalyzing the synthesis of inositol phosphorylceramide, and induces a strong growth defect in yeast. Resistance of yeast *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces marxianus*, and *Candida glabrata* to aureobasidin was conferred by mutation of *AUR1* gene (Hashida-Okado, Ogawa, et al., 1998; Hashida-Okado, Yasumoto, et al., 1998). Besides, mycophenolic acid is produced by several species of the genus *Penicillium*. It is a specific inhibitor of *IMH3* gene, which encodes IMP dehydrogenase. Resistance of the yeasts *Candida albicans* and *C. famata* to mycophenolic acid was conferred by overexpression of the native *IMH3* gene (Dmytruk et al., 2011).

In this dissertation, the mutant forms of the *AUR1* gene from *O. polymorpha* were created by substitution of alanine for cysteine at position 156. This mutant form of the *AUR1* gene with replacement A156C can be used as a marker for selection of aureobasidin-resistant transformants in *O. polymorpha*. Additionally, a plasmid with *IMH3* gene conferring resistance to mycophenolic acid from *O. polymorpha* was constructed. The transformants resistant to mycophenolic acid were obtained by introduction of an additional copy of *IMH3* gene into the genome of *O. polymorpha* wild-type strain. These results indicated that the mutated *AUR1* gene and the native

IMH3 gene can be successful applied as new dominant selectable markers in the yeast *O. polymorpha*. Besides, the *IMH3* gene was used as a selectable marker for constructing a plasmid to overexpress the *TAL1*, *TKL1*, and *AOX1* genes. Recombinant *O. polymorpha* strains overexpressing these genes demonstrated significantly higher ethanol production from xylose fermentation compared to the parental strain. This successful application indicates that the *IMH3* gene is an efficient selective marker. This is the first information on the use of *O. polymorpha* engineered *AURI* and native *IMH3* genes as dominant markers for selection of recombinant strains of *O. polymorpha*.

Furthermore, the third research topic in this dissertation is the production of ethanol from lignocellulosic biomass by enhancing xylose utilization. Energy supply issues in any country are regarded as a matter of national security and thus receive considerable attention. Traditional fossil fuels, being limited in supply and primarily located in geopolitically unstable regions, present high uncertainties in terms of supply. Therefore, the search for alternative energy sources has become a major global challenge. Bioethanol, also known as fuel ethanol, is a renewable energy source that offers the potential to reduce dependence on fossil fuels, thereby enhancing energy autonomy and security. Furthermore, it can be derived from the bioconversion of renewable plant materials, demonstrating substantial potential in bioenergy development, efficient utilization of agricultural waste resources, reduction of waste disposal pressures, and the promoting the sustainable use of biomass resources.

Biofuel ethanol can be utilized in vehicles through ethanol-gasoline blends (such as E10 or E85) or pure ethanol (such as E100). Low-concentration ethanol blends are compatible with standard gasoline engines, while high-concentration ethanol blends and pure ethanol necessitate the use of flexible fuel vehicles or specially designed ethanol fuel vehicles. The adoption of ethanol fuel can decrease reliance on fossil fuels, reduce carbon emissions, and enhance air quality (Wang et al., 2022).

Currently, nearly all ethanol is produced through microbial fermentation (such as *S. cerevisiae*) from food raw materials, typically sucrose or starch. This creates

competition with food resources for human consumption. To avoid such resource conflicts, the development of efficient technologies for producing fuel ethanol from inexpensive renewable resources, such as lignocellulosic residues from the agricultural and woodworking industries, is of significant economic and environmental importance. However, the cost of fuel ethanol derived from lignocellulosic waste is currently too high, and there is a lack of strains capable of simultaneously converting glucose and xylose into ethanol at elevated temperatures, which hinders industrial-scale implementation. Consequently, enhancing and optimizing microbial producers of ethanol from non-food raw materials is an urgent priority.

Previous studies in our lab successfully obtained recombinant strains of the naturally xylose-fermenting yeast species *O. polymorpha* BEP/ Δ cat8, significantly enhancing the efficiency of high-temperature alcoholic fermentation from xylose compared to the wild-type strain (Ruchala et al., 2017). However, some limiting factors in the metabolism of this pentose remain unclear and the role of transcription factors in xylose (and glucose) alcoholic fermentation is poorly understood (Alper & Stephanopoulos, 2007). Previous research found that transcription factors and sugar transporters play an important role in regulation of xylose alcoholic fermentation. While investigations into xylose alcoholic fermentation have been performed using *Scheffersomyces stipitis* and *Spathaspora passalidarum*, the specific sensing proteins responsible for this process remain unidentified (Jeffries & Van Vleet, 2009; Ribeiro et al., 2021). Therefore, in this work we focus on investigating the role of sugar sensors, including transcription factors and not transporting sugar sensors, in the process of xylose fermentation to shed light on their function in the *O. polymorpha* yeast. These observations could be of biotechnological importance for construction of more efficient ethanol producers from lignocellulosic hydrolysates.

Connection of work with scientific programs, plans, topics

The presented work was carried out as one of the parts of fundamental research in the Department of Molecular Genetics and Biotechnology of the Institute of Cell

Biology of the National Academy of Sciences of Ukraine on the topics: “Presidential Discretionary-Ukraine Support Grants” from Simons Foundation, Award No 1030281 and No 1290613. China-Ukraine Intergovernmental Exchange Project (8). National Natural Science Foundation of China (NSFC; No.32060034/No.32460051). China Scholarship Council for financial support (No. 201908520075).

The aim and objectives of the research

Aim

The objective of this work is to employ mutagenesis approaches to obtain *K. phaffii* mutant strains defective in cytosolic β -galactosidase degradation. Subsequently, the correlation between intracellular protein degradation and the autophagy pathway in selected mutants was investigated, providing a material foundation for uncovering autophagy defects and protein degradation mechanisms.

To achieve the goal, the following tasks were expected to be fulfilled in the dissertation work:

1) The parental strain GS200/LAC4 was subjected to mutagenesis using the mutagen MNNG, and transformants exhibiting a blue color on YPD with X-gal plates after methanol pre-cultivation were screened.

2) By analyzing β -galactosidase and alcohol oxidase (AOX) activities in selected mutants, we can determine whether their protein degradation occurs through the selective autophagy pathway or the pexophagy pathway.

3) The viability of selected mutants was determined by calculating the percentage of living cells and Phloxine B-stained dead cells after nitrogen starvation cultivation, confirming autophagy impairment. Additionally, biomass accumulation in different media was analyzed to assess the occurrence of growth defects.

Another aim of this dissertation is to develop new dominant selectable markers for future applications in metabolic engineering of the yeast *O. polymorpha*.

To achieve the goal, the following tasks were expected to be fulfilled in the dissertation work:

1) Two plasmids were constructed with two mutant forms of the *AUR1* gene from *O. polymorpha* by replacing the leucine residue with phenylalanine at position 53 along with replacement of histidine residue by tyrosine at position 72 or substitution of alanine for cysteine at position 156.

2) A plasmid was constructed with the *IMH3* gene from *O. polymorpha* conferring resistance to mycophenolic acid.

3) These plasmids were respectively transformed into the *Ogataea polymorpha* NCYC495 strain, and transformant growth was observed on plates containing aureobasidin and mycophenolic acid as antibiotics. Growth on the corresponding antibiotic plates indicated successful selection marker function, while absence of growth indicated ineffective selection markers.

4) Constructed *O. polymorpha* recombinant strains overexpressing the *TAL1*, *TKL1*, and *AOX1* genes, using the *IMH3* gene as a selectable marker, and studied their ethanol production during xylose alcoholic fermentation compared to the parental strain.

The third aim of this dissertation is to utilize metabolic engineering methods to construct high-yield *O. polymorpha* recombinant strains capable of enhancing the efficiency of ethanol fermentation from xylose.

To achieve the goal, the following tasks were expected to be fulfilled in the dissertation work:

1) Engineered *O. polymorpha* strains with enhanced expression of *AZF1*—a transcription factor homologous to *S. cerevisiae* *AZF1* involved in carbohydrate-responsive gene regulation—were constructed in wild-type and BEP/ Δ cat8 genetic backgrounds.

2) *O. polymorpha* strains with overexpression of the hexose transporter-like sensor gene *HXS1*—a close homologue of *S. cerevisiae* sensors *SNF3* and *RGT2*—were developed in wild-type and BEP/ Δ cat8 genetic backgrounds.

3) The ethanol production, sugar consumption, cell biomass, and activities of relevant enzymes related to xylose metabolism of the recombinant strains in both

xylose and glucose media were measured to identify the efficiency of ethanol production from glucose and, in particular, from xylose.

The object of research: 1. Mutant strains after MNNG mutagenesis in the methylotrophic yeast *K. phaffii*; 2. Engineered strain with mutated AUR1 gene and the native IMH3 gene as selective markers in *O. polymorpha* yeast; 3. Utilization of xylose for ethanol production in *O. polymorpha* yeast.

The subject of research: 1. Isolation of mutants defective in cytosolic β -galactosidase degradation in the methylotrophic yeast *K.phaffii*; 2. Development of new dominant selectable markers for the non-conventional yeasts *O. polymorpha*; 3. Construction of recombinant strains of *O. polymorpha* yeast capable of advanced fermenting xylose and glucose to ethanol.

Research methods

Several genetic, biochemical, and microbiological research methods were used to perform the work. The construction of recombinant vectors was carried out using methods of molecular biology, such as hydrolysis of DNA by restriction endonucleases, elution of DNA fragments from agarose gel, dephosphorylation of linearized vectors, ligation of the vector with an insert. To introduce recombinant plasmids into recipient cells (bacterial or yeast cells), transformation was performed by electroporation. Previously, to check the transformants, plasmid or genomic DNA of bacteria or yeast was isolated, respectively. Further, the obtained material was used to analyze the obtained transformants by PCR (polymerase chain reaction) methods. Biochemical characterization of recombinant and mutant strains was carried out by determining the activity of a number of enzymes in cell-free extracts. Also, the parameters of alcoholic fermentation of yeast strains on various substrates and sugar consumption were determined by HPLC. For simulating genome modification cassettes, processing results, etc. computer analysis methods, bioinformatics internet resources and software, electronic databases of known genes were used. Growth

characteristics of yeast cultures were analyzed by spectrophotometry. The mutagenesis method described by An et al. was modified (An et al., 1989). The viability assay methods to monitor autophagy were described by Noda (Noda, 2008). Images were taken using a fluorescence microscope in conjunction with a monochrome digital camera. AxioVision 4.5 and ImageJ software were used for photo processing.

Scientific novelty

Based on a transformant *K. phaffii* containing a vector for expressing the β -galactosidase gene *LAC4* from *Kluyveromyces lactis* fused to the fluorescent tag green fluorescent protein (GFP) under the control of the methanol-regulated promoter of gene *FLD1*, the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used to select the mutant strains with impaired degradation of β -galactosidase under X-gal staining. Multiple analyses confirmed the impairment of autophagy in the obtained β -galactosidase degradation deficient mutants. The results are important for exploring the impaired mechanisms of cytosolic protein degradation resulting from autophagy deficiency in the selected mutant *K. phaffii* strains, offering new insights for enhancing recombinant protein yields in future applications.

Genetically engineered approaches require suitable markers for the selection of recombinant strains with desired physiological characteristics. Among these, self-marker genes are particularly useful for transforming prototrophic industrial yeast strains. However, such markers have not yet been developed for *O. polymorpha*, despite substantial progress in molecular tools for this organism. This study provides the first evidence of the use of *O. polymorpha*-engineered *AUR1* and native *IMH3* genes as dominant markers for selecting recombinant strains of *O. polymorpha*.

Previous research reveals that transcription factors and sugar transporters play an important role in the regulation of xylose alcoholic fermentation. However, the role of sugar sensors in the process of xylose fermentation has not yet been studied in *O. polymorpha*. Therefore, the role of hexose sensor *HXS1* and transcription factor with sensing properties *AZF1* on xylose and glucose fermentation in the native xylose-

metabolizing yeast, *O. polymorpha*, were analyzed in this dissertation. The results indicated that overexpression of the *HXS1* and *AZF1* genes in both wild-type and advanced ethanol producer BEP/ Δ cat8 strains can elevate ethanol production from glucose, and especially from xylose. These findings are important for the construction of more efficient ethanol producers from lignocellulosic hydrolysates in the future.

Practical significance of scientific results

K. phaffii transferred from methanol to glucose medium, most of the enzymes involved in methanol utilization and already present in the cell undergo degradation and proteolysis. This is one of the main bottlenecks in the production of recombinant proteins using *K. phaffii* in industrial application. In this study, four β -galactosidase degradation-deficient mutant strains were obtained through MNNG mutagenesis. It was found that their impairment of protein degradation is indeed associated with the autophagy-deficient pathway, providing a new perspective for elucidating the protein degradation mechanism in methylotrophic yeast. The mutagenesis methods developed in this study can also be extended to obtain other mutant strains.

Additionally, the results indicated that the mutated *AURI* gene and the native *IMH3* gene can be applied as new dominant selectable markers in the yeast *O. polymorpha*. Besides, the *IMH3* gene was successfully used to construct an improved ethanol producer from xylose in *O. polymorpha*. These findings may be suitable for commercial applications of metabolically engineered strains with desired characteristics, as they prevent the transfer of genes conferring antibiotic resistance to pathogens and avoid the production of toxic or allergenic proteins by recombinant strains.

Furthermore, recombinant strains of *O. polymorpha* overexpressing the *HXS1* and *AZF1* genes (encoding hexose transporters and transcription factors, respectively), were constructed. These strains exhibited enhanced ethanol production capability during the alcoholic fermentation of glucose, especially xylose. The results indicate that the expression of relevant glucose and xylose sensing or transporter protein genes

helps improve the efficiency of converting xylose to ethanol. This aids in the further study of xylose metabolism-related genes. At the same time, the recombinant strains obtained in this study can serve as starter strains to improve the efficiency of ethanol fermentation from sugars derived from lignocellulosic hydrolysates.

Personal contribution of the acquirer

The postgraduate student developed a research plan for the fulfillment of specified tasks with the help of a scientific supervisor. The Ph.D. student and the scientific supervisor analyzed the experimental research results. The acquirer was engaged in the research work of methods and techniques to select the best of them in order to achieve the set goal of the experiment. However, the scientific supervisor was involved in this process if necessary. The preparation of scientific publications was carried out by a graduate student with the advisory support of a scientific supervisor, and the selection of a journal was carried out by a scientific supervisor. The results presented in the dissertation were obtained by conducting scientific research by a graduate student in cooperation with the co-authors of the publications.

The author of the dissertation expresses sincere gratitude to the scientific director, co-authors of the publications and all employees of the Institute of Cell Biology who participated in obtaining the research results presented in the dissertation.

Approbation of the results of the dissertation

The main provisions of the dissertation work are published in the form of scientific articles in specialized journals and presented in the form of abstracts of oral or poster presentations.

Among the conferences at which the obtained results were presented-1st International conference of young scientists of the Institute of Cell Biology and the University of Rzeszów “Current Issues in Cell Biology and Biotechnology” (Lviv, Ukraine, 2021), Conference of Young Scientists of Institute of Cell Biology (Lviv, Ukraine, 2022), Conference of Young Scientists of Institute of Cell Biology (Lviv,

Ukraine, 2024), 7th Congress of the All-Ukrainian public organization “Ukrainian Society of Cell Biology” with international representation (Lviv, Ukraine, 2024).

In total, 7 scientific works were published on the topic of the dissertation, including three articles in international editions indexed in the Scopus and Web of Science Core Collection databases, and four abstracts of reports in the materials of conferences, scientific symposia, and congresses.

The structure and scope of the dissertation

The dissertation contains the following sections: “Introduction”, “Literature review”, “Materials and research methods”, “Research results and their discussion”, “Analysis and generalization of the results”, “Conclusions” and “References”, “Appendix 1”. The dissertation is presented on 160 pages of printed text, of which the main part occupies 93 pages. The work contains 22 figures, five tables and one formula. The list of used literature includes 229 literature sources. One appendix is attached at the end of the work.

CHAPTER 1

LITERATURE REVIEW

1.1. Recombinant protein production in *K.phaffii*

K. phaffii is a methylotrophic yeast from the order Saccharomycetales and is classified as a non-traditional production host when compared to conventional, historically established hosts such as *E. coli* and *S. cerevisiae*. (Ata et al., 2021). It was described in the 1960s as *Pichia pastoris* by Ogata, capable of utilizing methanol as a sole carbon source and energy source (Ogata et al., 1969). In 1995, *P. pastoris* was reclassified as a member of the genus *Komagataella* (Yamada et al., 1995). The original name (*P. pastoris*) remains widely used in scientific literature.

K. phaffii has gained prominence as the most favored host cell due to its ability to produce recombinant proteins with glycosylation patterns similar to those found in mammalian cells (Pan et al., 2022). Furthermore, its remarkable capacity to achieve high cell densities in fermentation facilitates protein production yields. Thus, in this dissertation, our research is primarily focused on *K. phaffii*, which is a representative strain of methylotrophic yeast.

Its ability to utilize methanol stems from the fact that methanol can induce the expression of genes encoding alcohol oxidase (AOX), which is part of the first enzymatic step of the methanol utilization (MUT) pathway, catalyzing the oxidation of methanol to formaldehyde in *K. phaffii* (Cos et al., 2006). The enzyme encoded by AOX belongs to the group of glucose-methanol-choline oxidoreductases. Whereas, when glucose, glycerol, or ethanol is utilized as the carbon source, AOX is barely expressed. Further investigations have indicated that the expression of AOX is regulated at the transcriptional level. Importantly, its strong inducible promoter *PAOX1* exhibits remarkable efficiency in controlling the expression of heterologous genes (Bustos et al., 2022). Currently, two AOX genes (*AOX1* and *AOX2*) have been identified in *K. phaffii*. The protein encoded by *AOX2* exhibits 97% homology to and nearly identical specific catalytic activity as the protein encoded by *AOX1*.

Nevertheless, the promoter of the *AOX1* gene (*PAOX1*) is strongly induced by methanol, whereas *PAOX2* is extremely weak (Cereghino & Cregg, 2000).

Three types of *K. phaffii* host strains have been mainly exploited during the last decades, varying in their ability to exploit methanol: Type I: The Mut⁺ (methanol utilization plus) phenotype, characterized by intact *AOX1* and *AOX2* genes, enables *K. phaffii* strains to grow in methanol at wild-type rates. The vast majority of *K. phaffii* strains belong to the Mut⁺ phenotype, as they can efficiently utilize methanol as their sole carbon source. This makes them suitable for large-scale production and typically results in high levels of recombinant protein expression; Type II: The MutS (methanol utilization slow) strains, such as KM71 (*arg4 his4 aox1::ARG4*), the *AOX1* gene is knocked out and replaced by the *S. cerevisiae ARG4* gene. The cells can rely on a small amount of alcohol oxidase encoded by the *AOX2* gene to complete methanol metabolism. Therefore, these cells grow slowly in methanol medium and their recombinant protein expression levels are generally lower than those of Mut⁺; Type III: The Mut⁻ (methanol utilization minus) phenotype, both the *AOX1* and *AOX2* genes are knocked out. These strains, such as MC100-3 (*arg4 his4 aox1::ARG4 aox2::his4*), cannot perform methanol metabolism and thus cannot grow in methanol medium (Cos et al., 2005). Although its application in expression systems requiring methanol as an inducing carbon source is limited and the recombinant protein expression efficiency is relatively low, this phenotype is advantageous for long-term cultivation and selection. Therefore, the selection of appropriate strain types based on specific experimental requirements is crucial for optimizing recombinant protein production. In this study, the wild-type strain *K. phaffii* GS200 was used which belongs to the Mut⁺ phenotype.

1.2. Relationship between autophagy and cytosolic protein degradation in *K. phaffii*

Degradation of cytosolic proteins, triggered by glucose in *K. phaffii*, can occur by proteasomal degradation or autophagy, which significantly affects the yield of recombinant proteins. There are two types pathways of protein degradation: one is

proteasomal degradation occurring in the cytoplasm. Proteasomes mainly hydrolyze short-lived proteins, such as transcription factors, cell cycle regulators, and defective proteins. However, the vast majority of all cellular proteins are long-lived proteins that are degraded in vacuoles via autophagy-mediated degradation, which is the second pathway (Nazarko et al., 2008). These protein degradation mechanisms are highly conserved among yeast species. For instance, studies in *S. cerevisiae* have demonstrated that selective degradation of the cytosolic enzymes fructose biphosphatase and malate dehydrogenase occurs through similar pathways, involving proteasomal degradation, autophagy, and endocytosis (Menssen et al., 2012; Giardina & Chiang, 2013). Furthermore, *K. phaffii* has emerged as an exceptional model organism for studying autophagy mechanisms, owing to its distinctive methanol metabolism pathway. When transferred from methanol to glucose medium, it demonstrates pexophagy-the selective autophagy of peroxisomes. Researchers have extensively exploited this unique characteristic to unravel fundamental autophagy mechanisms, particularly in identifying and characterizing essential autophagy-related (ATG) genes and their functions (Bernauer et al., 2020). A notable example is the discovery of ATG11 and ATG30 genes through peroxisome degradation studies in *K. phaffii*, which revealed their critical roles as selective autophagy receptors (Kirkin, 2020). Combined with its amenability to genetic manipulation and rapid growth characteristics, *K. phaffii* has established itself as an invaluable model system that bridges fundamental autophagy research and biotechnology applications.

Increased expression of recombinant proteins in *K. phaffii* can be achieved through the inhibition of proteolytic degradation. To achieve this goal, several protease-deficient *K. phaffii* strains have been constructed through genetic modification. Notable examples include SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prb1*), and SMD1168 (*his4 pep4*), which can effectively reduce the enzymatic degradation of recombinant proteins by eliminating key protease genes. During large-scale fermentation cultivation, these protease-deficient strains have proven particularly valuable for obtaining functional heterologous proteins with reduced degradation

(Sreekrishna et al., 1997). The effectiveness of protease gene deletion has been demonstrated in various studies. For instance, Wu et al. constructed a *K. phaffii* strain with double knockout of *YPS1* and *PEP4* genes, which significantly improved protein yield. This modification increased the proportion of the complete fragment of human serum albumin and human parathyroid hormone tandem protein in the total protein from 30% to 80%, highlighting the potential of protease engineering (Wu et al., 2013). Nevertheless, these protease-deficient strains come with significant limitations. They typically exhibit compromised cellular functions, including poor viability, reduced transformation efficiency, decreased growth rates. These drawbacks are attributed to the essential roles that some proteases play in normal cellular processes, such as cellular homeostasis. While this approach can reduce protein degradation, it must be carefully balanced against potential negative impacts on host cell physiology. Consequently, protease-deficient strains are generally employed only when other strategies for enhancing protein production prove ineffective. These limitations have prompted researchers to investigate another major protein degradation pathway—cellular autophagy pathway.

Additionally, in *K. phaffii*, the autophagic degradation pathway has been identified as one of the predominant mechanisms for eliminating recombinant proteins; many heterologous proteins, such as human insulin precursors and antibody fragments, are degraded via this pathway. (Marsalek et al., 2019). Due to this fact, the autophagy-mediated vacuolar degradation significantly impacts recombinant protein production yields in *K. phaffii*. The mechanism of autophagy pathways and their related genes have received increasing attention. Such as key autophagy-related genes—including *ATG1*, *ATG8*, *PEP4*, *ATG5*, and *ATG7*—control the formation of autophagosomes and the proteolytic activity within vacuoles, where this degradation takes place, thus affecting the stability and yield of recombinant proteins (Bernauer et al., 2020). Up to now, some genes involved in autophagy and pexophagy, such as *ATG8*, *ATG30*, *ATG11*, *ATG24*, *VPS15*, *VPS47*, *YPT7*, *PEX3*, *PEX14*, *PEX14*, *HXS1*, *GCN1*, *GCN2* and *GSS1*, have been identified in *Ogataea polymorpha* and *K. phaffii* (Klionsky et al.,

2003; Geng & Klionsky, 2008; Farré et al., 2008; Polupanov & Sibirny, 2014; Sakai et al., 2006). Recent studies have demonstrated that disrupting these autophagy-related genes has the potential to enhance recombinant protein production. For example, Liu et al. investigated heterologous protein production in *Kluyveromyces marxianus* through radiation-induced mutagenesis. The engineered mutant T1 exhibited remarkable protein secretion efficiency, demonstrating a substantial ten-fold increase in yield compared to the parental strain. Comprehensive transcriptomic profiling revealed significant alterations in cellular processes, specifically characterized by enhanced intracellular vesicle trafficking and pronounced autophagy suppression. Molecular characterization identified a critical single cytosine deletion within the Mtc6p protein, which as a key factor contributing to autophagy reduction. Additional experiments confirmed that attenuating or deleting *MTC6P* consistently improved protein yields, suggesting that modulating autophagy-related genes could be an effective strategy for enhancing recombinant protein expression in yeast systems (Y. Liu et al., 2018).

To enhance protein production, it is essential to understand protein degradation mechanisms, particularly the autophagy-related pathways. However, the mechanisms of degradation of these and other intrinsic cytosolic proteins, as well as recombinant heterologous proteins with cytosolic localization in methylotrophic yeast, remain unclear. The complexity is further increased by the interconnected nature of various degradation pathways and their roles in cellular homeostasis. Therefore, the analysis of the mechanisms of degradation of cytosolic proteins in methylotrophic yeast, the selection of mutants with damage to the relevant mechanisms such as autophagy pathways, and the study of the productivity of cytosolic protein synthesis in such mutants has significant scientific interest and application potential (Dmytruk et al., 2020). Understanding these pathways and their regulation provides opportunities for enhancing recombinant protein productivity through selective disruption of autophagy-related genes. Therefore, autophagy-related genes and their regulatory mechanisms,

which are essential for developing effective strategies to improve recombinant protein yields, are introduced in this review.

1.3. Autophagy and Autophagy-related genes (ATG)

The growth status of cells is of great significance for the production of secreted proteins in bioreactors. In many instances, the concentration of the product in the extracellular fermentation broth is proportional to the cell biomass density in the culture. Autophagy, as a key regulatory pathway for cell growth, has a complex and intimate connection with cell metabolism, growth status, survival rate, and homeostasis (Ryter et al., 2013). Autophagy is a highly conserved subcellular degradation pathway in eukaryotes. It is a process in which cells degrade their own components through lysosomes or vacuoles to provide energy for the cells (Klionsky, 2007). The formation of autophagosomes with autophagy-related genes (ATG proteins) as the core component is a hallmark feature that distinguishes autophagy from other vesicle transport processes (Inoue & Klionsky, 2010). Along with apoptosis, autophagy has become another prominent research focus in the field of life sciences. Under normal physiological conditions, autophagy is maintained at a relatively low level. However, under stress conditions such as ultraviolet irradiation, low oxygen levels, and energy and nutrient metabolism challenges, cells can activate autophagy to ensure their survival. Therefore, this process is of great significance for maintaining cellular physiological metabolism and internal homeostasis.

Yeast, as an excellent model organism for studying autophagy, holds significant biological importance for further exploring autophagy in higher organisms. The commonly used yeast model strains mainly include *S. cerevisiae*, *K. phaffii*, and *Ogataea (Hansenula) polymorpha*. Among them, *S. cerevisiae* has been the most extensively studied model organism for autophagy research. In 1992, Yoshinori Ohsumi discovered the first yeast autophagy-related gene *ATG1* in autophagy-deficient strains of *S. cerevisiae*. This gene encodes a serine/threonine kinase that is essential for autophagy (Tsukada & Ohsumi, 1993). Subsequently, he and his team screened and identified 15 key autophagy genes by constructing *S. cerevisiae* deletion strains and

named them *ATG1* to *ATG15* (Ohsumi, 1999). Currently, 42 autophagy-related genes have been identified in yeast, and the majority of them are highly conserved in eukaryotes (Parzych et al., 2018). Among the numerous autophagy-related genes, yeast possesses 19 core autophagy genes known as “core Atg proteins”, which are comprised of the following genes: *ATG1-10*, *ATG12-14*, *ATG16-18*, *ATG29*, *ATG31*, and *ATG38*. (Suzuki et al., 2007). According to their functions, they can be divided into five major categories: the *ATG1* kinase complex (*ATG1*, *ATG13*, *ATG17*, *ATG29*, and *ATG31*); the PI3K kinase complex (*ATG6*, *ATG14*, *ATG38*, *VPS15*, and *VPS34*); the *ATG2-ATG18* complex (*ATG2*, *ATG18*); the ubiquitin-protease coupling system (*ATG3*, *ATG4*, *ATG5*, *ATG7*, *ATG8*, *ATG10*, *ATG12*, and *ATG16*); and the *ATG9* transmembrane protein transport system (*ATG9*) (Farré & Subramani, 2016). The discovery of the ATG gene family and the identification of their protein functions have brought significant convenience to autophagy research. These pioneering findings have revolutionized our understanding of autophagy by revealing the molecular components essential for autophagosome formation and regulation. The characterization of ATG genes has not only provided powerful genetic tools for studying autophagy but also enabled the identification of homologous genes in higher organisms. This provides an essential foundation for exploring the molecular mechanisms and signaling pathways of autophagy, advancing both fundamental studies in cellular processes (including heterologous protein production and metabolism) and therapeutic applications.

1.3.1. Classification of yeast autophagy pathway

Autophagy can be classified into selective and non-selective autophagy based on the specificity of degradation substrates (Wilfling et al., 2020). Selective autophagy is mainly induced by specific intracellular substrates, such as mitophagy, endoplasmic reticulum autophagy, and peroxisome autophagy and so on (Parzych & Klionsky, 2014). Non-selective autophagy is predominantly triggered by extracellular stimuli. For instance, it serves as a self-protection mechanism for yeast cells when they are subjected to starvation or nutrient deficiency (Ohsumi, 2013).

According to the way degraded substances enter vacuoles or lysosomes, cellular autophagy can be classified into three forms: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA) (Glick et al., 2010). Among them, microautophagy is defined as the process in which lysosomes directly engulf macromolecular proteins and damaged organelles, which are then degraded into small molecular substances by lysosomal enzymes (Schuck, 2020). Macroautophagy is a process where organelles and proteins targeted for degradation are enclosed within double-membrane structures called autophagosomes. Subsequently, these autophagosomes fuse with lysosomes/vacuoles for degradation (Feng et al., 2014). Chaperone-mediated autophagy mainly degrades proteins containing the KFERQ sequence. HSC70, as a chaperone protein, recognizes proteins with the KFERQ sequence and transports them to the lysosomal membrane. Subsequently, the target protein is transferred into the lysosome by the membrane receptor LAMP-2A and degraded within the lysosomal lumen. This process is highly specific, and degradation is only applicable to proteins with this specific sequence (Yao & Shen, 2023; Kaushik & Cuervo, 2018). Both microautophagy and macroautophagy can be selective or non-selective, while CMA is considered a form of selective autophagy (Li et al., 2021). At present, macroautophagy is the most extensively studied and thoroughly explored type of autophagy. It serves as the primary metabolic pathway for degrading accumulated proteins and damaged or redundant organelles, and it is also the predominant form of autophagy in yeast cells. In this dissertation, the term “autophagy”, unless otherwise specified, refers to macroautophagy.

1.3.2. The mechanism of the non-selective (general) macroautophagy pathway

Generally, non-selective autophagy is often referred to as macroautophagy, but they are not entirely equivalent. The concept of macroautophagy is broader, encompassing both non-selective and selective autophagic processes. Specifically, macroautophagy is characterized by the formation of double-membrane vesicles called

autophagosomes that engulf cellular components. When this process occurs randomly to degrade bulk cytoplasmic contents during starvation or stress, it is considered non-selective autophagy. However, macroautophagy can also be highly selective, targeting specific cellular components such as damaged organelles, protein aggregates, or pathogens through specialized receptor proteins and adaptor molecules. Therefore, while non-selective autophagy represents a major form of macroautophagy, it is more accurate to view macroautophagy as an umbrella term that includes both non-selective bulk degradation and various forms of selective autophagy.

For the purposes of this dissertation, subsequent discussions will focus on macroautophagy in its non-selective form, and the progression of this process will be describe. Generally, the process of non-selective autophagy in yeast is divided into the following steps: initiation of autophagy; elongation of autophagosome; formation of autophagosome; docking and fusion of autophagosome with vacuole; and degradation and recycling of substrates. As shown in Figure 1.1. These specific steps and their molecular mechanisms will be described in detail in the following sections.

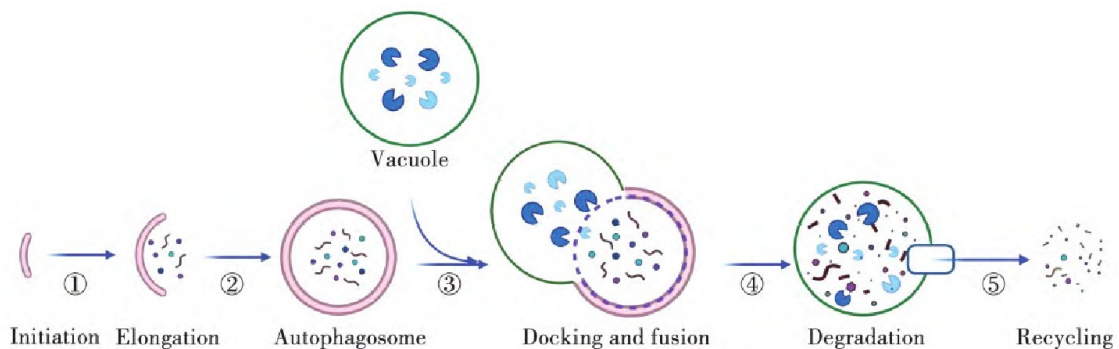


Fig. 1.1 The process of macroautophagy (Peña-Oyarzún et al., 2022)

The first step of autophagy involves the initiation of signaling cascades that trigger the autophagic response. The initiation of autophagy is induced by multiple pathways, including the TOR (Target of rapamycin) pathway, PI3K (Phosphatidylinositol-3-kinase) pathway, AMPK (AMP-activated protein kinase) pathway, and PKA (Protein kinase A system) pathway (Wang & Zhang, 2019). The TOR pathway is a key

regulatory pathway for cells in response to adverse environmental stimuli such as starvation and hypoxia. When yeast cells are nutrient-rich, the TOR protein is in an activated state. The rapamycin-targeted kinase complex 1 (TORC1) and its target recognition cofactor, the regulatory-associated protein of TOR (RAPTOR), hyperphosphorylate Atg13 (Wang & Zhang, 2019; Noda, 2017). Atg13 is a serine-rich protein that contains phosphorylation sites and other key regions, and serves as a direct substrate of TORC1. The hyper-phosphorylation of Atg13 prevents its association with other autophagy-initiating proteins, thereby inhibiting the occurrence of autophagy. (Alers et al., 2014).

Coversely, when yeast cells are starved or stimulated by rapamycin, TORC1 becomes inactive and Atg13 is rapidly dephosphorylated, thus inducing the occurrence of autophagy (Dossou & Basu, 2019). This process is associated with the activation of protein phosphatase 2A (PP2A)-Cdc55 and PP2A-Rts1. Their synergistic effect leads to the full dephosphorylation of Atg13, promoting the occurrence of autophagy (Banreti et al., 2012; Yeasmin et al., 2016). Dephosphorylated Atg13 exhibits enhanced interaction capability with Atg1 and Atg17. Under all circumstances, Atg17 forms a stable complex with Atg29 and Atg31 in a 1:1:1 ratio, known as the Atg17-Atg29-Atg31 complex (Liu & Klionsky, 2016). Ultimately, a pentameric complex centered around Atg1 is formed, comprising Atg1-Atg13-Atg17-Atg29-Atg31, known as the Atg1 kinase complex (Noda & Fujioka, 2015). Atg1, as a conserved kinase, serves as an upstream key initiator of autophagy induction and plays a crucial role in initiating the autophagy process (Noda & Fujioka, 2015).

Following the formation of Atg1 kinase complex, the next crucial step in autophagy is the assembly of autophagosome at a specific site called the phagophore assembly site (PAS). Located near the vacuole, PAS recruits a large number of proteins required for autophagy in a hierarchical manner, obtains lipids, and continuously extends to form a ring structure. Eventually, it closes to form an autophagosome with a double-layer membrane (Hollenstein & Kraft, 2020). The localization of PAS to the vacuole is mediated by the vacuolar membrane protein Vac8. Vac8 interacts with

Atg13 at the PAS site and anchors PAS to the vacuole during autophagosome formation (Hollenstein et al., 2019). Once PAS is localized to the vacuole, it undergoes assembly in a hierarchical fashion, where Atg proteins are sequentially recruited to promote autophagosome formation (Lorin et al., 2013).

In yeast, the Atg1 kinase complex is the first factor recruited to PAS. The formation of autophagosomes is stringently regulated by this complex. In the complex, Atg1, as the core protein, facilitates the recruitment of other downstream proteins. Atg13 is linked to the vacuolar membrane protein Vac8 to localize PAS to the vacuole. Atg17 functions as a scaffold protein and plays a crucial role in the assembly of autophagy-related proteins (Shibutani et al., 2015). Subsequently, the transmembrane protein Atg9 is recruited to PAS, aiding in autophagosome nucleation and serving as a “membrane carrier” to participate in the formation of the autophagosome membrane. Atg9 is the sole fully characterized membrane protein, possessing six transmembrane domains. It shuttles between the PAS site, mitochondria, endoplasmic reticulum (ER), and Golgi apparatus, and plays a crucial role in autophagosome precursor nucleation and autophagosome membrane formation (Shirahama-Noda et al., 2013). The circulation of Atg9 requires PI3K to form PI3P. The existence of this lipid triggers Atg9 to depart from the PAS site and enables the sequential recruitment of Atg2 and Atg18 (Hitomi et al., 2023; Nascimbeni et al., 2017). Thereafter, the class III PI3K complex I containing Atg14 is recruited, inducing autophagosome precursor nucleation. PI3K accumulates abundantly at PAS and phosphorylates the 3-OH on the PI inositol ring to generate PI3P. The accumulation of a significant amount of PI3P can recruit its binding proteins Atg2 and Atg18. Atg2, Atg4, and Atg18 are all associated with the extension and expansion of autophagosomes (Nascimbeni et al., 2017).

With the recruitment and coordination of these proteins, autophagosome assembly initiates at the PAS site. Subsequently, the autophagosomes undergo expansion and elongation. The elongation of yeast autophagosomes is reliant on two ubiquitin-like (UBL) binding systems: the Atg8 ubiquitin-like protein binding system and the Atg12 ubiquitin-like protein binding system (Yin et al., 2020).

Atg12 is the first ubiquitin-like protein identified in yeast. The Atg12 binding system includes Atg5, Atg7, Atg10, Atg12, and Atg16 (Karow et al., 2020). Among them, Atg7 and Atg10 serve as E1 ubiquitin-activating and E2 ubiquitin-conjugating enzymes respectively, and are implicated in the modification of the substrate Atg12 protein (Karow et al., 2020). The Atg12 binding system plays a crucial role in recruiting other factors and facilitating the extension of autophagosomes (Mizushima, 2020). Subsequently, Atg5-Atg12 combines with Atg16 to generate the Atg5-Atg12-Atg16 complex. This complex catalyzes the binding of ubiquitin-like protein Atg8 to PE, thereby forming a complex that participates in the expansion of the autophagosome membrane (Xiong et al., 2018).

The Atg8 binding system consists of Atg3, Atg4, Atg7, and Atg8 (Roberto et al., 2020). The Atg4 cysteine protease can cleave Atg8 to expose glycine, which then binds to the E1 ubiquitin-activating enzyme Atg7 and the E2 ubiquitin-conjugating enzyme Atg3. Ultimately, under the action of the E3 ubiquitin ligase, the cleaved Atg8 binds to PE to form the Atg8-PE complex (Roberto et al., 2020). The Atg8-PE complex is localized on the inner and outer membranes of autophagosomes and exerts a crucial role in both the extension and closure of autophagosome membranes (Nieto-Torres et al., 2021).

The final step of autophagy involves the fusion of autophagosomes with vacuolar membrane and degradation of substrates. Autophagosomes undergo expansion and extension and ultimately close to form a complete double-membrane structure enclosing substances awaiting degradation. However, the constituent elements of the autophagosome membrane have not been precisely confirmed (Lőrincz & Juhász, 2020). Subsequently, driven by SNARE proteins, the outer membrane of the autophagosome double membrane fuses with the vacuolar membrane, further giving rise to an autophagic body with a single-membrane structure. Following autophagosome fusion with the vacuolar membrane, the inner vesicle (autophagic body) is released into the vacuolar lumen. Here, Atg15, the only known vacuolar phospholipase, plays a critical role in the late-stage autophagy process. Transported to

the vacuole via the multivesicular body (MVB) pathway, Atg15 becomes activated by Pep4 and Prb1, whereupon it functions as a phospholipase B to degrade autophagic body membranes (Kagohashi et al., 2023). This membrane degradation is essential, as it exposes the autophagic cargo to vacuolar hydrolases for complete breakdown. In the absence of Atg15, autophagic bodies accumulate within the vacuole, significantly impeding both autophagic flux and cargo degradation. Eventually, under the action of hydrolases within the vacuole, the autophagic body and its contents are digested and degraded into small molecular substances and released (Adnan et al., 2019). The degraded substances are further converted to generate basic metabolites, which are transported to the cytoplasm and act as new proteins and lipids to protect cells under stress conditions and maintain the internal homeostasis of cells in an adverse environment.

Through systematic investigation of the autophagy pathway, from the initiation of PAS formation to the final degradation of autophagic bodies in vacuoles, we can identify key regulatory points and genes involved in protein degradation. This knowledge provides valuable insights for genetic engineering strategies aimed at reducing target protein degradation and improving protein yield in industrial production. By manipulating autophagy-related genes and pathways, it becomes possible to optimize cellular protein homeostasis, potentially leading to enhanced protein production efficiency while maintaining cell viability under production conditions. In addition to general autophagy, selective autophagy participates in the degradation of specific organelles and together with general autophagy forms a complete system for intracellular material degradation, thus playing an important role in improving protein production.

1.4. The mechanism of the selective autophagy pathway

Selective and non-selective autophagy share the same core molecular mechanism; however, selective autophagy is distinguished by its requirement for specific receptors, which are dispensable in non-selective autophagy (Jin et al., 2013). Selective

autophagy is a highly specific process that recognizes and degrades distinct substrates, including protein complexes, damaged organelles, and invading microorganisms. Depending on the type of organelle involved, different types of selective autophagy are distinguished, with representative examples including pexophagy (peroxisomal autophagy), mitophagy (mitochondrial autophagy) and ribophagy (ribosome autophagy).

1.4.1. Pexophagy (peroxisomal autophagy) pathway

The main function of peroxisomes is to metabolize methanol through alcohol oxidase, catalase, and formaldehyde dehydrogenase. Additionally, peroxisomes can break down fatty acids, formamide, and hydrogen peroxide. Therefore, the primary role of peroxisomes is to remove toxic substances from cells. Due to the important role of peroxisomes in cells, cells strictly control the number of peroxisomes based on environmental factors (Gabaldón, 2010). When *K. phaffii* grows on methanol, oleic acid, or organic amines, peroxisomes can occupy up to 80% of the cell volume. During this time, when the carbon source for *K. phaffii* changes to glucose or ethanol, the peroxisomes are engulfed by vacuoles. This process is called pexophagy. After pexophagy occurs, peroxisomes occupy less than 5% of the cell volume.

K. phaffii is the only species that can undergo different modes of pexophagy in response to varying culture media composition (Tuttle & Dunn, 1995). It has rapid rates of peroxisome production and degradation, and well-characterized genome making it an ideal model organism for investigating selective peroxisome autophagy mechanisms (De Schutter et al., 2009). Moreover, *K. phaffii* is also a commonly used host for heterologous protein expression, primarily due to the strong transcriptional activity of the alcohol oxidase promoter (*pAOX*) of the alcohol oxidase localized within peroxisomes (Cregg, 2007). The induction of pexophagy downregulates AOX promoter activity, consequently attenuating heterologous protein expression (Potvin et al., 2012). Thus, elucidating the molecular mechanisms of pexophagy provides critical

insights into the regulation of recombinant protein production and its inhibitory pathways.

K. phaffii exhibits two different modes of peroxisome autophagy: micropexophagy and macropexophagy (Tuttle & Dunn, 1995). Micropexophagy is triggered when the carbon source shifts from methanol to glucose. During micropexophagy, peroxisomes initially move in close proximity to the vacuole, after which the vacuole flattens and develops arm-like extensions of new vacuolar membrane components. The vacuole, newly formed membrane components, and a specialized structure called the micropexophagy apparatus (MIPA) work collectively to engulf the peroxisomes. Finally, vacuolar proteases degrade the peroxisomes (Sakai et al., 1998). Micropexophagy is characterized by the simultaneous vacuolar engulfment of multiple peroxisomes (Manjithaya et al., 2010). Macropexophagy is induced when the carbon source shift from methanol to ethanol. The process is characterized by the sequential assembly of autophagosomal components around individual peroxisomes (Dunn et al., 2005; Till et al., 2012), culminating in the formation of a double-membrane autophagosomal structure. Upon completion of sequestration, the autophagosome undergoes fusion with the vacuole, facilitating the delivery of its peroxisomal for hydrolytic degradation. In contrast to micropexophagy, macropexophagy processes peroxisomes individually, with each autophagosome sequestering a single peroxisome.

At the beginning of this process, since *K. phaffii* metabolizes glucose or ethanol faster than methanol, more energy is generated when its carbon source changes from methanol to glucose or ethanol. This excess energy increases the phosphorylation level of a specific receptor, Atg30, in *K. phaffii*. Subsequently, Atg30 localizes to the peroxisome membrane proteins Pex3 and Pex14, and recruits Atg11 and Atg17 to form the phagophore assembly site (PAS). This structure gradually expands into either the sequestering arm of micropexophagy or the autophagosomes of macropexophagy. During micropexophagy, a specific membrane structure called MIPA forms with the participation of Atg8, Atg9, and Atg26 (Farré et al., 2013). After the vacuolar

sequestering arm and newly formed MIPA surround the peroxisome, fusion with the vacuole occurs under the action of Atg1, Atg24, and Vac8, initiating the degradation phase. In the macrophagy process, Atg6 first localizes to the vacuolar region, and when Atg8 binds to one of the Atg6 proteins, it links to PAS, which then grows into the autophagosomal membrane. These components extend the autophagosomal membrane under the action of Atg9, ultimately forming the autophagosome. Subsequently, Atg24 binds to Atg17 to fuse with the vacuole, similarly undergoing degradation under the action of Atg1, Atg24, and Vac8 (Oku et al., 2006).

Both macro- and microphagy of peroxisomes are accomplished through the coordinated action of multiple autophagy-related proteins, and elucidating the quantitative relationships of these proteins' cooperative actions has become a new research focus. Preventing peroxisome autophagy in *K. phaffii* under high-energy conditions can maintain the expression of foreign genes driven by the alcohol oxidase promoter while also providing energy for heterologous protein expression and accumulation of high-value chemicals. At the same time, in mutants with defects in pexophagy-related genes, the degradation of peroxisomes (and, accordingly, peroxisomal proteins) is impaired, which may benefit the elevation of protein yield. Zhang et al. prevented peroxisome autophagy in *K. phaffii* under high-energy conditions by adding oxygen carriers such as hexane (J. G. Zhang et al., 2008) and controlling cellular maintenance energy (J. Zhang et al., 2008), which increased the accumulation of S-adenosyl methionine and the expression of calf intestinal alkaline phosphatase light chain in recombinant *K. phaffii* (Zhang et al., 2009). These studies provide valuable guidance for this thesis demonstrating that protein yield can be regulated through the control of pexophagy process.

1.4.2. Mitophagy (mitochondrial autophagy) pathway

Mitochondrial autophagy was first proposed by Lemasters in 2005, which mainly refers to the process where under stimuli such as ROS, nutrient deficiency, and cellular aging, mitochondria in cells undergo depolarization damage. The damaged

mitochondria are specifically engulfed by autophagosomes and fuse with lysosomes (or vacuoles in yeast), thereby completing the degradation of damaged mitochondria and maintaining cellular homeostasis (Lemasters, 2005).

In yeast, mitochondria may be transported to the vacuole in different ways. The first way is the classic starvation / rapamycin-induced pathway which mediated by TOR and results in macroautophagy. Atg32, one of the first discovered receptor proteins capable of mediating mitochondrial autophagy, localizes to the outer mitochondrial membrane and contains a WxxL motif for direct interaction with Atg8. This protein also interacts with Atg11 at the peri-vacuolar phagophore assembly site (PAS), where autophagosomes form to engulf and deliver mitochondria to the vacuole for degradation (Okamoto et al., 2009; Kanki et al., 2009). The second way which induced autophagy in the stationary phase. This pathway relies on the autophagosomal identification of mitochondria via the mitochondrial Aup1 protein and results in macroautophagic disposal. Tal et al. discovered and defined Aup1 (ancient ubiquitous protein 1), a protein phosphatase homolog in yeast that interacts with the autophagy-related protein kinase Atg1p. While the deletion of Aup1 does not affect starvation-induced non-selective macroautophagy, it is essential for stationary phase mitochondrial autophagy when lactate is used as a carbon source. In cells with Aup1 gene mutations, both mitochondrial autophagy levels and cell viability significantly decrease, indicating that AUP1 plays a pro-survival role in Aup1-mediated mitochondrial autophagy (Tal et al., 2007).

Additionally, starvation/rapamycin can also induce microautophagy of mitochondria, a process in which recognition of the mitochondrial protein Uth1 results in the direct engulfment of mitochondria by the vacuole. Kissová et al. made the first discovery of genetic regulation of mitochondrial autophagy through their research on the Uth1 gene. The Uth1 molecule contains a “SUN” (Sim1p, Uth1p, Nca3p, and Sun4p) domain and localizes to the outer mitochondrial membrane. Under normal conditions, the effects of starvation and rapamycin lead to autophagic clearance and mitochondrial degradation, while the deletion or mutation of the Uth1 gene results in

ineffective clearance of damaged mitochondria (Kissová et al., 2004). A characteristic of Uth1 protein-mediated mitochondrial autophagy is the direct contact and possible fusion between mitochondria and vacuoles, similar to non-selective microautophagy.

Although the direct relationship between mitophagy and protein yield has not been as extensively studied as processes like pexophagy, emerging evidence suggests that mitophagy may regulate protein biosynthesis through indirect mechanisms (Ashrafi and Schwarz, 2013). Specifically, mitophagy maintains mitochondrial homeostasis and functional integrity through selective degradation of damaged mitochondria. A well-functioning mitochondrial network can provide sufficient ATP supply (Herzig and Shaw, 2018), thereby furnishing the essential energy required for protein translation. This optimization of energy metabolism may influence total protein yield under specific physiological or pathological conditions.

1.4.3. Ribophagy (ribosome autophagy) pathway

Kraft et al. (Kraft et al., 2008) demonstrated that under starvation conditions, ribosomes are degraded in the vacuole via different pathways (Fig.1.2). One pathway involves the non-selective uptake of ribosomes, which are engulfed and transported to the vacuole. The other pathway is a specific autophagy process known as ribophagy, which selectively degrades the 40S and 60S ribosomal subunits. Additionally, due to the close structural association between ribosomes and the endoplasmic reticulum (ER) or mitochondria, ribosomes can also be degraded via a bypass autophagy pathway during ER-phagy or mitophagy. Screening of yeast mutants revealed that ribophagy relies on the catalytic activity of the ubiquitin-specific protease Ubp3 and its cofactor Bre5 (Kraft et al., 2008). However, the regulation of the large ribosomal subunit (60S) is dependent on the Ubp3/Bre5 ubiquitin protease, whereas the 40S small subunit is not, suggesting that the large and small ribosomal subunits are subjected to distinct degradation pathways, although the exact mechanisms remain unclear. Further studies have shown that efficient ribophagy depends on the Ubp3-Bre5-Ufd3-Cdc48 complex, which eliminates specific ubiquitinated targets through deubiquitination (Ossareh-

Nazari et al., 2010). The Ubp3 complex induces the deubiquitination of the lysine 74 residue on Rpl25, which can also be ubiquitinated by the ribosome-associated E3 ligase Ltn1. There exists an antagonistic effect between Ltn1 and the Ubp3 complex, which dynamically regulates the activity of ribophagy by competing for the same site on Rpl25, further supporting the specificity of this mechanism (Kraft et al., 2008; Ossareh-Nazari et al., 2010). Therefore, the balance between ubiquitination and deubiquitination of Rpl25 serves as the primary molecular basis for ribophagy, although its precise regulatory mechanism remains unclear (Beese et al., 2019).

In their study on starvation-induced ribophagy, Wyant et al. (Wyant et al., 2018) discovered that nuclear fragile X mental retardation-interacting protein 1 (NUFIP1) is closely associated with the initiation of ribophagy and serves as a key receptor mediating this process. NUFIP1 forms a heterodimer with zinc finger HIT domain-containing protein 3 (ZNHIT3) (Quintern et al., 2016), and promotes the binding of early autophagosomes, facilitating the transfer of ribosome-containing autophagosomes to the lysosome (Wyant et al., 2018). Ribosome transport to the vacuole depends on the involvement of ATG1 and ATG7 in yeast (Cebollero et al., 2012). Further analysis confirmed that NUFIP1 contains four LC3B-interacting regions (LC3-interacting regions, LIRs), and based on the interaction between NUFIP1 and LC3B, Wyant et al. (Wyant et al., 2018) hypothesized that NUFIP1 could be a selective autophagy receptor for an unknown substrate in the cytoplasm. It has been established that NUFIP1 is a critical protein in starvation-induced ribosome degradation. NUFIP1 translocates from the nucleus to the cytoplasm, where it directly interacts with LC3B to transport ribosomes into autophagosomes, a process that may be independent of direct regulation by nutrients and mTORC1 (Wyant et al., 2018).

Ribophagy, a selective autophagy pathway targeting ribosomes, fundamentally influences protein yield through multiple regulatory mechanisms. At the molecular level, this process enhances protein synthesis efficiency by selectively eliminating damaged or non-functional ribosomes that could otherwise impair translational accuracy and output. The quality control function of ribophagy maintains an optimal

ribosomal pool composition, directly contributing to maximal protein production capacity. Moreover, under stress conditions, ribophagy-mediated ribosome turnover serves as a critical determinant of protein synthesis rates by modulating the availability of functional ribosomes and recycling ribosomal components for the assembly of new translation machinery (Mathis et al., 2017). During cellular growth and proliferation, the dynamic regulation of ribosome abundance through ribophagy creates a homeostatic balance that optimizes protein synthesis efficiency relative to cellular energy status and metabolic demands (An et al., 2019). Notably, recent evidence suggests that perturbations in ribophagy-dependent ribosome quality control can lead to significant reductions in protein yield, highlighting the direct relationship between ribophagy function and protein production capacity (Gretzmeier et al., 2017). However, many questions remain unresolved, such as whether changes in ribosome homeostasis under mTORC1 inhibition promote its interaction with NUFIP1-ZNHIT3, and whether these changes involve post-translational modifications, protein ubiquitination, or the interaction between proteins and ribosomes, which still require further clarification. Therefore, a deep understanding of the specific molecular mechanisms of ribophagy is of significant scientific value for identifying effective therapeutic targets based on ribosomal cell homeostasis.

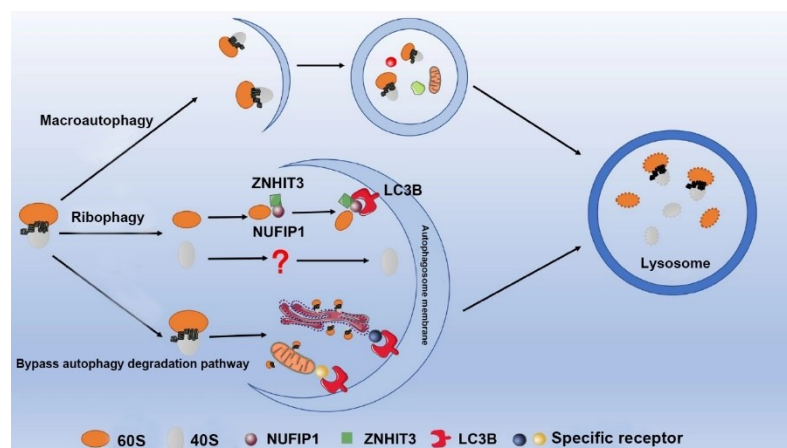


Fig. 1.2. The process of ribophagy (Zhao et al., 2022)

In addition to the mentioned above, other forms of selective autophagy include lipophagy (autophagy of lipids), nucleophagy (autophagy of the nucleus), glycophagy (autophagy of glycogen granules), zymophagy (autophagy of zymogen granules), and

ER-phagy (endoplasmic reticulum autophagy) pathway and so on. Selective-autophagy represents a sophisticated cellular quality control mechanism that specifically targets and degrades distinct cellular components. These processes are mediated by specific receptor proteins that recognize designated substances and connect them to the core autophagy machinery. Unlike macroautophagy, selective autophagy maintains cellular homeostasis through precise recognition and elimination of specific substrates, thereby playing crucial roles in stress response, organelle quality control, and cellular adaptation to environmental changes.

1.4.4. Cytoplasm to vacuole targeting (Cvt) pathway

The Cytoplasm-to-Vacuole Targeting (Cvt) pathway represents a specialized form of selective autophagy that shares core molecular machinery with general autophagy while exhibiting distinct characteristics and functions. Unlike bulk autophagy, which functions as a non-selective, starvation-induced degradative process, the Cvt pathway operates constitutively under nutrient-rich conditions as a biosynthetic trafficking route. This pathway demonstrates remarkable cargo selectivity, specifically transporting certain vacuolar hydrolases, such as precursor aminopeptidase I (prApe1), to the vacuole while excluding bulk cytoplasm (Shintani et al., 2002).

The Cvt pathway proceeds through four sequential and highly coordinated stages. First, during cargo recognition and complex assembly, precursor Ape1 (prApe1) oligomerizes into dodecamers that further aggregate to form a higher-order Ape1 complex. The receptor protein Atg19 specifically recognizes this complex through the prApe1 pro-peptide. Other vacuolar hydrolases, including α -mannosidase (Ams1) and aminopeptidase IV (Ape4), are recruited through their interaction with distinct binding domains of Atg19 to form the complete Cvt complex. Next, the scaffold protein Atg11 facilitates targeting of this complex to the Pre-autophagosomal Structure (PAS). The third stage involves vesicle formation, where Atg19 binds Atg8-PE, enabling the sequestration of the Cvt complex within a double-membrane phagophore. Finally, the completed Cvt vesicle fuses with the vacuole, releasing a single-membrane Cvt body

into the vacuolar lumen. Following fusion, the Atg15 lipase degrades the Cvt body membrane, exposing the cargo to vacuolar hydrolases. This leads to the degradation of Atg19 and Atg8, removal of the prApe1 pro-peptide, and subsequent enzyme activation. Through this sophisticated trafficking mechanism, the Cvt pathway selectively delivers and activates multiple specific cargo proteins including prApe1, Ams1, and Ape4 to maintain vacuolar function (Umekawa & Klionsky, 2012). This process represents one of the key mechanisms for maintaining cellular functionality and protein homeostasis in yeast cells.

Understanding these pathways is particularly significant for biotechnology applications, as modulation of selective autophagy could potentially enhance protein production in industrial strains by preventing unnecessary degradation of cellular components and optimizing cellular resources.

Beyond the previously described autophagy pathway, cytoplasmic enzyme and protein stability are regulated by multiple complementary mechanisms. For example, redox homeostasis is maintained through thioredoxin (Trx1/Trx2)-mediated repair, which reverses oxidative modifications caused by reactive oxygen species (ROS). This mechanism not only preserves protein function but also significantly impacts structural stability. While ROS-mediated cysteine oxidation leads to protein destabilization, thioredoxin-mediated repair effectively mitigates this oxidative damage (Bao et al., 2007). Furthermore, allosteric regulation through metabolites (such as ATP, NAD⁺) and cofactors (such as Fe-S clusters, heme) stabilizes tertiary structure via long-range conformational effects, as exemplified by NAD⁺-dependent Adh1 and iron-sulfur cluster-containing Aco1 (Talib & Outten, 2021). Additionally, signal transduction networks dynamically regulate the proteostasis network in response to environmental changes, including stress signaling pathways such as heat-activated Hsf1 (inducing HSP expression) and the TOR pathway (sensing nutrient stress). Cytoplasmic pH changes mediated through structural modifications or lysosomal enzyme leakage also contribute to this regulation (Pincus, 2020). These mechanisms operate synergistically within an integrated network, enabling cells to dynamically

adapt to fluctuating environmental conditions and metabolic demands while maintaining protein homeostasis in yeast.

1.5. Ethanol production from lignocellulose biomass

Bioethanol is considered as a clean and renewable fuel derived from biomass, mainly sourced from plant materials. It is a widely accepted alternative for meeting the growing demand for green fuels in the transportation sector, helping to reduce carbon emissions (Sirajunnisa & Surendhiran, 2016). The production process generally includes the conversion of starch-rich plant materials into monosaccharides, which are subsequently fermented into ethanol by microorganisms such as yeast. Based on feedstocks types, bioethanol is classified into 1G, 2G, 3G, and 4G biofuels (Alvira et al., 2010). First-generation bioethanol feedstock is mainly edible food crops such as rice, wheat, barley, potato, corn, sugarcane etc. The second-generation bioethanol feedstocks contain lignocellulose biomass or residues that are abundant in nature. The third and fourth generation of biofuel sources are microalgae, which are converted into biofuel by algae-to-biofuels technology (Lamichhane et al., 2021).

Current bioethanol production mainly comes from first-generation sources, with Brazil utilizing vast amounts of sugarcane and the USA relying predominantly (95%) on corn for bioethanol production. However, this route is often referred to as the “food versus fuel” debate due to directly competing with food consumption, thus necessitating an increase in food prices (Islam et al., 2019; Amerit et al., 2023). Consequently, there is an increasing focus on sustainable biofuel production that utilizes raw materials (lignocellulosic biomass) for bioethanol, which does not compete with food sources-specifically, second-generation biofuels.

Plant biomass, also known as lignocellulosic biomass, is the most abundant biomass resource on Earth and is primarily composed of three key components: cellulose, accounting for 30-50% of the dry weight; hemicellulose, making up 20-40%; and lignin, comprising 15-25% of the dry weight, along with small amounts of acids, salts, and minerals (Nishiyama et al., 2002). Cellulose is a homogeneous polymer

formed by the linear linkage of glucose units through β -1,4-glycosidic bonds. Interactions between cellulose fibers occur via hydrogen bonding, resulting in the formation of both crystalline and amorphous regions. Hemicellulose, on the other hand, is a heteropolymer composed of various monosaccharides, including pentoses and hexoses. Lignin is an amorphous aromatic polymer characterized by a molecular structure that is rich in oxygenated phenylpropane units and their derivatives (Saha, 2003; Olofsson et al., 2008).

Generally, the process of converting complex lignocellulosic substrates into ethanol is performed in four steps: the first is physical and chemical pretreatment of biomass; the second is enzyme hydrolysis and saccharification; the third is fermentation of both pentoses and hexoses using microorganisms; the last step is recovery of the product (Lamichhane et al., 2021). However, the main barrier in this process for bioethanol production lies in the pretreatment with enzymatic hydrolysis and simultaneous fermentation of both hexose and pentose sugars. In this dissertation, we focus on addressing the latter issue of exploring microorganisms capable of utilizing both hexose and pentose to produce ethanol, which would benefit the industrial application of second-generation ethanol.

Currently, *S. cerevisiae* is one of the most commonly used microorganisms for industrial ethanol production from hexoses, due to its ability to withstand extreme industrial conditions, such as high osmotic pressure, high alcohol concentration, and low pH (Hong & Nielsen, 2012). Unfortunately, it cannot utilize xylose, which is the second most abundant sugar after glucose in lignocellulose biomass, due to lacking the enzymes that catalyze the initial stages of the pentose catabolism. In the last decades, metabolic engineering approaches for introducing heterologous xylose utilization pathways and optimizing internal metabolisms have been carried out to develop efficient xylose-fermenting *S. cerevisiae* strains. However, the best engineered strains of *S. cerevisiae* are still inferior to glucose fermentation in certain parameters of xylose fermentation, and typically initiate xylose utilization after glucose depletion, leading to incomplete xylose consumption (Sibirny, 2023; Dmytruk et al., 2017). As a result,

researchers have shifted their focus to microorganisms that can naturally ferment xylose.

Among the yeasts capable of naturally fermenting xylose, attention has primarily been focused on the *Scheffersomyces (Pichia) stipitis* and the most thermotolerant yeast known, *Ogataea polymorpha*. *S. stipitis* can effectively ferment both glucose and xylose; however, it has much lower ethanol tolerance than *S. cerevisiae* and requires oxygen for growth. *O. polymorpha*, which can grow and ferment glucose and xylose at temperatures of up to 50°C, shows relatively high ethanol tolerance. However, the wild-type strains of *O. polymorpha* produce very low amounts of ethanol from xylose. Even though several methods of metabolic engineering and classical selection have been carried out, the ethanol production from xylose by recombinant strains of *O. polymorpha* is still lower than that from glucose fermentation (Ruchala et al., 2017; Dmytruk et al., 2017).

Consequently, no microorganisms have been obtained that completely fulfill the diverse requirements for the industrial production of second-generation ethanol. As industrially feasible lignocellulose biorefinery requires a microbe that not only can withstand the harsh conditions in the lignocellulosic hydrolysate (e.g. low pH, osmotic stress and inhibitory compounds) but also is able to process all the sugars in the feedstock, such as hexose (C6) and pentose (C5) sugars (Hahn-Hägerdal et al., 2007; Buschke et al., 2013). To achieve this goal, it is essential to enhance xylose metabolism, one of its primary bottlenecks. Therefore, a comprehensive understanding of xylose metabolic mechanisms and pathways is crucial for developing efficient xylose-utilizing strains. This knowledge would enable targeted metabolic engineering strategies to overcome current limitations, optimize xylose utilization efficiency, and ultimately improve ethanol production from lignocellulosic biomass. Such improvements are particularly significant for advancing second-generation bioethanol production towards industrial viability and sustainability.

1.6. Xylose metabolism to produce ethanol in yeast

Hydrolysis is used to release sugars from lignocellulosic biomass. In the resulting monosaccharide mixture, glucose constitutes 60%-70% of the total sugars. Xylose, as the second most abundant sugar, varied in content depending on the type of feedstock, averaging approximately 30% (Brigham et al., 2018). Converting the two most abundant sugars can increase both the environmental and economic benefits of lignocellulose as a source for biofuels. Therefore, to enhance the efficiency of ethanol production from xylose and achieve efficient co-utilization of glucose and xylose, it is crucial to understand the metabolic pathways of xylose and their regulatory mechanisms (Kwak et al., 2019; Jansen et al., 2017).

During the last decades, two different initial xylose metabolism pathways have been successfully introduced into *S. cerevisiae* by heterologous expression. These are the xylose isomerase (XI) pathway of bacteria and fungi which catalyzes the isomerization of D-xylose to D-xylulose directly, and the XR/XDH pathway consisting of a xylose reductase (XR) and a xylitol dehydrogenase (XDH) enzyme (Fig.1.3).

The XR/XDH pathway is a two-step reaction, commonly found in yeasts and fungi, where D-xylose is first reduced to D-xylitol, which is then oxidized to D-xylulose. The exogenous genes associated with the XR-XDH pathway are primarily derived from the yeast *Pichia stipitis*. However, the differing coenzyme preferences of XR and XDH result in either a deficiency of NAD^+ or an excess of NADPH, leading to an imbalance in redox metabolism. This imbalance causes the accumulation of intermediate products such as xylitol, thereby hindering the conversion efficiency of sugar to alcohols. Although extensive coenzyme engineering research has been conducted, including adjustments to the XR/XDH activity ratio, modifications of cofactor preferences, and regulation of the intracellular NADH/NADPH ratio, this deficiency has not been effectively resolved (Broach, 2012; Rolland et al., 2000; Colombo et al., 1998; Osiro et al., 2018; Ozcan et al., 1998; Vanhalewyn et al., 1999).

The XI pathway, which facilitates the conversion of xylose to xylulose in a single step without the involvement of coenzymes, has been established in *S. cerevisiae*

significantly later than the XR-XDH pathway. This delay is largely attributed to the considerable challenges associated with the screening process for the xylose isomerase (XI) gene, which requires high-level expression for optimal activity in the yeast. Currently, the XI pathway has become the primary xylose metabolic pathway used in *S. cerevisiae* strains for second-generation ethanol production. This pathway is mainly derived from the cow rumen metagenome, the anaerobic fungus *Piromyces sp.*, the bacterium *Clostridium phytofermentans*, and the rumen fungus *Orpinomyces*. Following directed evolution techniques to enhance its activity, the optimized gene can be expressed in yeast for adaptive evolution. Additionally, increasing the copy number of *XYLA* has been shown to further enhance the xylose metabolic capacity of *S. cerevisiae* (Broach, 2012; Peeters et al., 2006; Osiro et al., 2019; Longo et al., 2012; Yan Liu et al., 2018). In the previous decade, numerous researchers have attempted to enhance ethanol production in *S. cerevisiae* by introducing xylose metabolism pathways. For example, Jo et al. engineered a novel strain based on *S. cerevisiae* containing xylose-metabolic genes (*XYL1*, *XYL2*, and *XYL3*) from *Scheffersomyces stipitis*. They developed a synthetic isozyme system of xylose reductase (XR), creating a DXS strain that coexpresses both wild-type and mutant XR. When evaluated through batch fermentations using a glucose/xylose mixture, this strain demonstrated impressive performance, achieving an ethanol productivity of 1.85 g/L·h and a yield of 0.427 g/g. (Jo et al., 2017). Coimbra et al. initially constructed strain CAT-1-XIT by introducing D-xylose isomerase (*xylA* gene, XI) from *Streptomyces coelicolor*. The recombinant strain was further enhanced with additional copies of homologous genes encoding xylulose kinase (XK) and transaldolase (TAL1). Subsequently, a plasmid (pRS42K::XI) containing *xylA* from *Piromyces sp.* was transferred to CAT-1-XIT, followed by adaptive evolution. After 10 subcultures, the evolved CAT-1-XIT (pRS42K::XI) strain demonstrated efficient xylose utilization, consuming 74% of the available D-xylose and producing 12.6 g/L ethanol with a yield of 0.31 g ethanol/g D-xylose (Coimbra et al., 2023).

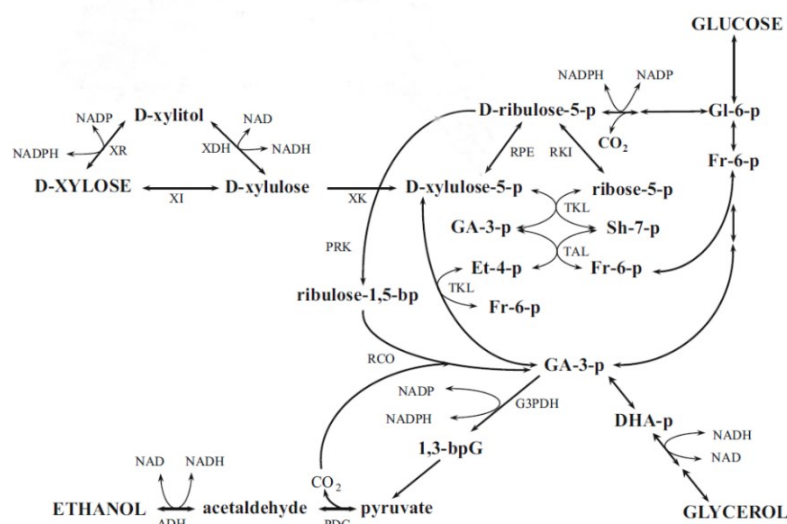


Fig. 1.3. Metabolism of xylose and glucose to produce ethanol. XR: xylose reductase; XDH: xylitol dehydrogenase; XI: xylose isomerase; XK: xylulokinase; TKL: transketolase; TAL: transaldolase; G3PDH: glyceraldehyde-3-phosphate dehydrogenase; PDC is pyruvate decarboxylase; ADH: alcohol dehydrogenase (Ruchala J. et al., 2020)

However, the attempts of metabolic engineering mentioned above are far from meeting industrial demands. Therefore, natural xylose-metabolizing yeasts like *Spathaspora passalidarum*, *Scheffersomyces stipites*, *Kluyveromyces marxianus* and *Ogataea polymorpha* attract interest of many researchers (Long et al., 2012; Su et al., 2015). Among native xylose-fermenting yeasts, the thermotolerant species *O. polymorpha* (growth up to 50°C) is of special interest as it is suitable for simultaneous saccharification and fermentation (SSF) process. Wild-type strains of *O. polymorpha* show robust growth on both glucose and xylose; however, it accumulate nearly 200 times less ethanol from xylose than from glucose (Voronovsky et al., 2005). The advanced *O. polymorpha* ethanol producers from xylose were obtained using methods of metabolic engineering and classical selection before (Ruchala et al., 2017; O. Kurylenko et al., 2018; Dmytruk et al., 2008; Kurylenko et al., 2021). Although such recombinant strains were characterized by 40-fold improved ethanol production from xylose as compared to the wild-type strain, xylose uptake was slow and incomplete.

It is known that for xylose-utilizing yeasts, glucose appeared to be the preferred sugar, being consumed first during mixed sugar fermentation (Saloheimo et al., 2007; Hua et al., 2019). Many researchers have endeavored to reveal the differences in the “omics” between the evolved strains (with high xylose utilization capacity) and their parents (with low xylose utilization capacity), as well as the differences between the strains cultured in xylose and in glucose (Shen et al., 2012; Hou et al., 2016; Sato et al., 2016; Myers et al., 2019). The results suggested that limited xylose fermentation rate is due to lack of a signaling pathway to recognize xylose as a carbon source and regulate the cells to convert to a state that promotes xylose utilization.

When biosensors were applied to *S. cerevisiae* strains engineered for xylose uptake with the XR/XDH pathway and a xylose transporter, high xylose concentrations triggered the same signal as low glucose concentrations did. This indicated that xylose resulted in the opposite signal to that of glucose and may trigger a starvation response rather than a fermentation response (Osiro et al., 2019). Extensive studies on glucose signaling pathways and their controls of glucose metabolism showed that efficient hexose transporters and glycolysis, which are factors for efficient xylose metabolism, depend on activation of glucose signaling pathways (Wu et al., 2020). Positive effects of activating the glucose sensing system in xylose-fermenting strains by upregulating the cAMP-PKA and Rgt2/Snf3-Rgt1 pathways were also found in recent work (Wu et al., 2020), suggesting that extracellular glucose signals can promote xylose utilization (Wang et al., 2015). In other words, glucose signaling pathways are not only fundamental to glucose metabolism but are also crucial for enabling the efficient metabolism of other sugars, such as xylose. Consequently, the activation of glucose signaling pathways is essential for optimizing xylose utilization. Therefore, understanding the regulation mechanisms of sugar sensing and signaling pathways, particularly their roles in xylose utilization, is crucial for developing efficient xylose-utilizing strains.

1.7. Sugar sensing and regulation in yeast for improved xylose fermentation

In the presence of glucose, metabolism of *S. cerevisiae* cells is switched to a fermentative mode even when they are exposed to oxygen (Diaz-Ruiz et al., 2011; Pfeiffer & Morley, 2014). This is called Crabtree effect. Subsequently, glucose transport and metabolism are upregulated. Simultaneously, catabolic activities involved in utilization of alternative carbon sources, including gluconeogenesis and respiration, are suppressed (Rolland et al., 2006). This metabolic switch results from a crosstalk between several glucose-signaling pathways. In yeast, there are three main glucose-signaling pathways: the Snf3p/Rgt2p pathway, the Snf1/Mig1p pathway and the cAMP/protein kinase A (PKA) pathway (Osiro et al., 2019). Recent studies have investigated the effects of engineering these three main signaling pathways on xylose metabolism and fermentation in *S. cerevisiae* (Brink et al., 2021). The following will describe these signaling pathways and their roles in detail.

In the Rgt2/Snf3-Rgt1 pathway (also called the “sensor/receptor-repressor (SRR)” pathway) that regulates glucose uptake into the cells, high and low levels of extracellular glucose are sensed by the membrane-spanning proteins Rgt2 and Snf3, respectively (Ozcan et al., 1996). The signal is then relayed into the cell, leading to the phosphorylation dependent degradation of Mth1 and Std1, which are co-repressors necessary for the DNA-binding of Rgt1. The elimination of Mth1 and Std1 exposes Rgt1 to phosphorylation, which releases it from gene promoters and derepresses the expression of target genes, including the ones encoding hexose transporters (Broach, 2012; Busti et al., 2010). Recent work confirmed that Rgt2 and Snf3 have a very limited capacity to sense xylose and thereby to trigger the expression of hexose transporter genes. Based on these findings, Wu et al. deleted the *RGT1* gene in a xylose-utilizing *S. cerevisiae* strain and demonstrated that this deletion positively affects xylose utilization (Wu et al., 2020).

S. cerevisiae Snf3/Rgt2 receptors belong to the family of the *HXT* (Hexose transporter) proteins that regulates glucose uptake into the cells. However, unlike the multiple functional hexose carriers (Hxt1-17 and Gal2) in *S. cerevisiae*, they have lost

the ability to transport glucose and evolved into specialized membrane sensors that detect extracellular glucose levels (Ozcan et al., 1998; Boles & Hollenberg, 1997; Wieczorke et al., 1999). Furthermore, these non-transporting hexose sensors appear to be yeast-specific and have not been identified in higher eukaryotes. Moreover, they are present in the majority non-conventional yeast species, as exemplified by the *Kluyveromyces lactis* Rag4 (Betina et al., 2001), *Candida albicans* Hgt4 (Brown et al., 2006), *Hansenula polymorpha* Hxs1 (Stasyk et al., 2008), and *Pichia pastoris* Gss1 (Polupanov et al., 2012). They may represent a novel class fungal hexose transporting receptors or transceptors that participate in sugar signaling for catabolite repression and are interconnected with the PKA pathways.

The Snf1/Mig1p pathway is the main glucose repression pathway that negatively regulates expression of the genes involved in the utilization of alternative carbon sources (Hedbacker & Carlson, 2008; Kayikci & Nielsen, 2015; Coccetti et al., 2018). Snf1 is a protein kinase that responds to glucose starvation and, as a central element integrating glucose repression signaling and cellular energy levels, regulates growth and metabolism by controlling the expression and/or phosphorylation of transcription factors such as Mig1, Rgt1, and Atg1 (Qiu et al., 2023). The Snf1 kinase complex has a well-recognized dual role in glucose repression, both as an activator and as a repressor. In addition to regulating *HXTs* and glucose-repressed genes, it is also involved in a plethora of cellular processes important for cell proliferation, aging, energy homeostasis, autophagy, and stress response (Mitchelhill et al., 1994; Ashrafi et al., 2000; Wang et al., 2001; Sanz et al., 2016; Zhang & Cao, 2017). Mig1 serves as a key transcriptional repressor of the glucose repression mechanism in *S. cerevisiae* and controls expression of the genes encoding proteins functioning in metabolism and transport of alternative carbon sources such as maltose, galactose, and sucrose (Nehlin & Ronne, 1990; Treitel et al., 1998). Still, the mechanism by which the Snf1-Mig1 pathway is regulated at the molecular level is not entirely elucidated. However, it was shown that differences in the amount of hexose transporter molecules (e.g., Hxt7) in the cell could cause cell-to-cell variability in the activation of Snf1-Mig1 pathway. This

model suggested a formerly unrecognized regulatory step of the Snf1-Mig1 pathway at the level of Mig1 dephosphorylation and pointed to the transport of Mig1 in and out of the nucleus as a major source of variability between individual cells (Welkenhuysen et al., 2017).

Therefore, the effects of this pathway on xylose utilization have been studied. First, research confirmed that this pathway does not response to xylose (Brink et al., 2016) and that deletion of *SNF1* accelerated xylose utilization when strains were grown in mixed sugar environments (Cai et al., 2018). Second, the disruption of Mig1, which is phosphorylated by Snf1 and inhibits the transcription of hexose transporter genes, was shown to have little effect on xylose fermentation (Roca et al., 2004) or on glucose-xylose co-fermentation (Cai et al., 2018). However, deleting both *MIG1* and *MIG2* increased xylose consumption rates by 12% under constant fermentation (Roca et al., 2004). Moreover, the overexpression of a mutant of Mig1 was found to promote xylose utilization of a recombinant strain, although the mechanism responsible remains unclear. Finally, deleting both *MIG1* and *SNF1* has been found to accelerate glucose utilization but slow xylose utilization (Cai et al., 2018). These inconsistent results suggest that the effect of the Snf1 regulatory pathway on xylose utilization is complex, and additional research is required to reveal the involved regulatory mechanisms.

The cAMP/PKA pathway is another important glucose-responsive signaling pathway, which operates in the following way: (1) The transmembrane protein, Gpr1, undergoes an allosteric effect when extracellular glucose or sucrose binds to it. Then, the Gpr1 stimulates the transition of the small G protein Gpa2 from an inactive state (binding with GDP) to an active state (binding with GTP). (2) The GTP-bound Gpa2 activates adenylate cyclase Cyr1 which catalyzes the conversion of ATP to cAMP and subsequently increases the intracellular levels of cAMP. (3) cAMP binds to the regulatory subunit Bcy1 of PKA and exposes the active site of Tpk1/Tpk2/Tpk3, thus activating PKA. Additionally, the cAMP-PKA pathway is also regulated by an intracellular protein, Ras. Intracellular glucose and its metabolites stimulate the GDP-bound Ras (inactive) to convert to GTP-bound Ras (active). The active Ras can also

increase Cyr1 activity and consequently, PKA activity. Additional proteins that affect the pathway are two cAMP phosphodiesterases, Pde1 and Pde2, that catalyze cAMP to AMP to prevent the overactivation of PKA. The active PKA up-regulates glycolysis and down-regulates gluconeogenesis at both transcriptional and translational levels (Ozcan et al., 1996; Thevelein & de Winde, 1999).

Wu et al. expressed the constitutively active mutants Gpa2^{G132V} and Ras2^{G19V}, respectively, or deleted both *PDE1* and *PDE2* genes, which increase PKA signaling by catalyzing the conversion of cAMP to AMP to perturb this pathway. These operations were found to improve xylose utilization and ethanol production (Wu et al., 2020). In a recent study by Sato et al., adaptive evolution and reverse engineering was used to discover previously unknown epistatic interactions between different genes that, when deleted, improved xylose uptake and utilization: *HOG1*, *IRA2*, *ISU1* and *GRE3* (Sato et al., 2016). Their results showed that combinations of these deletions led to improved growth, xylose consumption and specific ethanol productivity in strains engineered with the XI pathway during anaerobic conditions. They also observed that *isu1*Δ and *hog1*Δ enabled aerobic xylose respiration (Sato et al., 2016).

IRA2 and *HOG1* are known to be connected to the sugar and stress signaling networks (cAMP/PKA pathway and MAP kinase (MAPK) cascades respectively) (Tanaka et al., 1990; Reiser et al., 1999) and are therefore along the lines of the importance of the signaling networks for solving the xylose paradox. The *ISU1* gene, which encodes a mitochondrial Fe-S cluster scaffold protein (Mühlenhoff et al., 2003), had not been previously connected to xylose metabolism, but was recently pinpointed by two independent studies (Sato et al., 2016; dos Santos et al., 2016). As for *GRE3*, it may primarily improve the XI pathway by decreasing the xylitol concentration and thus XI inhibition (Träff et al., 2001). Furthermore, Qiu et al. confirmed that deletion of *HOG1*, *KSSI* (encoding an MAPK involved in the signal transduction pathways that control starvation and pheromone response), and *SMK1* (encoding an MAPK involved in signal transduction pathways controlling sporulation), combined with overexpression of *GCN4* (encoding a global transcriptional activator of amino acid

biosynthetic genes) shortened the strain lag phase in media containing xylose as the sole carbon source (Qiu et al., 2023).

Moreover, transcription factors that may force cells to improve xylose utilization have also been studied to identify regulatory targets. Michael et al. investigated the effects of knocking out eight transcription factors : such as Cat8 (catabolite repression), Hap4 (heme activator protein), Adr1 (alcohol dehydrogenase II synthesis regulator), Msn2 and Msn4 (multicopy suppressor of SNF1 mutation 2 and 4), Gis1 (G1g1-2 suppressor), Aft2 (activator of Fe (iron) transcription), and Usv1 (up in starvation). Their results showed that knockout of *CAT8* altered metabolic gene transcription levels and reduced the euclidean distance to the goal state by 60%. And this also resulted in decreased carbon flow to both biomass and ethanol (Michael et al., 2016). While in our previous results, we found that deletion of the *CAT8* ortholog (identified in *O. polymorpha* genome) in both the wild-type strain and an advanced xylose-to-ethanol producer led to 50% and 30% increases in ethanol production from xylose, respectively. When *CAT8* was overexpressed under the strong constitutive GAP promoter of glyceraldehyde-3-phosphate dehydrogenase, the engineered strains showed decreased ethanol production in xylose medium while maintaining unchanged glucose alcoholic fermentation capacity, suggesting a specific role of *CAT8* in xylose alcoholic fermentation (Ruchala et al., 2017). Additionally, the deletion of *HAP4* led to a 1.8-fold increase in ethanol production from xylose compared to its parental strain (Dzanaeva et al., 2021). Although the current mechanisms are not fully precise, the transcriptome engineering model has great potential to become a powerful research tool for identifying novel transcription factors in the future. It not only helps to discover new regulatory elements involved in xylose metabolism but also provides innovative strategies for enhancing xylose utilization. Understanding these transcriptional networks and their regulatory mechanisms will facilitate the rational design of more efficient strains for improved ethanol production from xylose.

The above review primarily focuses on the glucose sensing system in *S. cerevisiae*, while in the methylotrophic yeast *O. polymorpha*, researchers have also

studied its sugar sensing system in recently years. Through functional complementation studies on 2-deoxyglucose (2-DG) resistant mutants, researchers discovered a hexose transporter homolog named *OpGcr1* (Glucose Catabolite Repression) in *O. polymorpha*, which functions as a glucose carrier and may represent the first identified member of a new class of yeast transceptors that sense and transport glucose (Stasyk et al., 2018). And the *O. polymorpha* genome revealed two additional hexose transporter homologs besides *OpGcr1* (Stasyk et al. 2008). One is *OpHxs1* (hexose sensor), which shared the highest sequence homology with non-transporting glucose sensors found in yeast, such as Snf3 and Rgt2 from *S. cerevisiae*. The other protein, *OpHxt1* (hexose transporter), showed the strongest similarity to established yeast glucose facilitator proteins. Studies revealed distinct functions of *OpHxs1* and *OpHxt1* in both *S. cerevisiae* and *O. polymorpha*. When overexpressed in a hexose transporter-deficient *S. cerevisiae* strain, *OpHxs1* failed to support growth on glucose or fructose, indicating it lacks transport function. In contrast, *OpHxt1* demonstrated functional transport activity in this heterologous system. In *O. polymorpha*, *Hxs1* was expressed at moderate-low levels and played a crucial role in glucose-induced expression of *Hxt1*, a low-affinity transporter. The sensor function of *Hxs1* depended on its C-terminal region, similar to other yeast non-transporting sensors. Additionally, a R203K substitution in *Hxs1* resulted in constitutive signaling activity. While *Hxs1* was not essential for glucose repression or glucose-induced pexophagy, its mutation caused a temporary but significant impairment in AOX repression when cells were exposed to fructose. This effect likely resulted from reduced fructose transport in the mutant strain. These findings indicate that in the Crabtree-negative yeast *O. polymorpha*, *Hxs1* functions as the sole transporter-like sensor in the hexose induction pathway, while the efficiency of hexose uptake influences the intensity of hexose catabolite repression (Stasyk & Stasyk, 2019).

However, there are no reports on the role of *Hxs1* in xylose recognition and utilization. In particular, the function of this gene in glucose and xylose alcoholic fermentation remains unexplored. Given that no specific sensing proteins involved in

xylose alcoholic fermentation have been identified to date, investigating whether Hxs1, as a glucose sensor, can also sense xylose and promote xylose fermentation to ethanol represents an intriguing scientific question, which will be addressed in this thesis.

Extensive research has been conducted on glucose-related sensing systems, leading to comprehensive understanding of glucose transporters, transcription factors, and their regulatory networks in *S. cerevisiae*. In contrast, studies focusing on xylose-related regulatory elements are relatively limited, creating a significant knowledge gap in our understanding of xylose metabolism. Despite the implementation of numerous successful metabolic engineering strategies, including the introduction of xylose metabolic pathways and optimization of key enzymes, xylose utilization by recombinant *S. cerevisiae* still significantly lags behind its performance on glucose. Therefore, research attention should be redirected towards naturally xylose-metabolizing yeasts such as *O. polymorpha*, which possess inherent ability to efficiently utilize xylose. In-depth exploration of xylose sensing systems in these organisms may lead to the discovery of novel genes and regulatory mechanisms involved in xylose transport and its transcriptional regulation (Chen & Nielsen, 2013; Dai et al., 2015). Finally, comprehensive understanding of these molecular mechanisms and their transcriptional regulation would have profound implications for developing efficient bioprocesses. Such knowledge could enable the rational design of more effective strains, where carbon flux can be precisely regulated and efficiently redirected toward desired products such as bioethanol, ultimately advancing the field of second-generation biofuel production.

1.8. Summary

This review focuses on the major protein degradation pathways in yeast, with particular emphasis on autophagy-mediated protein degradation. The review systematically examines various autophagic pathways, categorizing them based on their substrate specificity, and provides detailed insights into their underlying mechanisms and associated related genes. Through comprehensive analysis of these

distinct autophagic mechanisms, we explore their relationship with protein degradation, aiming to identify key genes involved in these processes. This understanding of autophagy-mediated protein degradation pathways may provide novel strategies for optimizing recombinant protein production in *K. phaffii* expression systems, particularly through targeted manipulation of autophagy-related genes to enhance protein accumulation and cellular productivity.

Additionally, advancements in second-generation ethanol production from lignocellulosic biomass are highlighted, with a focus on molecular engineering and the selection of enhanced ethanol-producing strains capable of xylose utilization. Special emphasis is placed on the initial stages of xylose metabolism and its regulatory mechanisms, particularly within sugar-sensing signal pathways. Microorganisms optimized for fermenting lignocellulosic hydrolysates are required to exhibit high productivity, ethanol tolerance, and the capacity to concurrently metabolize multiple carbon substrates. These foundational works of previous researchers have established a robust basis for this study, facilitating our efforts to construct xylose-fermenting strains with enhanced ethanol production efficiency.

CHAPTER 2

MATERIALS AND RESEARCH METHODS

2.1. Research materials

Chemical compounds, reagents, and enzymes manufactured by Sigma (USA), Fluka AG (Switzerland), Promega (USA), Roth (Germany), Difco (USA), Reanal (Hungary), Fermentas (Lithuania) were used in the work. Chemical reagents of domestic production had the qualifications of “hch” and “osch”.

2.2. Strains of microorganisms

The yeast strains and the *E. coli* bacterial strains used in this study are listed in Table 2.1.

Table 2.1

Microorganism strains used in this study

Strain name	Description	Source
Yeasts		
GS200	<i>his4 arg4</i>	(Waterham et al., 1996)
GS200/LAC4	PralF-pr <i>FLD1-LAC4</i> -GFP	(Zazulya et al., 2023)
SMD1163	<i>his4 pep4 prb1</i>	(Gleeson et al., 1998)

NCYC495	<i>leu1-1</i>	(Gellissen, 2000)
2EthOH- /XYL1m/XYL2/XYL3/BrPA/ △cat8	<i>cat8::hphNT1</i> , pX1M-Z-X2 (<i>GAPp-XYL1mod-AOXt</i> , <i>GAPp-XYL2-XYL2t</i>), pGLG61/XYL3 (<i>GAPp-XYL3-AOXt</i>), resistant to brompyruvate	(Ruchala et al., 2017; Kurylenko et al., 2014)
MNNG-1; MNNG-2; MNNG-3; MNNG-4	Mutans by MNNG from <i>K.phaffii</i>	Section 3.1.1
WT/AUR1-2	pUC57/AUR1-2	Section 3.2.1
WT/IMH3	pUC57/IMH3	Section 3.2.2
BEP/△cat8/IMH3/TAL1/TKL1 /AOX1	pUC57/IMH3/prGAP_TAL1_ prGAP_TKL1_prGAP_AOX1	Section 3.2.3
WT/ AZF1	pUC19/prGAP_AZF1/NTC (<i>GAPp- AZF1-GAPt</i>)	Section 3.3.1
WT/ HXS1	pUC19/prGAP_HXS1/NTC (<i>GAPp- HXS1-GAPt</i>)	Section 3.3.1
BEP/△cat8/AZF1	pUC19/prGAP_AZF1/NTC (<i>GAPp- AZF1-GAPt</i>)	Section 3.3.1
BEP/△cat8/HXS1	pUC19/prGAP_HXS1/NTC (<i>GAPp- HXS1-GAPt</i>)	Section 3.3.1

<i>E. coli</i>		
DH5α	<i>lacZ</i> ΔM15, <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (_{rk-} , _{mk+}), <i>supE44</i> , <i>relA1</i> , <i>deoR</i> , Δ(<i>lacZYAargF</i>)U169	(Neidhardt, 2016)

2.3. Plasmids

The plasmids used in this study are presented in Table 2.2.

Table 2.2

List of vectors used in the dissertation work

Name	Reference for construction
PraIF-pr <i>FLD1-LAC4</i> -GFP	(Zazulya et al., 2023)
pUC57/AUR1-1	Section 3.2.1
pUC57/AUR1-2	Section 3.2.1
pUC57/IMH3	Section 3.2.2
pUC57/IMH3/TAL1/TKL1/AOX1	Section 3.2.3
pTkZr	(Kurylenko et al., 2018)
pTaZr	(Kurylenko et al., 2018)
pUC19/prGAP_AZF1/NTC	Section 3.3.1
pUC19/prGAP_HXS1/NTC	Section 3.3.1

2.4. Primers

The primers used in this study for the creation and verification of the correctness of the constructed vectors are presented in Table 2.3.

Table 2.3

List of primers used for conducting the research

Primer name	Nucleotide sequence of the primer 5'→3'
SM166	TGCTCTAGAATGTCGACAGAAGCTCGAGATCAC
SM167	TTTGCGGCCGCTTATTGTCCATGGCTATGTACA
AZF1_F	AAAGCGGCCGCGCATGCCGATGATATACTC
AZF1_R	AAAGCGGCCGCTCAAACCTCCATGACCG
Ko1036	AGTCATACGTGTAGGTTTTTGGCG
RV131	GCGCAATAGGGTCTCTTTC
RV132	GCATTAATTGATCCACCTTTG
OK219	CCG GAATTC GCG CAA TAG GGT CTC TTT C
OK220	GAAAAATATAAAAATTCCGTAGGGAATCCAAGC CAACACGTCTAAAAATGTGCAAGTGTAGGATGCAAA TAGCTG
OK221	CAGCTATTTGCATCCTACACTTGCACATTTTTA GACGTGTTGGCTTGGATTCCCTACGGAATT TTA TATTTTTC
OK222	CCGGAATTCGTGCCTAATCAAATGCATTC
OK223	GGCGAATTTGTGAAACATGAGGTGTAC

OK224	GTACACCTCATGTTTCACAAATTCGCC
pUC_F	GTTGTAAAACGACGGCCAGT
pUC_R	CACACAGGAAACAGCTATGAC
OK225	CGGGGTACCCAATTCGTCTTCTTCAACAGAGTC
OK227	CGGGGTACCGTTCACATCAGAAAGGTCAAGCTG
OK248	CGGGCCCGTCGACTGCAGTCGACGGGGCCCGGGATCCCAAT
OK249	CAAGCTTGCATGCAGGCCTTTTTTCGTCCGACCTGTTTGG ATC
OK250	GAAAAAGGCCTGCATGCAAGCTTG
OK251	GTCGACTGCAGTCGACGGGGCCCG
OK252	GAGGCCTGCATGCAAGCCAATTATCATTAATAATCACTC
OK253	CTATGACCATGATTACGCCAAGATCGTTCCTGAAAACCTC
OK254	AATTGGCTTGCATGCAGGCCTC
OK255	GTCGACTGCAGTCGACGGGGCCCG

2.5. Nutrient media

Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 1% glucose) or YNB (0.67% Yeast Nitrogen Base with ammonium sulphate, 0.05% yeast extract) mineral medium or YNB (0.17% Yeast Nitrogen Base without ammonium sulphate, 0.05% yeast extract). Methanol (1% w/v) and glucose (2% w/v) were used as carbon sources. For the auxotrophic strain growth, the final concentration of amino acids were added: L-histidine - 40 mg/L, L-arginine - 40 mg/L. The selection of transformants of *O. polymorpha* was carried out on a medium with norzeothricin (100mg/L). Media were sterilized by autoclaving, aqueous solutions of labile

compounds were filtered through nitrocellulose filters (pore size 0.2 μm) and added to the media before use. All the solid media contained agar (2% w/v). Yeasts were grown on agar media on Petri dishes in a thermostat or cultured in liquid media with shaking of 220 rpm at 28 °C.

Escherichia coli DH5 α bacteria were grown at 37°C on a rich (LB) medium (Ausubel et al., 1988). For the selection of plasmid-containing bacteria, ampicillin was used at a concentration of 100 mg/L.

2.6. Chemical mutagenesis with MNNG

The mutagenesis method described by An et al. was modified (An et al., 1989). In this study, yeast cells were pre-cultivated in YPD medium overnight to a density of 3×10^6 cells/mL. The MNNG was dissolved in 50 mM phosphate buffer at pH 6. The cell suspension with MNNG at a final concentration of 0.1 mg/mL was shaken for 30 minutes at 28°C. Then, the cells were washed twice with 50 mM phosphate buffer at pH 7 and cultured in YNB with 1% methanol for 3 hours at 28°C to activate β -galactosidase. Finally, 50-100 μL of cells were spread on YPD plates containing X-Gal (final concentration 150 mg/L). After 2-3 days of incubation at 28°C, the blue mutant colonies were selected.

2.7. Biochemical methods

2.7.1. Obtaining cell-free extracts

Yeast cells were cultured overnight in YPD medium, followed by washing with distilled water. Subsequently, cells were transferred to YNB medium supplemented with glucose (2% w/v) and methanol (1% w/v), respectively, and incubated for 18 hours at 28 °C. Then the cells pelleted by centrifugation at 3000 g for 3 minutes at 4°C in Eppendorf microtubes. The supernatant was collected, and the cells were washed twice with 1 mL of distilled water under the same centrifugation conditions. Cell-free extracts were prepared using glass beads ("Ballotini"). The washed cell pellet was

resuspended in 1M Tris-HCl buffer (pH 7.5) containing 100 mM PMSF (phenylmethylsulfonyl fluoride) and 100 mM DTT (Dithiothreitol) to a final cell concentration of 50-100 mg/mL. The suspension was transferred to eppendorf tubes and mixed with glass beads (0.45-0.5 mm diameter) at a ratio of 3:4 (v/v) before freezing. Cell disruption was performed by vortexing for 15 minutes at 4°C with intermittent cooling on ice every 5 minutes. The cell-free extract was obtained by centrifugation of the homogenate at 14000 rpm for 20 minutes at 4°C. The supernatant was collected for further analysis and stored at -20 °C.

2.7.2. Determination of protein concentration in cell-free extracts by the Lowry method

Reagents: A-2% solution of Na_2CO_3 in 0.1 M NaOH; B-0.5% solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium citrate; C-1 mL of solution B mixed with 49 mL of solution A; D-Folin reagent diluted with water to a final concentration of 1 N.

To 100 μL of the test solution, 1 mL of solution C was added, and the mixture was left to stand at room temperature for 10 minutes. To the resulting mixture, 100 μL of Folin reagent was quickly added, vigorously mixed, and the absorbance was measured at 750 nm in a 1 cm cuvette after 40 minutes reaction in the dark. The calibration curve was constructed using a solution of BSA (bovine serum albumin) at known concentrations of 25-800 $\mu\text{g/mL}$ (Lowry et al., 1951). By constructing a standard curve using values at $\text{OD}_{750\text{nm}}$ under different concentrations, and subsequently obtaining the standard curve equation, the protein concentration of the sample will be determined according to the standard curve equation.

2.7.3. Determination of enzyme activity

β -galactosidase activity was determined in a reaction mixture with the following composition (1 mL): 0.9 mL of Z-buffer (60 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10 mM KCl, 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) was added to each sample and

adjusted to a final protein concentration of 0.2 mg/100 μ L using distilled water. The mixture was mixed and incubated for 5 minutes at 28°C. The reaction was initiated by adding 0.2 mL of ONPG (4 mg in 1 mL of Z-buffer), and the start time was recorded. When the solution turned yellow (indicating the formation of o-nitrophenol), the reaction was stopped by adding 0.5 mL of 1 M sodium carbonate. The optical density was measured at 420 nm using 1 cm cuvette. β -galactosidase activity was calculated using the formula:

Equation 2.1

$$A = \frac{OD_{420} \times 1.7}{P \times V \times t}$$

where:

OD₄₂₀ is the optical density of the product at 420 nm;

P is the protein concentration (mg/mL);

V is the volume of the reaction mixture (mL);

t is the reaction time (min).

Alcohol oxidase (AOX) activity was determined in a reaction mixture with the following composition (1 mL): 50 mM K-P buffer, pH 7.5, 820 μ L; methanol (dilute 250 times), 100 μ L; peroxidase (1 mg/mL), 30 μ L; o-dianisidine (2.5 mg/mL), 30 μ L. The reaction was started by the addition of 20 μ L permeabilized cells with a concentration of 50 mg/mL to the reaction mixture while added 20 μ L H₂O was added as the control. After 5-20 minutes when the pink color developed, the reaction was stopped by adding 260 μ L of concentrated HCl. The activity was determined photometrically based on optical absorption with a Helios Epsilon spectrophotometer at a wavelength of 525 nm in a 1 cm-wide cuvette.

The permeabilized cells were prepared by mixing cells with 50 mM K-P buffer containing 1 mM PMSF and 0.5% CTAB, and the mixture were vortexed 15 minutes

at 28 °C. After then washed the permeabilized cells in 50 mM K-P buffer with 1 mM PMSF. The final concentration of the permeabilized cells is 50 mg/mL.

2.7.4. Measurement of the biomass accumulation

Cell biomass (in units of optical density (OD_{600nm})) was determined by optical absorption of diluted suspensions by photometry on a Helios Epsilon spectrophotometer at a wavelength of 600 nm in a 1 cm wide cell. To estimate growth kinetic parameters, the mutant strains were pre-cultured in YPD medium overnight and washed with distilled water then transferred to YNB containing either methanol (1% w/v) or glucose (2% w/v) as sole carbon source. For biomass assays, cell suspensions were collected daily (every 24 hours).

2.7.5. Detecting the fluorescence under the microscopy

Images were taken using a fluorescence microscope (Axio Imager A1; Carl Zeiss MicroImaging, Jena, Germany) in conjunction with a monochrome digital camera (Axio Cam MRm; Carl Zeiss MicroImaging). AxioVision 4.5 (Carl Zeiss MicroImaging) and ImageJ software were used for photo processing.

2.7.6. Yeast viability assays to monitor autophagy

The mutant strains were grown in YPD medium for 18 to 24 hours at 28°C with shaking at 220 rpm. In this case, the GS200 and SMD1163 were used as positive and negative control strains, respectively. The cells were washed twice with sterilized water and inoculated into the nitrogen starvation YNB medium (0.17% yeast nitrogen base without ammonium sulfate and amino acids with 2% glucose and 0.05% yeast extract) to initiate general autophagy at 28°C with shaking at 220 rpm. After 0, 1, and 2 days of cultivation, the cells were spread on YNB plates containing ammonium sulfate and 2% glucose. The number of colonies was counted after 2 to 3 days of incubation. The

cell percentage viability was calculated by comparing the colony numbers to the baseline measurement taken on day 0.

Phloxine B staining was used to assess yeast viability in this study. Phloxine B staining reflects the percentage of dead cells because the plasma membrane barrier was disrupted in dead cells which the dye penetrated into the cell and stained the cytosolic materials. The method described by Noda was minimally modified (Noda, 2008). The measured yeasts were pre-cultured in YPD media for 24 hours at 28°C. Then transferred into YNB without nitrogen media. After 0 and 1 days of cultivation under nitrogen starvation conditions, yeast cells strains were stained with phloxine B solution at a final concentration of 2 µg/mL. A fluorescence microscope with a red filter was used to estimate stained dead cells. The GS200 and SMD1163 were used as negative and positive control strains, respectively.

2.8. Basic molecular genetic methods

2.8.1. Extraction of total DNA from yeast cells

This method is a modified version of the protocol developed by Johnston for *S. cerevisiae*, which utilizes lyticase for cell spheroplasting, RNase A for RNA degradation, and alcohol for DNA precipitation (Johnston, 1994). It is specifically designed for plasmid DNA isolation from yeast cells and was optimized in the course of this study.

Cells were cultured in 3 mL of liquid selective medium at 37°C until reaching the late logarithmic phase (OD 4-6, wavelength 600 nm, 1 cm cuvette). The biomass was harvested by centrifugation and resuspended in 0.3 mL of 50 mM EDTA buffer (pH 8.0). Lyticase (50 IU) was then added to the suspension, and the mixture was incubated at 37°C for 60 minutes. After incubation, the cells were pelleted by centrifugation at 12,000 rpm for 2 minutes. The supernatant was discarded, and 0.3 mL of lysing solution (0.2% SDS, 50 mM EDTA) was added to the pellet. After resuspending the pellet, the microtubes were heated at 65°C for 15–30 minutes, then cooled to room temperature. To the incubation mixture, 0.1 mL of 3M potassium acetate (pH 5.2) was

added, vortexed vigorously, and placed on ice for 5 minutes. The mixture was then centrifuged at 12,000 rpm for 3 minutes. The supernatant was transferred to clean microtubes containing 0.3 mL of isopropanol and left to stand for 10 minutes. Subsequently, the samples were centrifuged at 12,000 rpm for 3 minutes, the supernatant was discarded, and the pellet was washed with 70% ethanol and air-dried. The resulting DNA was dissolved in 100 μ L of TE buffer, followed by the addition of 5 μ L of RNase A (10 mg/mL). The mixture was incubated at 37°C for 20 minutes. Then, 100 μ L of buffer-saturated phenol was added to the incubation mixture. Following centrifugation at 14,000 rpm for 15 minutes, the aqueous phase was transferred to a fresh microcentrifuge tube. 0.1 volume of 3M potassium acetate (pH 5.2) and 2 volumes of 96% ethanol were added. The mixture was incubated at -20°C for 15 minutes, then centrifuged again at 14,000 rpm for 15 minutes. The resulting pellet was washed with 70% ethanol, air-dried, and the nucleic acid was dissolved in 30 μ L of TE buffer and stored at -20°C.

2.8.2. Plasmid DNA extraction from *E. coli* cells

E. coli transformant cells were cultured in 1.5 mL of LB medium containing 100 mg/L ampicillin for 14–16 hours, and sedimented by centrifugation (10,000 rpm, 3 min) in plastic tubes twice to completely remove the rest of the nutrient medium. The cell pellet was resuspended in 0.15 mL of TE buffer (pH 7.5) with gentle agitation. Then, a mixture of 0.15 mL of 0.2 M NaOH and 1% SDS were added, and the mixture was incubated at room temperature for 15 minutes. Following this, 0.15 mL of 2M potassium acetate was added, gently mixed, and the mixture was incubated on ice for 15 minutes. The protein precipitate was removed by centrifugation at 10,000 rpm for 20 minutes at room temperature, and the supernatant (0.4 mL) was transferred to clean test tubes. To the supernatant, 0.7 volume (0.3 mL) of isopropanol was added, mixed, and centrifuged again at 10,000 rpm for 20 minutes at room temperature. The precipitate DNA and RNA pellet was washed with 70% ethanol, dried, and

resuspended in 40 μ L of sterile distilled water or TE buffer. The DNA solution was then stored at -20°C .

Preparative quantities of plasmid DNA were isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA), according to the manufacturer's protocol.

2.8.3. Construction of vectors

The method of DNA cleavage by restriction enzymes relies on the ability of class II restriction endonucleases to cleave DNA at specific sites. In this study, we used the methodology described earlier (Sambrook et al., 2001), following the instructions provided by the enzyme manufacturers: Fermentas (Lithuania), NEB (USA), and Promega (USA).

To dephosphorylate the sticky ends of linearized vector DNA and prevent self-ligation, alkaline phosphatase CIP produced by NEB (USA) was used following the reaction conditions recommended by the manufacturer.

Ligation of linearized DNA fragments was performed according to a previously described method with some modifications. Bacteriophage T4 DNA ligase was employed for the ligation, and the reaction was conducted in the buffer provided with the proprietary ligase preparation from Promega (USA), following the manufacturer's instructions. To isolate recombinant plasmids, the ligation mixture was purified by ethanol precipitation or using a Qiagen PCR purification kit (Qiagen, USA) before being transformed into *E. coli* cells via electroporation (Sambrook et al., 2001).

2.8.4. Transformation of yeast

The transformation of *O. polymorpha* was carried out by the electroporation method as described earlier (Faber et al., 1994). The method is based on the ability of yeast cells to absorb exogenous DNA under the influence of an electric pulse. Following the transformation of *O. polymorpha* yeast, the cell mixture was incubated

for one hour at 37°C in 1 mL of YPD medium before plating on antibiotic-containing agar. The transformation of *E. coli* bacteria was conducted using standard electroporation (Sambrook et al., 2001).

2.8.5. Basic molecular genetics techniques

The isolation and purification of plasmid DNA, preparation and transformation of competent *E. coli* cells, electrophoresis of DNA in agarose gel, elution of DNA fragments from agarose gel, digestion of DNA with restriction enzymes, ligation of linearized DNA fragments, and amplification of DNA fragments using polymerase chain reaction (PCR) were performed as described by Sambrook (Sambrook et al., 2001). DNA purification was performed using Qiagen columns (USA) with the Qiagen PCR Purification Kit. For amplifying DNA fragments via PCR, synthetic oligonucleotide primers from IDT Technologies or Sigma (USA) were used.

2.9. Conditions for alcoholic fermentation of yeasts

To conduct alcoholic fermentation of glucose and xylose, the yeast biomass of *O. polymorpha* was cultivated in a complete medium (YPD/YPX) containing 1% yeast extract, 2% peptone, and 2% glucose or xylose for two days on an orbital shaker (220 rpm) at 37°C. The cells were harvested by centrifugation and washed with distilled water. The biomass (2 mg/mL of cells) was transferred to a mineral medium (YNB) supplemented with 10% xylose or 10% glucose. Alcoholic fermentation was carried out on an orbital shaker at 37°C under limited aeration (140 rpm) for 5 days. All experiments were performed in triplicate at minimum.

The yeast biomass was measured nephelometrically using a Helios γ spectrophotometer ($\lambda = 600$ nm, using 1 cm cuvette), with the dry weight calculated based on a calibration curve.

2.10. Software for searching and analyzing nucleotide and amino acid sequences

In this study, the databases for *O. polymorpha* - <http://genome.jgi-psf.org/Hanpo2/Hanpo2.home.html> and *S. cerevisiae*- <http://www.yeastgenome.org/> were used. The analysis of the DNA nucleotide sequence was performed using the following programs: NEBcutter V2.0 (<http://tools.neb.com/NEBcutter2/>) and Oligonucleotide Properties Calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>), and the software package available at <http://www.bioinformatics.org/sms/>. For the comparative analysis of amino acid and nucleotide sequences, programs available at <http://www.ebi.ac.uk/Tools/msa/clustalw2/> were used. Similarity searches of amino acid sequences were conducted using the BLAST web service from the National Center for Biotechnology Information (Bethesda, MD, USA) at <http://www.ncbi.nlm.nih.gov/BLAST/>.

2.11. Determination of analyte concentration

The concentrations of ethanol, xylose, glucose, and xylitol in the medium were determined using high-performance liquid chromatography (PerkinElmer, Series 2000, USA) with an ion-exchange column Aminex HPX-87H (Bio-Rad, Hercules, USA). A 4 mM H₂SO₄ solution was used as the mobile phase, with a flow rate of 0.6 mL/min and a column temperature of 35°C. The concentration of ethanol in the medium was also determined using an Alcotest kit, based on the principle that ethanol is oxidized by alcohol oxidase in the presence of atmospheric oxygen to acetaldehyde and hydrogen peroxide. The latter, in a coupled peroxidase reaction, oxidizes a chromogen to a colored product, which is measured photometrically (Gonchar et al., 2001).

2.12. Statistical analysis of experimental data

Statistical processing of the results was carried out using standard methods. All experiments were performed in triplicate with three independent replicates. Each point in the graphs and diagrams represents the mean value (M) calculated from three measurements in one of several independent experiments. The standard error of the mean (m) was calculated using the standard deviation (σ). In the figures, error bars represent the standard error of the mean. Comparison between two variables was performed using t-test. The difference between values was considered statistically significant when $p < 0.05$. The results of the experiments were presented as mean \pm standard deviation.

CHAPTER 3

RESEARCH RESULTS AND THEIR DISCUSSION

3.1. Screening of mutants defective in β -galactosidase degradation in methyltrophic yeast *Komagataella phaffii*

The β -galactosidase synthesized in the recombinant methanol-grown strain *K. phaffii* GS200/LAC4 under *FLD* promoter can be visualized on plates with X-Gal by the development of a blue color. After shifting the GS200/LAC4 cells to a glucose-containing medium with X-Gal, the colonies remain white due to the degradation of the enzyme. In this study, the chemical mutagen MNNG was used to select mutants with defective degradation of β -galactosidase. After mutagenesis, four mutant strains MNNG-1, MNNG-2, MNNG-3 and MNNG-4 were selected on the YPD plate with X-Gal which remained blue after shift of methanol-grown cells to glucose plates. To confirm the autophagy-deficient phenotype, the selected mutants were first pre-cultured on methanol or glucose and then transferred to YPD plates containing X-Gal.

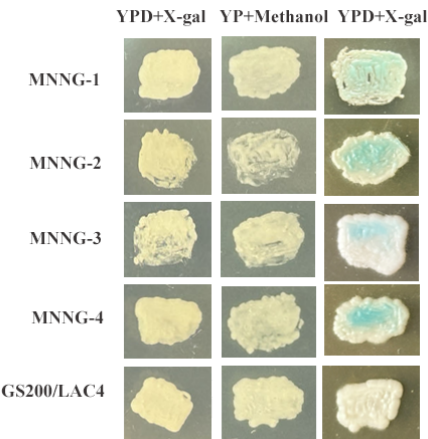


Fig. 3.1. The selected mutants were pre-cultured on methanol or glucose separately, and then transferred to YPD plates containing X-Gal. The mutant strains with defective β -galactosidase degradation appear blue on YPD medium containing X-

Gal after pre-culturing in methanol. However, they remained white on this medium when pre-cultured on glucose.

The result suggested that these four mutant strains are defective in β -galactosidase degradation and therefore appeared blue on YPD medium containing X-Gal after pre-culturing in methanol compared to the parental strain. However, they remained white on this medium when pre-cultured on glucose (Fig. 3.1).

3.1.1. Biochemical characterization of the selected mutants

Changes in β -galactosidase activity and fluorescence of hybrid β -galactosidase-GFP protein under the microscope were analyzed in the selected *K. phaffii* mutants. The specific β -galactosidase activity under the conditions of methanol-induction for 1 day and subsequent shift to glucose cultivation for 18 hours were investigated. The mutant strains exhibited a 1.4- to 4-fold increase in β -galactosidase activity compared to the parental strain GS200/LAC4 (Fig. 3.2).

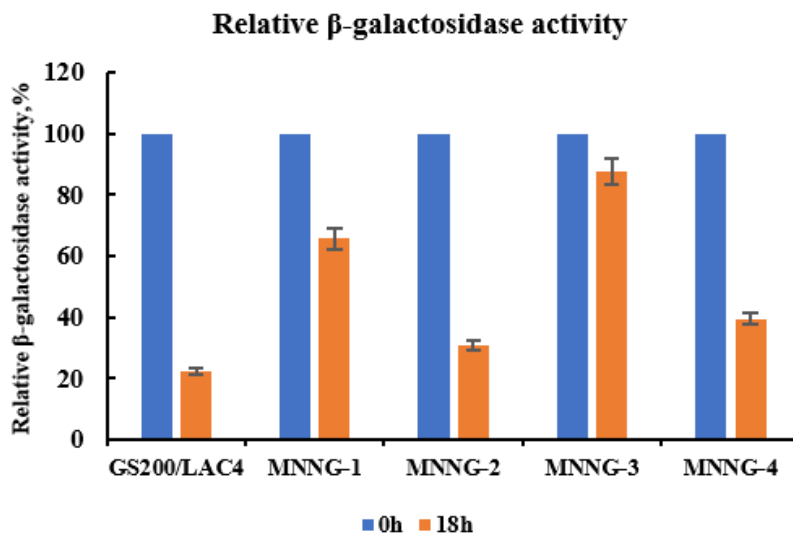


Fig. 3.2. Specific activity of β -galactosidase after 1 day of methanol induction with subsequent shift to glucose in GS200/LAC4 and MNNG mutants of *K. phaffii*.

Samples were collected immediately after shift from methanol to glucose (0 h) and 18 h cultivation on glucose.

To visualize the impaired degradation of β -galactosidase, the selected mutants were subjected to fluorescent analysis under the fluorescence microscope, as the enzyme is fused with GFP. During cultivation on methanol, β -galactosidase is actively synthesized and accumulated in the cytosol of yeast cells. Selected strains were grown in YNB medium supplemented with methanol (1% w/v) as a carbon source for 1 day at 30°C. A green glow of the cells was observed using a green light filter. Then, cells were shifted to glucose medium (2% w/v) to stimulate the degradation of this enzyme. Fluorescence microscopy images clearly demonstrated that degradation of β -galactosidase was impaired in the mutant strains, which exhibited higher fluorescent compared to the parental strain (Fig. 3.3). These fluorescence staining results are consistent with the β -galactosidase activity results described above (Fig. 3.2), indicating that there is indeed β -galactosidase degradation defect in these mutant strains compared to the parental strain.

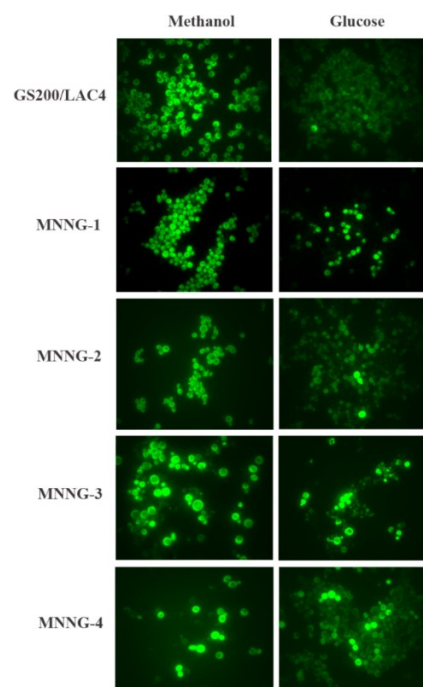


Fig. 3.3. Fluorescence images were obtained from *K. phaffii* GS200/LAC4 and MNNG mutants that were grown in liquid YP medium with methanol (1% w/v) for 24

h and transferred to either YP medium with methanol (1% w/v) or glucose solution (2% w/v). Images were taken 24 h after the transfer. The strains produced cytosolic LAC-GFP fusion protein.

Pexophagy is a selective autophagy process that degrades damaged or superfluous peroxisomes in the yeast vacuole. In *K. phaffii*, pexophagy occurs when cells are shifted from methanol to glucose-containing medium (Bernauer et al., 2020). Alcohol oxidase (AOX) is a key peroxisomal matrix protein. In the selected mutants, pexophagy was studied by analyzing the residual AOX activity after shifting the cells from methanol to glucose.

The results demonstrated that AOX activity in SMD1163 (defected in vacuolar proteases strain) was 6.9-fold higher than that in GS200/LAC4 after incubation in glucose (Fig.3.4). MNNG-1 and MNNG-3 exhibited approximately 3- and 6-fold higher AOX activity compared to GS200/LAC4, suggesting that mutations in these strains may be linked to the selective pexophagy pathway. While MNNG-2 and MNNG-4 showed an almost complete loss of AOX activity in glucose-containing medium, indicating that the mutations in these two strains are not associated with the pexophagy pathway (Fig. 3.4).

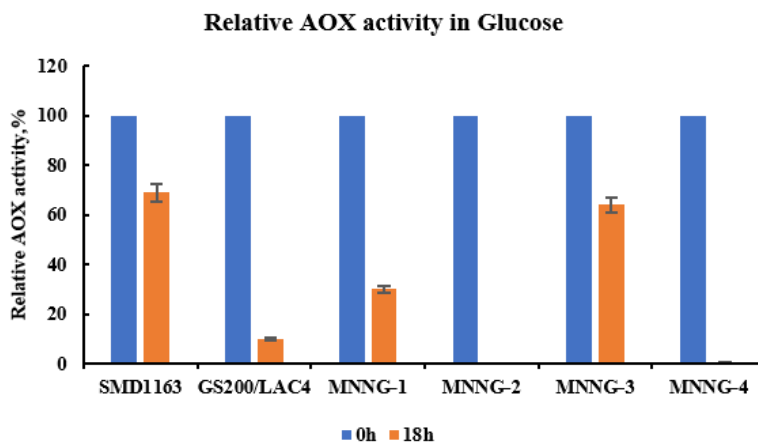
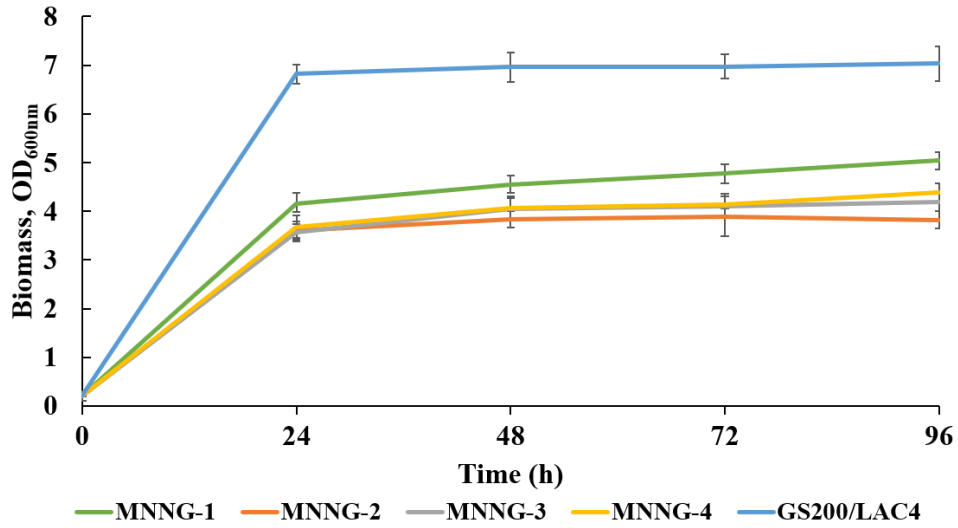


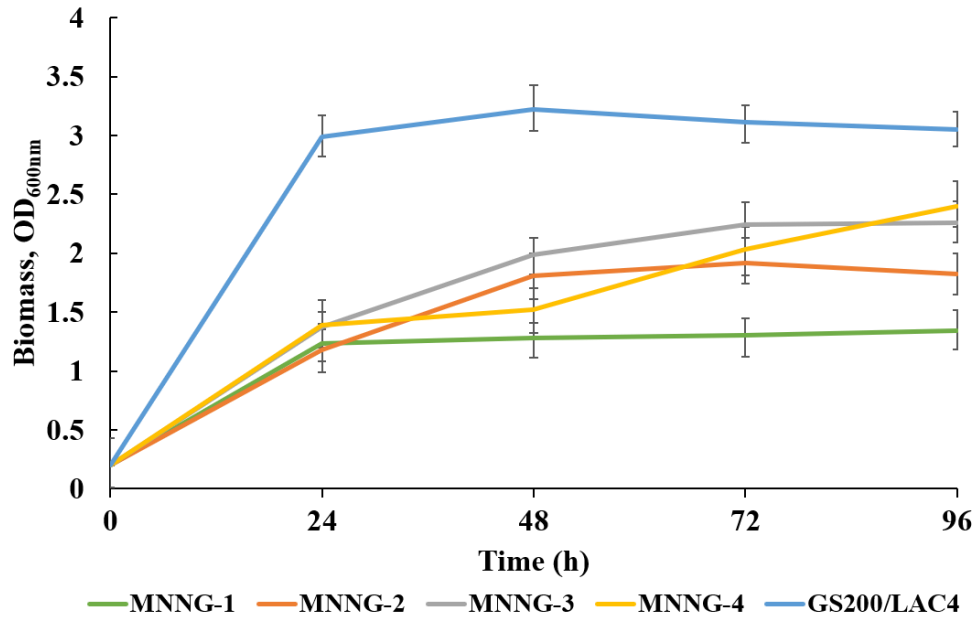
Fig. 3.4. Specific activity of AOX after 1 day of methanol induction with subsequent shifting to glucose in GS200/LAC4, SMD1163 and MNNG mutants of *K.*

phaffii. Samples were collected immediately after shifting from methanol to glucose (0 h) and after 18 h of cultivation on glucose.

Biomass accumulation is a key characteristic, particularly for strains used in recombinant protein production, as typically protein production correlates positively with cell biomass. Additionally, a key function of autophagy is to generate metabolic substrates for cellular growth through activation of autophagic pathways under adverse conditions. Impairment of the autophagy pathway results in compromised growth of the strain under identical conditions. In this study, the growth kinetics of the mutant strains were assessed in mineral media YNB supplemented with glucose (2% w/v) and methanol (1% w/v) (Fig. 3.5.). The maximum biomass accumulation of the mutants ranged from OD₆₀₀ 3.5 to 5.0 after 96 hours of cultivation in glucose-containing medium, while the parental strain reached OD₆₀₀ 6.8 at 24 hours (Fig. 3.5A). Among the tested mutants, MNNG-1 showed the highest biomass accumulation at OD₆₀₀ 5.0. In contrast, MNNG-1 exhibited the lowest biomass accumulation at OD₆₀₀ 1.3 when cultivated in methanol-containing medium (Fig. 3.5B). MNNG-2, MNNG-3, and MNNG-4 followed a similar trend, accumulating between OD₆₀₀ 1.7 and 2.4 after 96 hours of cultivation in methanol. The parental strain, however, reached OD₆₀₀ 3.2 at 48 hours in methanol medium. (Fig. 3.5B). These results demonstrate that the selected mutant strains exhibited reduced growth compared to the parental strain in media containing either glucose or methanol as the sole carbon source. This indicates that the selected mutant strains indeed possess autophagy defects relative to the parental strain, which consequently affected its growth performance.



A. Glucose



B. Methanol

Fig. 3.5. Biomass accumulation of GS200 and MNNG mutants. Cells of tested strains were pre-grown in YPD medium and then transferred to YNB supplemented with 2% w/v glucose (A) and 1% methanol (B) as sole carbon sources, respectively. The initial OD_{600nm} value of the biomass was 0.2.

3.1.2. Viability assays to monitor autophagy with nitrogen starvation

The physiological roles of autophagy in yeast are diverse and include supplying amino acids that are generated as a result of the degradation of cytosolic materials (Tsukada & Ohsumi, 1993; Onodera & Ohsumi, 2005). These roles became evident from studies analyzing autophagy-deficient mutants. One of the common phenotypes of autophagy-deficient mutants is the loss of viability that occurs during incubation in starvation medium. Therefore, cellular viability is one of the best indicators for assessing the completion of the entire autophagic process (Noda, 2008).

According to our previous results, the inactivation of β -galactosidase occurs in vacuoles through an autophagy pathway (Dmytruk et al., 2021; Zazulya et al., 2023). It was interesting to investigate whether the selected mutants with impaired degradation of β -galactosidase also have defects in the process of autophagy. Thus, the viability of selected mutants was assessed under nitrogen starvation, which triggers the autophagy. The wild-type strain GS200 of *K. phaffii*, and the SMD1163 strain, which has a defective vacuolar proteinase, were used as the positive and negative control, respectively. After 1 day of incubation under nitrogen starvation conditions, the number of colonies on the plate was counted, and relative percentage viability was calculated by comparing it to day 0 (cell culture grown in nitrogen-complete medium) as the baseline. As shown in Figure 3.6., the wild-type strain GS200 exhibited higher viability, with nearly 130 colonies, compared to the negative control SMD1163 strain, which had around 20 colonies after 1 day of nitrogen starvation. This indicates that the viability of SMD1163 is approximately 16% of that of the wild-type strain. The number of colonies in all selected mutant strains was lower than that of the wild-type strain, with MNNG-4 showing no colonies at all after 1 day starvation (Fig.3.6.). The viability of the selected mutants ranged from 37% to 0%, indicating impairments in autophagy.

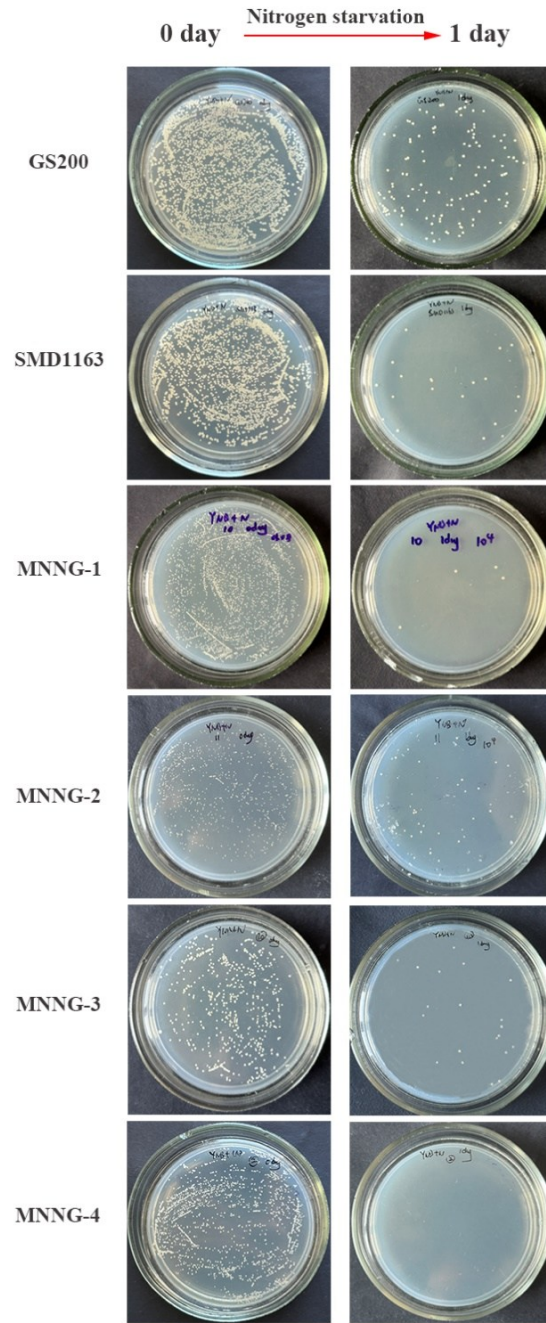


Fig. 3.6. Viability assay to monitor autophagy in GS200, SMD1163 and MNNG mutants. Cells of tested strains were pre-grown in YPD and then transferred to YNB without nitrogen to initiate autophagy. After 1 day of incubation under nitrogen starvation conditions, the number of colonies on the plate was counted, and percentage viability was calculated by comparing it to day 0 (cell culture grown in nitrogen-complete medium) as the baseline. The wild-type strain GS200 of *K. phaffii*, and the SMD1163 strain, which has defective vacuolar proteinases, were used as the positive and negative control, respectively.

The obtained results were confirmed using phloxine B viability assay. Phloxine B, a red dye and fluorescein derivative, is excluded from living yeast cells but penetrates and stains cytosolic materials when the cell dies and the plasma membrane barrier is disrupted (Noda, 2008). This method was used to isolate *S. cerevisiae* autophagy-defective mutants on starvation medium (Tsukada & Ohsumi, 1993). In this study, phloxine B was used to assess the viability of strains subjected to nitrogen starvation and those without treatment. After one day of nitrogen starvation, the mutant strains showed nearly 100% dead cells, similar to the SMD1163 strain, which has defective vacuolar proteinases, while the wild-type GS200 still retained approximately 50% viability (Fig. 3.7). Hence, we can conclude that general autophagy is indeed impaired in the selected mutants.

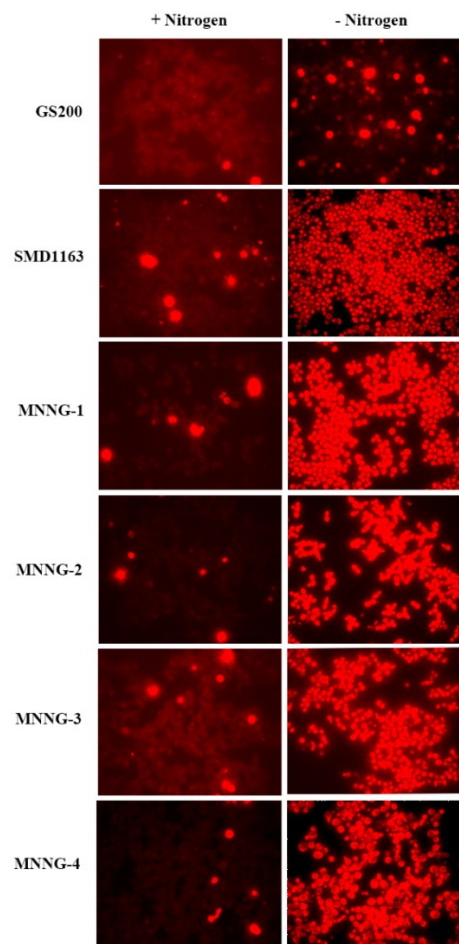


Fig. 3.7. Phloxine B viability assays to monitor autophagy in GS200, SMD1163 and MNNG mutants. Cells of tested strains were pre-grown in YPD and then

transferred to YNB without nitrogen to initiate autophagy. After one day of cultivation, nitrogen-starved cells were stained with phloxine B (final concentration of 2 $\mu\text{g/mL}$). A fluorescence microscope with a red filter was used to estimate stained dead cells. The wild-type strain GS200 of *K. phaffii*, and the SMD1163 strain were used as the negative and positive control, respectively.

As a result of this work, four mutants with impaired degradation of cytosolic β -galactosidase were selected. It is interesting to note that the mutants MNNG-1 and MNNG-3, most probably, also exhibited defects in selective autophagy and pexophagy, while the mutants MNNG-2 and MNNG-4 were characterized solely by defects in selective autophagy of cytosolic β -galactosidase. Genes could be homologous to identified *ATG* genes which are involved in degradation of both β -galactosidase and alcohol oxidase degradation while those involved in selective autophagy of only cytosolic β -galactosidase, most probably, occurred in the new, not identified genes till now. The mutations in the isolated strains will be identified through genome sequencing. The identified genes will help shed light on the mechanisms of selective degradation of cytosolic proteins.

The results of this research presented in this sub-chapter were published by the Ph.D. student in an experimental article:

Zuo M, Dmytruk OV, Dmytruk KV, Kang YQ, Sibirny AA.(2025). Isolation of mutants defective in cytosolic β -galactosidase degradation in the methylotrophic yeast *Komagataella phaffii*. *Cytology and genetics*, Vol. 59, No. 1, pp. 71–78. <https://doi.org/10.3103/S0095452725010104>.

The results of this subsection are presented in Abstract: **Zuo M**, Dmytruk OV, Dmytruk KV , Sibirny AA. Screening for mutant strains with autophagy defects of cytosolic protein β -galactosidase in the methylotrophic yeast *komagatella phaffii*. // 7th Congress of the All-Ukrainian public organization “Ukrainian Society of Cell Biology”

with international representation. 11-13 September, Lviv, Ukraine. – 2024. – P.29.

Zuo M, Dmytruk OV, Dmytruk KV , Sibirny AA. Searching the gene involved in the autophagy of cytosolic and peroxisomal proteins in methylotrophic yeast *Komagatella phaffii*. // Conference of Young Scientists. May 20, Lviv, Ukraine. – 2024. – P.3.

3.2. Development of new dominant selectable markers for the non-conventional yeasts *Ogataea polymorpha*

Genetically engineered approaches are widely used for construction of new strains with modified metabolic pathways and for studying basic aspects of eukaryotic cell biology. The growing potential of such approaches requires additional suitable markers for selection of recombinant strains with desired physiological characteristics.

In this part of the dissertation, we developed novel dominant selectable markers for future applications in metabolic engineering of the yeast *O. polymorpha*.

3.2.1. Selection of *O. polymorpha* strains resistant to aureobasidin

It was reported that resistance of yeast *S. cerevisiae* to aureobasidin was conferred by mutation of *AUR1* gene required for sphingolipid synthesis. Point mutations of *AUR1*, which cause substitution of Leu-137, His-157 or Phe-158 by Phe, Tyr or Tyr, respectively, have been identified as dominant mutations that confer resistance to antibiotic (Heidler & Radding, 1995; Hashida-Okado et al., 1996). In this study, to obtain *O. polymorpha* strains resistant to aureobasidin, two mutant forms of the *AUR1* gene were created by replacing the leucine residue with phenylalanine at position 53 along with replacement of histidine residue by tyrosine at position 72 (L53 CTT→F53 TTT; H72 CAT→Y72 TAT) or substitution of alanine for cysteine at position 156 (A156 GCA→C156 TGT). The substitutions L53F and H72Y in *AUR1* gene of *O. polymorpha* are corresponding to the L137F and H157T in *AUR1* of *S. cerevisiae*, and

the substitution of A156C in *AUR1* of *O. polymorpha* corresponds to the G240C in *AUR1* of *S. pombe* (Fig. 3.8.).

AUR1_Sp	1	MS-A--LSTIKKRRLAACNRASQYKLETSINEMPTFRLRN---TKWSWTHLQYVFLAGNL
AUR1_Sc	1	MANPFSRWFLUSERPPNCHV---ADLETSLDEHQTLLKVQKYKPALSDWV---HYDFLGSIM
AUR1_Op	1	-----
AUR1_Sp	55	IEACIVIESEGFW-GKFGIACLLAIALTVELTRCIFEPAIVITWAILFYSOREFIPRWR
AUR1_Sc	56	IEVFI--TNEPAPWIFKILFYCFLETLFITEATSOFFENALPILTQVALYETSSYFPDORR
AUR1_Op	1	-----MSQFENYGLPVLCWVALFFSSKEIPLQLR
AUR1_Sp	114	PPIWVRVLPITLENILYGSNLSLLISKTHHSILDILAWVPYGVMHYSAPFLISEILFTFAP
AUR1_Sc	114	PPITVKVLPVAVETILYGDNLSDLATSTNSFLDILAWLPYGLFHFAGAPFVVAAILFVFGP
AUR1_Op	30	PPITVKVLPFGMETILYGDNLSCLLASATCTFLDVLAWIPYGLHFSLPFVVAAMIFLAP
AUR1_Sp	174	EGTLPVWARTFGYMNLFGVLIQMA-FPCSPPWYENNYGLEPATYAVRGSPGGLARIDALF
AUR1_Sc	174	ETVILQGYAFAFGYMNLFGVIMONV-FPAAPPWYKILYGLQSANYDMHGSPGGLARIDKLL
AUR1_Op	90	EKTIRILYSFTFGYMNIVGVITQNLLEACAPPWYKVLHGLEKANYSMKGSPGGLARIDKLL
AUR1_Sp	233	GTSIYTDGFSNSPVVFGAFPSLHAGWAMLEALFISHVFPYRRCFYGYVLWLCWCTMYLT
AUR1_Sc	233	GINMYTAFSNSSVIFGAFPSLHSGCATMEALFFCYCFPKLPFLFIAYVCWLWSTMYLT
AUR1_Op	150	GIDMYTSAFINSPLIFGALPSLHSCASLDALWLCYLFPRETPLWCYVCWLWSTMYLT
AUR1_Sp	293	HHYFVDLVGGMCLAITCEVFAQKLRLPQLQTGKILRWYEFVIEGHGISEKTSNSLARTG
AUR1_Sc	293	HHYFVDLMAGSVLSYVIEQYTKYTHLPVIVDTSLFCRWSYTSIEKYDISKS---DPLAADS
AUR1_Op	210	HHYFVDLTFGCSLAIAAEYSTKLKAG-KLPINNKFRCWSYCHLDLEDEVKE---DPLVTDN
AUR1_Sp	353	SPYPLGRDSFTQNPNAVAFMSGNNMELANTLHEWSVGSSEEFLEFSPAADLIDRPASTT
AUR1_Sc	350	-NDI-----ESVPLSNLEHDEDENMAD-EE--SVSPSLFEGSTSVS
AUR1_Op	266	-SEI-----IEDD--LDVLDV-----LFEDTSIEM
AUR1_Sp	413	SSIIFD-ASHLP*----
AUR1_Sc	387	RSSATSIISLGVKRA*
AUR1_Op	289	RDIS-----TA*

Fig. 3.8. The alignment of *AUR1* gene from several yeast species. Conserved sequences are on black background. Amino acid residues substituted in *Ogataea polymorpha* gene during this study are marked in red. Abbreviations of yeasts species: Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Op, *O. polymorpha*.

The retrieval of *AUR1* gene sequence was performed using the *O. polymorpha* NCYC495 leu1.1v2.0 database (<http://genome.jgi-psf.org/Hanpo2>). The genomic DNA of *O. polymorpha* strain NCYC495 was used as a template to amplify the *AUR1*

gene coding region with L53F and H72Y substitutions, together with its own promoter and terminator using primers OK219/OK220 and OK221/OK222. The resulting fragments were fused by overlap-PCR using primers OK219 and OK222. The *AUR1* gene coding region with A156C substitution along with its own promoter and terminator was amplified from genomic DNA of *O. polymorpha* NCYC495 strain using primers OK219/OK223 and OK224/OK222. The resulting fragments were coupled by overlap-PCR using primers OK219/OK222. Amplified fragments (1.7 kb) were digested with *Eco*RI and cloned into an *Eco*RI-linearized pUC57 vector. As a result, plasmids pUC57/AUR1-1 and pUC57/AUR1-2 were constructed (Fig.3.9.).

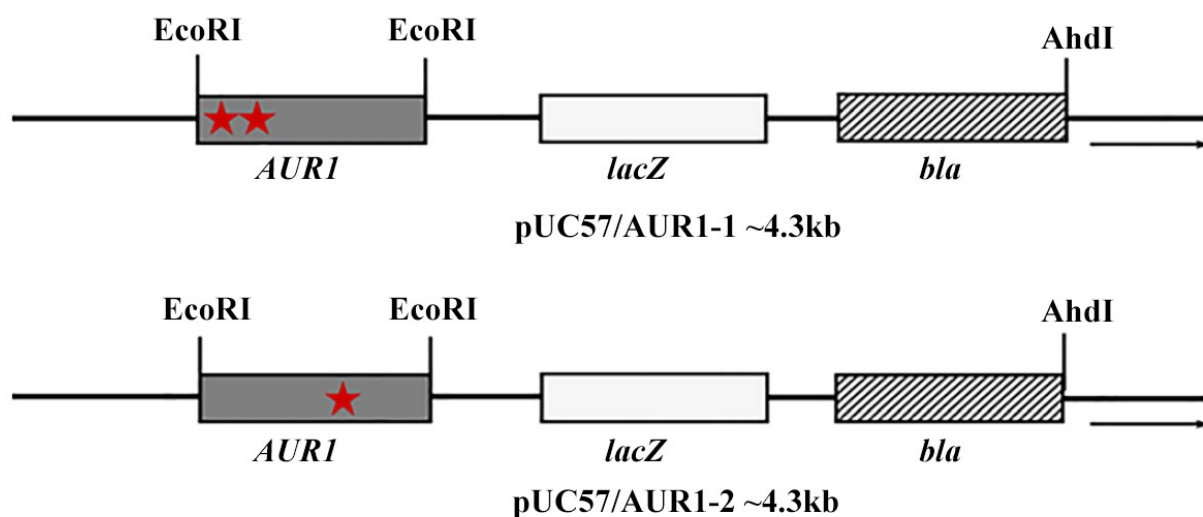


Fig.3.9. Linear schemes of plasmids pUC57/AUR1-1 and pUC57/AUR1-2. The *AUR1* gene is designed as grey box. The introduced point-mutations in *AUR1* gene are marked with asterisks. Gene *bla* for selection of bacterial transformants on the ampicillin containing medium and fragment of gene *lacZ* from *Escherichia coli* are designed as dashed and white boxes, respectively.

Constructed plasmids after linearization with *Nde*I restriction endonuclease were introduced into *O. polymorpha* wild-type strain by electroporation. Transformants were selected on YPD medium supplemented with aureobasidin (0.3 mg/L). As a result of transformation with plasmid pUC57/AUR1-2, we have obtained colonies of transformants on the third day of cultivation. The frequency of transformation was 40

transformants per microgram of DNA (Fig. 3.10A). After transformation with the plasmid pUC57/AUR1-1 no colonies on the medium with aureobasidin were observed.

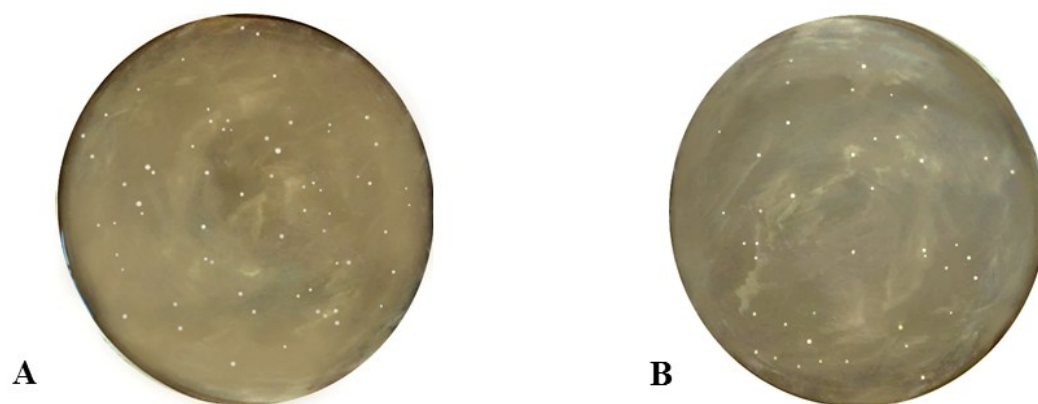


Fig.3.10. A) Colonies obtained on YPD medium supplemented with aureobasidin (0.3 mg/L) on the third day after transformation of *O. polymorpha* wild-type strain using plasmid pUC57/AUR1-2 (1.5 μ g of DNA per sample). B) Colonies obtained on YNB medium supplemented with mycophenolic acid (40 mg/L) on the fifth day after transformation of *O. polymorpha* wild-type strain using plasmid pUC57/IMH3 (2 μ g of DNA per sample).

The results indicate that the mutant form of the *AUR1* gene with replacement at position 156 can be used as a marker for selection of aureobasidin-resistant transformants in *O. polymorpha*.

3.2.2. Construction of *O. polymorpha* strains resistant to mycophenolic acid

In the yeast *C. famata*, the introduction of additional copies of *IMH3* gene increased the resistance to mycophenolic acid. The *IMH3* gene was successfully used as a dominant selective marker in clinical isolates of *C. famata*, *C. albicans* and *C. tropicalis* (Dmytruk et al., 2011). In this study, for construction of a plasmid with a gene conferring resistance to mycophenolic acid, the genomic DNA of *O. polymorpha* NCYC495 was used as a template to amplify the coding region of *IMH3* gene with its own promoter and terminator using primers OK225/OK227. The amplified fragment

(3.0 kb) was digested with KpnI and cloned into KpnI-linearized pUC57 vector. The constructed plasmid was named pUC57/IMH3 (Fig.3.11).

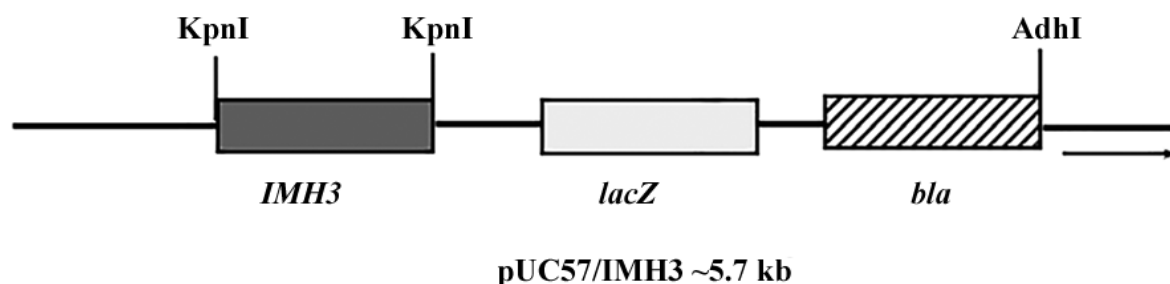


Fig.3.11. Linear scheme of plasmid pUC57/IMH3. The *IMH3* gene conferring resistance to mycophenolic acid is designed as grey box. Gene *bla* for selection of bacterial transformants on the ampicillin containing medium and fragment of gene *lacZ* *Escherichia coli* are designed as dashed and white boxes, respectively.

The constructed plasmid after linearization by NdeI was introduced into *O. polymorpha* wild-type strain by electroporation. Transformants were selected on YNB medium with mycophenolic acid (40 mg/L) on the fifth day of cultivation. The frequency of transformation was 20 transformants per microgram of DNA (Fig.3.10B).

Here, we conclude that the introduction of an additional copy of *IMH3* gene into genome of *O. polymorpha* wild-type strain was sufficient to obtain transformants resistant to mycophenolic acid.

3.2.3. Construction of *O. polymorpha* strains overexpressing *TAL1*, *TKL1* and *AOX1*

At the next stage of this study, *IMH3* gene was used as selectable marker for construction of a plasmid for overexpression of *TAL1*, *TKL1* and *AOX1* genes, encoding cytosolic transaldolase, cytosolic transketolase and peroxisomal alcohol oxidase, respectively.

For simultaneous overexpression of *TAL1* and *TKL1* genes, a BamHI-restriction fragment bearing the *GAP1* promoter fused with the ORF of the *TAL1* gene was isolated from the plasmid pTaZr and cloned into the NarI-linearized and dephosphorylated vector pTkZr (Kurylenko et al., 2018). The resulting plasmid was named pUC57/TAL1/TKL1. The 4.9-kb DNA fragment harbouring both genes was PCR amplified with the primers OK248 and OK249, from the plasmid, pUC57/TAL1/TKL1. Primers OK250 and OK251 were used for amplification of the plasmid pUC57/IMH3 carrying *IMH3* gene as selectable marker. The resulting fragments were fused by Gibson Assembly and introduced into genome of *E. coli* Dh5 α by electroporation. The obtained colonies were tested for the presence of a cassette for expression of *TAL1* and *TKL1* genes using primers OK 248 and OK 249. The resulting plasmid was named pUC57/TAL1/TKL1/IMH3. For constitutive expression of *AOX1* gene, the 2.9-kb DNA fragment harbouring this gene under control of strong constitutive promoter *GAP1* was PCR amplified with the primers OK252 and OK253, from the plasmid pUC57/AOX1. Primers OK254 and OK255 were used for amplification of the plasmid pUC57/TAL1/TKL1/IMH3. The obtained colonies were tested for the presence of a cassette for expression of *AOX1* gene using primers OK 252 and OK 253. The resulting plasmid was named pUC57/IMH3/TAL1/TKL1/AOX1 (Fig.3.12.).

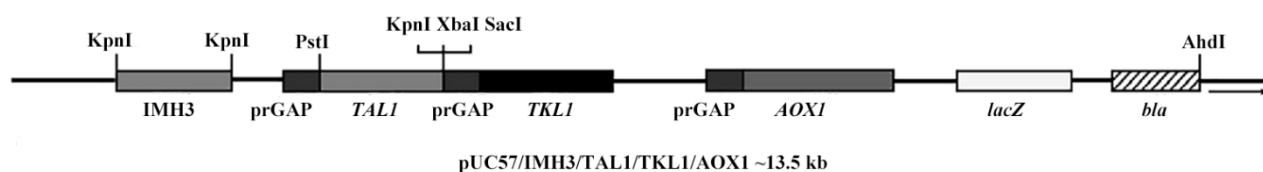


Fig.3.12. Linear scheme of plasmid pUC57/IMH3/TAL1/TKL1/AOX1. The *IMH3* gene conferring resistance to mycophenolic acid, *TAL1* and *AOX1* are designed as grey boxes. The *TKL1* is designed as a black box. Gene *bla* for selection of bacterial transformants on the ampicillin containing medium and fragment of gene *lacZ* *Escherichia coli* are designed as dashed and white boxes, respectively.

The constructed plasmids were introduced into genome of *O. polymorpha* recombinant strain BEP/ Δ cat8 obtained by methods of metabolic engineering (Kurylenko et al., 2018). Transformants were selected on YNB medium with mycophenolic acid (40 mg/L) on the fifth day of cultivation. Selected transformants were examined by diagnostic PCR using pairs of primers OK256/OK257, OK256/OK259 and OK256/OK260.

The obtained *O. polymorpha* recombinant strains overexpressing TAL1, TKL1 and AOX1 genes were studied for their ethanol production during xylose alcoholic fermentation as compared to the parental strain (Fig.3.13.). The resulting strain BEP/ Δ cat8/TAL1/TKL1/AOX1 exhibited higher ethanol production from xylose fermentation, accumulating 39% more ethanol relative to the parental strain BEP/ Δ cat8 at 43 h.

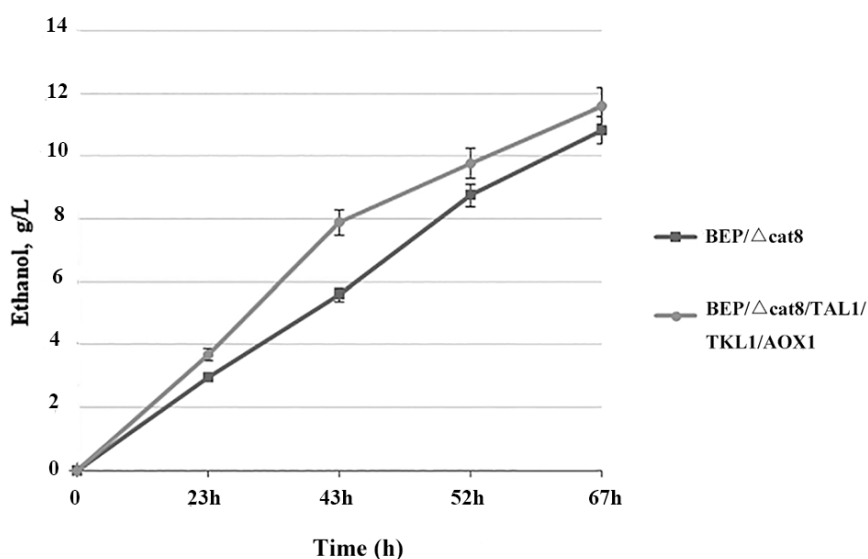


Fig.3.13. Ethanol production during xylose alcoholic fermentation by *Ogataea polymorpha* strain overexpressing *TAL1*, *TKL1* and *AOX1* genes relative to the parental BEP/ Δ cat8 strain. Data are shown as mean of three independent experiments. Bars in the figure indicate ranges of the standard deviation.

The results of this research presented in this sub-chapter were published by the Ph.D. student in an experimental article:

Bratiichuk D, Kurylenko O, Vasylyshyn RV, **Zuo M**, Kang YQ, Dmytruk K, Sibirny AA. (2020). Development of new dominant selectable markers for the nonconventional yeasts *Ogataea polymorpha* and *Candida famata*. *Yeast*, 37(9-10):505-513.

3.3. The role of hexose transporter-like sensor *HXS1* and transcription activator involved in carbohydrate sensing *AZF1* in xylose and glucose fermentation in the thermotolerant yeast *Ogataea polymorpha*

In recent years, it has been found that transcription factors and sugar transporters play an important role in regulation of xylose alcoholic fermentation. However, the role of sugar sensors in the process of xylose fermentation remains unexplored. The hexose transporter-like sensor gene *HXS1* in *O. polymorpha* was described earlier, which was not involved in glucose repression or catabolite inactivation though its deletion led to significantly impaired transient transcriptional repression in response to fructose (Stasyk et al., 2008). The role of this gene in xylose metabolism and in glucose and xylose alcoholic fermentation has not been studied. The *S. cerevisiae* *AZF1* gene was identified as sugar- and oxygen-responsive transcription factor. The *S. cerevisiae* *azf1Δ* mutant cannot grow on glycerol. This transcription factor as [*AZF1*⁺] conformer was shown to form a prion (Newcomb et al., 2002; Stein et al., 1998; Stewart et al., 2021). Nevertheless, the role of the *AZF1* gene in xylose fermentation in the native xylose-metabolizing yeasts has never been investigated previously.

In this part of the dissertation, we investigated the roles of two genes in *O. polymorpha*: the hexose sensor gene *HXS1* and the gene *AZF1*, which encodes a homolog of the *S. cerevisiae* transcription factor with sensing properties. The functions of both genes in xylose and glucose alcoholic fermentation were elucidated.

3.3.1. Overexpression of the hexose transporter-like sensor gene *HXS1* and the transcription activator *AZF1*, which is involved in carbohydrate sensing, in both the wild-type and the best recombinant strain of *O. polymorpha* with enhanced ethanol production

To further increase the efficiency of alcoholic xylose fermentation, the corresponding genes were overexpressed in the genome of wild-type and recombinant strain EthOH-/XYL1m/XYL2/XYL3/BrPA/cat8Δ of *O. polymorpha*, a previously obtained advanced ethanol producer from xylose isolated by our laboratory. This strain is abbreviated as BEP/Δcat8 hereinafter in this dissertation (Ruchala et al., 2020).

Vectors for overexpression of the genes *HXS1* and *AZF1* were constructed as described below. To overexpress the *HXS1* and *AZF1* gene under control of the strong constitutive *GAP1* promoter of the glyceraldehyde-3-phosphate dehydrogenase gene. *O. polymorpha* genome database (<https://mycocosm.jgi.doe.gov/Hanpo2/Hanpo2.home.html>) was used for retrieval of the *HXS1* and *AZF1* genes sequences. The *O. polymorpha* *AZF1* gene homolog is much shorter than its counterpart from *S. cerevisiae* (1911 vs 2745 bp, respectively); the deduced proteins exhibit 26.5% identity and 34.7% similarity. Vector pUC19-GAPpr-GAPt-natNT2 was obtained as described by Semkiv (Semkiv et al., 2019). The 574 bp DNA fragment corresponding to the strong constitutive promoter of the *GAP1* gene was amplified from genomic DNA of *O. polymorpha* strain NCYC495 and 209 bp DNA fragment corresponding to the terminator of the *GAP1* gene was amplified from the same matrix. Then, these two fragments were joined by overlap-PCR, digested with restriction endonucleases BamHI and SalI, and cloned into the corresponding sites of the plasmid pUC19. The gene natNT2 conferring resistance to nourseothricin was amplified from the plasmid pRS41N and used as a selection marker (Taxis & Knop, 2006).

The 1917 bp long DNA fragment corresponding to the ORF of the gene *HXS1* was amplified from the genomic DNA of NCYC495 strain using primers SM166 and SM167, digested with restriction endonucleases XbaI and NotI, and cloned into the

corresponding sites of the plasmid pUC19-GAPpr-GAPt-natNT2. The resulting vector was named pUC19-GAPpr-HXS1-GAPt-natNT2, abbreviated as pHXS1 in this dissertation (Fig.3.14).

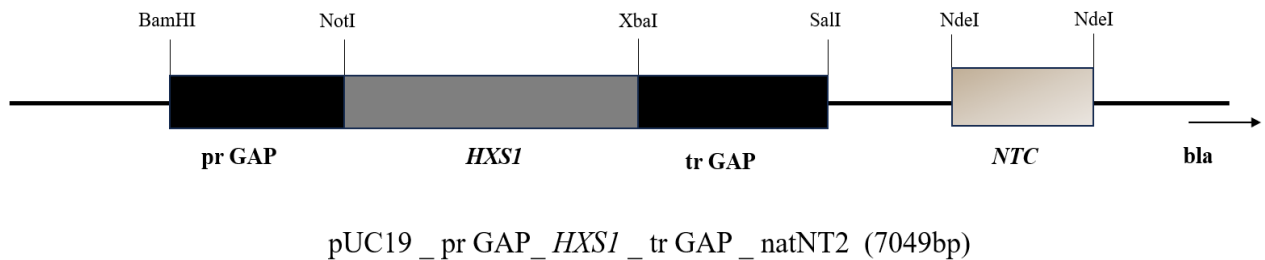


Fig. 3.14. Linear diagram of the plasmid vector pUC19_prGAP_*HXS1*_trGAP_natNT2: prGAP - promoter of the glyceraldehyde-3-phosphate dehydrogenase gene; trGAP - terminator of the glyceraldehyde-3-phosphate dehydrogenase gene; NTC - gene for resistance to nourseothricin; *HXS1* - hexose transporter-like sensor gene in *O. polymorpha*.

The 1911 bp DNA fragment corresponding to the ORF of the gene *AZF1* was amplified from the genomic DNA of *O. polymorpha* strain NCYC495 strain using primers AZF1_F and AZF1_R, digested with restriction endonuclease NotI and cloned into the corresponding site of the pre-dephosphorylated pUC19-GAPpr-GAPt-natNT2 vector. The resulting vector was named pUC19-GAPpr-*AZF1*-GAPt-natNT2, abbreviated as pAZF1 in this dissertation. Figure 3.15 illustrates this plasmid construction map. All plasmids in this study were verified by hydrolysis with restriction endonucleases and PCR assay.

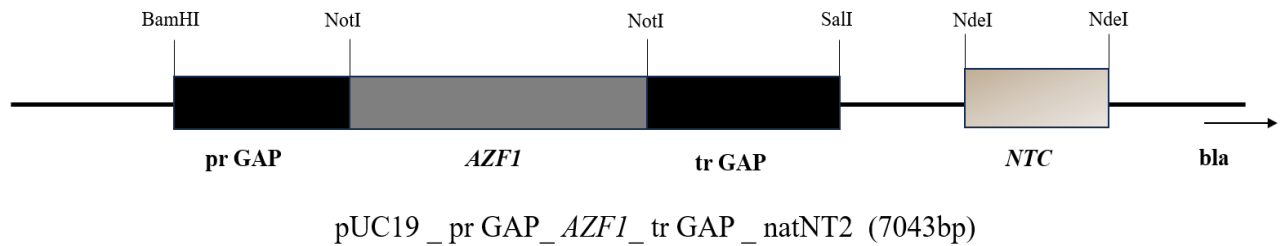


Fig. 3.15. Linear diagram of the plasmid vector pUC19_prGAP_*AZF1*_trGAP_natNT2: prGAP - promoter of the glyceraldehyde-3-phosphate dehydrogenase gene; trGAP - terminator of the glyceraldehyde-3-phosphate dehydrogenase gene; NTC - gene for resistance to nourseothricin; *AZF1* - transcription activator gene in *O. polymorpha*.

To obtain recombinant strains with *HXS1* or *AZF1* overexpression, vectors pHXS1 and pAZF1 were linearized with restriction endonuclease BamHI and used for transformation of *O. polymorpha* best ethanol producer strains BEP/ Δ cat8 and wild-type strain NCYC495 by electroporation. Plasmids were randomly integrated into the genome of these two strains. Transformants were selected on a solid YPD medium supplemented with 150 mg/L of nourseothricin. Selected transformants were stabilized through a 1-day cultivation in non-selective media, followed by transfer to selective media (YPD supplemented with 150 mg/L of nourseothricin). Then, the four transformants were examined by diagnostic PCR using primers Ko1036/SM167 for BEP/ Δ cat8/*HXS1* and WT/*HXS1*, primers RV131/RV132 for BEP/ Δ cat8/*AZF1* and WT/*AZF1*.

3.3.2. Characterization of alcoholic fermentation of the obtained *O. polymorpha* recombinant strains with enhanced expression of *HXS1* and *AZF1* genes

Stable recombinant strains with overexpression of the *HXS1* and *AZF1* genes were used to investigate their ability to produce ethanol under limited aeration conditions (140 rpm). The cultivation was carried out in liquid mineral medium YNB containing 10% xylose and 10% glucose, at 37 °C.

Heterologous expression of the *HXS1* gene was achieved under the regulation of the constitutive *GAP1* promoter derived from the glyceraldehyde-3-phosphate dehydrogenase gene in strain BEP/ Δ cat8. The results demonstrated that the *HXS1* activated the production of ethanol from glucose-medium. Specifically, after 72 hours of fermentation, the strain BEP/ Δ cat8/*HXS1* produced 37 g/L of ethanol, while parental strain BEP/ Δ cat8 only produced 34 g/L of ethanol. Additionally, the overexpression of *HXS1* also activated ethanol production from xylose medium. After 72 hours of fermentation, strain BEP/ Δ cat8/*HXS1* generated 13 g/L of ethanol, in contrast to parental strain BEP/ Δ cat8 which produced only 9.1 g/L of ethanol (Fig. 3.16a, 3.16c and Table 3.1). The biomass in both glucose and xylose media was shown to be higher compared to the parental strain (Fig. 3.16b, 3.16d) indicating superior growth characteristics of BEP/ Δ cat8/*HXS1* in both carbon sources. Of note, this sensor is predicted to induce expression of functional hexose transporters in *O. polymorpha* (Stasyk et al., 2008; 2012).

Subsequently, *HXS1* was overexpressed in the wild-type strain NCYC495, designated as WT/*HXS1*. The engineered strain demonstrated enhanced ethanol production in xylose medium (0.83 g/L versus 0.49 g/L in wild-type), while maintaining comparable performance in glucose medium (Fig. 3.17a, 3.17b and Table 3.1). Substrate utilization revealed accelerated consumption of both xylose and glucose by WT/*HXS1* compared to the wild-type strain (Fig. 3.17c, 3.17d and Table 3.1), although cells biomass yields remained comparable across both strains and carbon sources (Fig. 3.17e, 3.17f and Table 3.1). These observations suggest that the *HXS1*

sensor may regulate xylose metabolism through modulation of intracellular enzymatic pathways involved in xylose catabolism and fermentation. Thus, its overexpression potentially alleviates metabolic bottlenecks in xylose utilization. Collectively, these findings demonstrate that *HXS1* overexpression on the background of both wild-type and BEP/ Δ cat8 strains contributes to enhanced ethanol production efficiency.

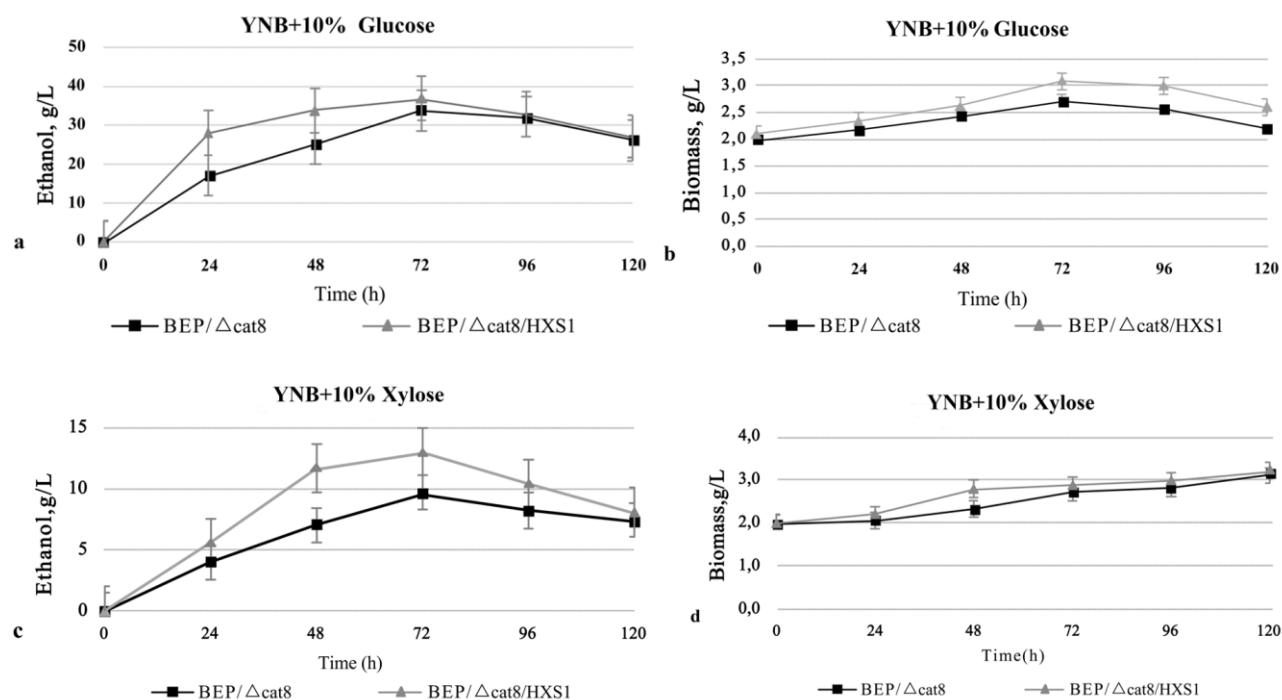


Fig. 3.16. Ethanol production (a, c) and biomass accumulation (b, d) during 10% glucose and 10% xylose alcoholic fermentation of *O. polymorpha* wild type BEP/ Δ Cat8 and recombinant strains with *HXS1* gene overexpression at 37°C. Data are shown as mean of three independent experiments.

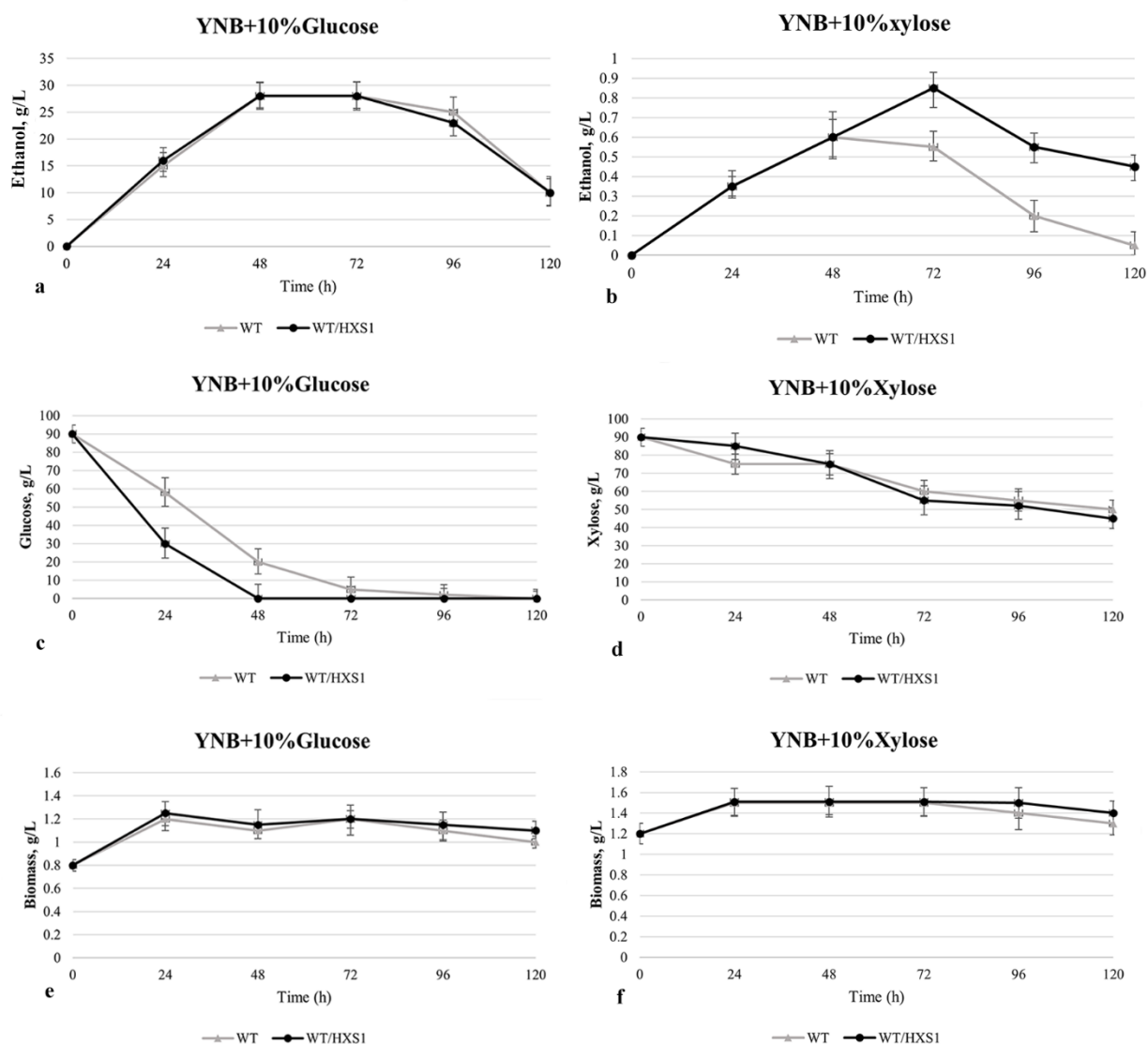


Fig. 3.17. Ethanol production (a, b), sugar consumption (c, d) and biomass accumulation (e, f) during 10% glucose and 10% xylose alcoholic fermentation of *O. polymorpha* wild type and recombinant strains with *HXS1* gene overexpression at 37 °C. Data are shown as means of three independent experiments.

The overexpression of *AZF1* significantly enhanced alcoholic fermentation of xylose in the wild-type (WT) genetic background, resulting in a two-fold increase in ethanol production after 48h in WT/*AZF1* recombinant strain (Fig.3.18b). This enhancement was also analysed glucose fermentation (Fig.3.18a). Additionally, when *AZF1* was overexpressed in the BEP/ Δ cat8 background (an advanced ethanol producer), ethanol accumulation increased by approximately 10% in glucose medium (Fig.3.18a). Moreover, in the xylose medium, ethanol production was enhanced by

more than 30%, reaching 11 g/L after 72 hours as shown in Fig. 3.18b, with detailed fermentation parameters presented in Table 3.1.

Furthermore, substrate utilization was substantially improved in the recombinant strain. The BEP/ Δ cat8/*AZF1* strain exhibited enhanced sugar consumption rates compared to the parental strain, achieving 90% glucose utilization and 70% xylose utilization by 72h (Fig.3.18c and Fig.3.18d). Biomass accumulation was also significantly improved in the recombinant strain. During glucose fermentation, BEP/ Δ cat8/*AZF1* reached maximum biomass at 48h. In xylose fermentation, the strain demonstrated consistently higher biomass yields throughout the fermentation period, reaching a peak optical density (OD₆₀₀) of 3.0 after 120h (as shown in Fig.3.18e and Fig.3.18f).

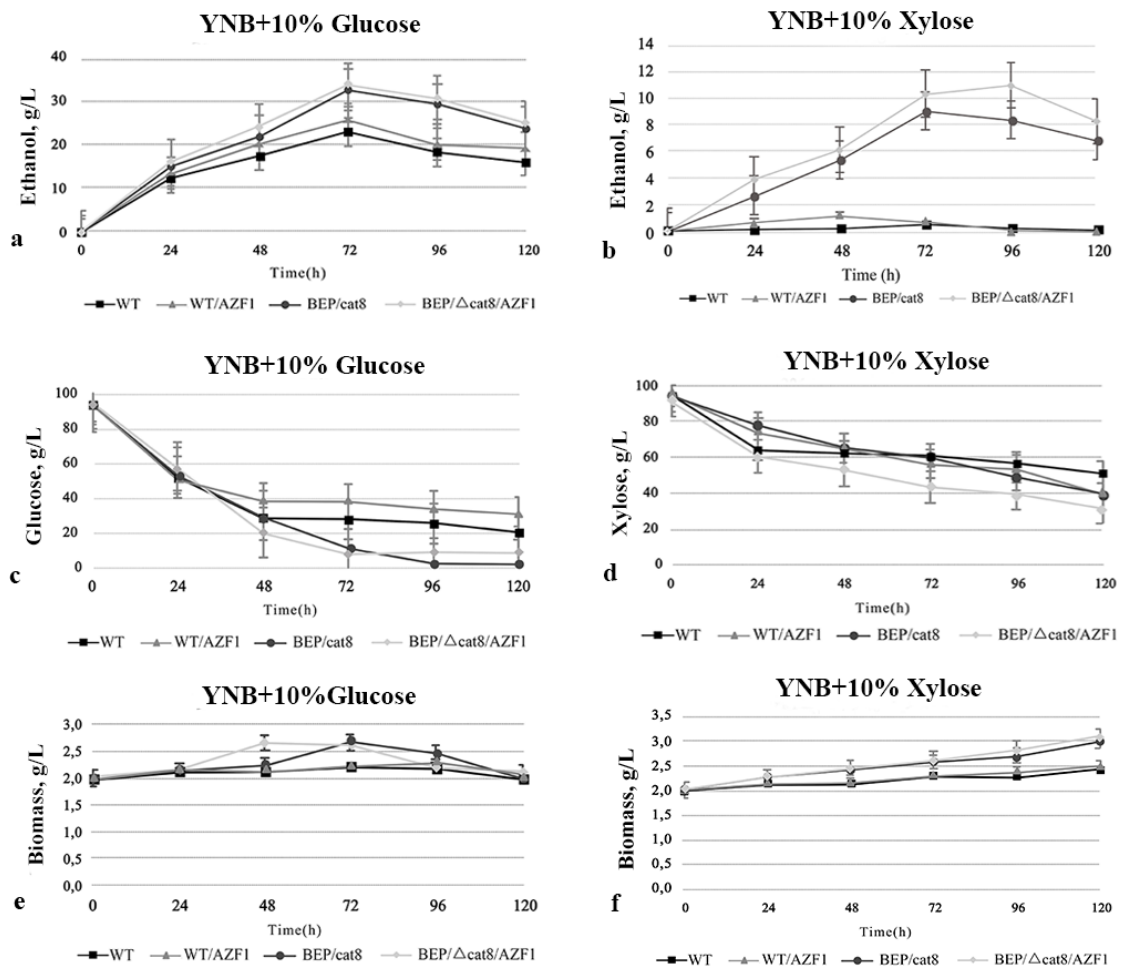


Fig. 3.18. Ethanol production (a, b), sugar consumption (c, d) and biomass accumulation (e, f) during 10% glucose and 10% xylose alcoholic fermentation of *O. polymorpha* wild type, BEP/ Δ cat8 and recombinant strains with *AZF1* gene overexpression at 37 °C. Data are shown as means of three independent experiments.

In addition, growth tests were conducted in the background of wild-type and BEP/ Δ cat8 strains with *AZF1* overexpression. The results demonstrated that throughout all tested stages, both BEP/ Δ cat8/*AZF1* and WT/*AZF1* strains exhibited superior growth on xylose and glucose compared to BEP/ Δ cat8 and WT strains. These findings indicate that *AZF1* overexpression can enhance the growth compared to the wild-type and BEP/ Δ cat8 strains. (Fig. 3.19a and 3.19b).

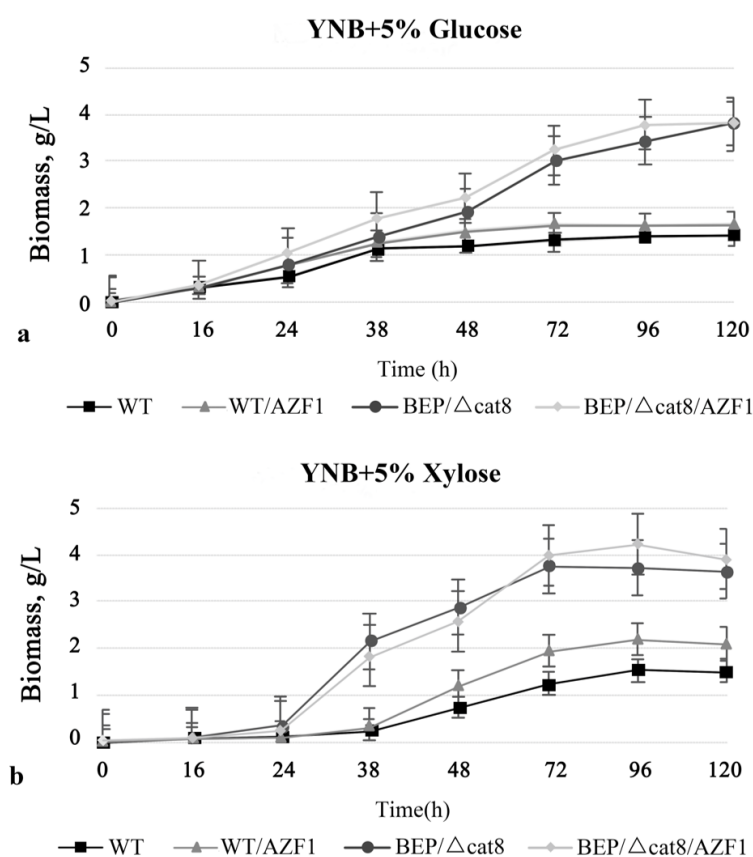


Fig. 3.19. Growth test of the *O. polymorpha* wild type, BEP/ Δ cat8 and recombinant strains with *AZF1* gene overexpression on different media, cultivation at 37 °C. Data are shown as means of three independent experiments.

Ethanol yield, ethanol specific production rate and ethanol productivity during xylose alcoholic fermentation for all studied recombinant strains are also listed in Table 3.1. The highest ethanol yield of 354 mg per g of consumed xylose was observed in strain BEP/ Δ cat8/*AZF1*, whereas strain BEP/ Δ cat8/*HXS1* has shown the highest ethanol specific production rate (63mg/g biomass/h) and ethanol productivity (181 mg/L/h).

Table 3.1.

Main parameters of xylose fermentation at 37 °C by the tested *O. polymorpha* strains with *HXS1* and *AZF1* genes overexpression

Strain	Ethanol (g/L)	Ethanol yield (mg/g of consumed xylose)	Ethanol specific production rate (mg·g biomass ⁻¹ ·h ⁻¹)	Ethanol productivity (mg·L ⁻¹ ·h ⁻¹)
	0.49±0.03	15±2	4±1	7±1
WT/ <i>AZF1</i> ^a	1.20±0.05	41±8	10±3	25±2
WT/ <i>HXS1</i> ^a	0.83±0.07	25±4	8±1	12±3
BEP/ Δ cat8 ^b	9.10±0.10	154±20	49±11	126±22
BEP/ Δ cat8/ <i>AZF1</i> ^c	11.02±0.11	354±31	40±19	114±19
BEP/ Δ cat8/ <i>HXS1</i> ^b	13.04±0.12	352±29	63±17	181±27

^a Data of ethanol yield (mg/g of consumed xylose), ethanol specific production rate (mg·g biomass⁻¹·h⁻¹), ethanol productivity (mg·L⁻¹·h⁻¹) and ethanol concentration (g/L) are represented on YNB medium supplemented with 10% of xylose on 48 h of fermentation

^b 72h of fermentation

^c 96h of fermentation

The specific activities of key enzymes involved in xylose metabolism and ethanol fermentation, including xylose reductase (XR), xylitol dehydrogenase (XDH), pyruvate decarboxylase (PDC), and alcohol dehydrogenase (ADH), were analyzed in the transformants. The metabolic pathway of xylose utilization requires its initial conversion to xylulose and subsequent phosphorylation to xylulose 5-phosphate for entry into the pentose phosphate pathway (PPP). This conversion is catalyzed sequentially by xylose reductase (XR) and xylitol dehydrogenase (XDH), facilitating the transformation of xylose to xylitol and subsequently to xylulose. In the ethanologenic pathway, pyruvate decarboxylase (PDC) serves as a crucial enzyme catalyzing the decarboxylation of pyruvate to acetaldehyde and CO₂, followed by the reduction of acetaldehyde to ethanol via alcohol dehydrogenase (ADH) (Ruchala J. et al., 2020).

To elucidate the metabolic impact of gene overexpression, enzymatic activities were assessed in the parental strains and the recombinant strains BEP/ Δ cat8/*HXS1*, BEP/ Δ cat8/*AZF1*, WT/*AZF1* and WT/*HXS1* during xylose fermentation at 37 °C. The results demonstrated that overexpression of either *HXS1* or *AZF1* in the BEP/ Δ cat8 genetic background significantly enhanced the specific activities of xylitol dehydrogenase and pyruvate decarboxylase (Table 3.2). Moreover, consistent with previous observations, the BEP/ Δ cat8 strain exhibited elevated specific activities of these enzymes compared to the wild-type strain. These findings suggest that the enhanced ethanol production observed in strains overexpressing *HXS1* and *AZF1* can

be attributed to the increased activities of xylitol dehydrogenase and pyruvate decarboxylase. However, the underlying molecular mechanisms governing these metabolic alterations remain to be elucidated and warrant further investigation.

In conclusion, our findings demonstrate that the *HXS1* sensor plays a crucial role not only in glucose utilization but also in xylose metabolism and alcoholic fermentation of both sugars. Similarly, the transcription factor *AZF1*, a homolog of *S. cerevisiae* *AZF1*, significantly enhances ethanol production from both glucose and xylose, with particularly pronounced effects on xylose fermentation in *O. polymorpha*. Collectively, these results establish that the genes encoding the hexose sensor *HXS1* and the carbohydrate-sensing transcription factor *AZF1* in the thermotolerant yeast *O. polymorpha* represent promising molecular tools for further metabolic engineering. The enhanced ethanol yields achieved through overexpression of these genes highlight their potential utility in developing more efficient strains for industrial-scale lignocellulosic bioethanol production.

Table 3.2.

Activities of the enzymes xylose reductase (XR), xylitol dehydrogenase (XDH), alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) in the tested *O. polymorpha* strains with the *HXS1* and *AZF1* genes overexpression. Activities were measured on 72 h of fermentation on xylose at 37 °C

Strain	Activity U/ mg of protein			
	XR	XDH	ADH	PDC
NCYC 495 WT	0.99±0.05	0.62±0.03	0.97±0.05	0.33±0.02
WT/AZF1	0.89±0.04	0.50±0.02	0.76±0.03	0.53±0.02
WT/HXS1	0.85±0.04	0.67±0.03	0.81±0.03	0.37±0.02
BEP/ Δ cat8	0.84±0.04	1.05±0.05	1.07±0.05	0.69±0.03

BEP/ Δ cat8/ AZF1	0.87 \pm 0.04	1.42 \pm 0.06	0.70 \pm 0.03	0.92 \pm 0.05
BEP/ Δ cat8/ HXS1	0.87 \pm 0.04	1.47 \pm 0.07	1.06 \pm 0.05	0.83 \pm 0.04

The results of this subsection are highlighted as part of the work in the manuscript and abstract:

Semkiv MV, Ruchala J, Tsaruk AY, Zazulya AZ, Vasylyshyn RV, Dmytruk OV, **Zuo M**, Kang YQ, Dmytruk KV, Sibirny AA. (2022). The role of hexose transporter-like sensor hxs1 and transcription activator involved in carbohydrate sensing azf1 in xylose and glucose fermentation in the thermotolerant yeast *Ogataea polymorpha*. *Microb Cell Fact.*, 21, 162. Q1. doi: 10.1186/s12934-022-01889-z.

Zuo M, Vasylyshyn RV, Sibirny AA. The role of transcription activator involved in carbohydrate sensing azf1 in xylose and glucose fermentation in the thermotolerant yeast *ogataea polymorpha*. // Conference of Young Scientists of Institute of Cell Biology, June 8, Lviv, Ukraine. – 2022. – P.19.

CHAPTER 4

ANALYSIS AND GENERALIZATION OF THE RESULTS

One of the most striking achievements of modern biotechnology is the rapid development of recombinant protein production for various economic and medical applications. Methylophilic yeasts, defined as organisms capable of utilizing methanol as a sole carbon source, have emerged as highly effective expression systems for both native and heterologous proteins of industrial importance.

K. phaffii, as a typical representative of methylophilic yeast, offers significant advantages over other systems for heterologous protein production. These advantages include strong and strictly regulated promoters of genes encoding methanol metabolism enzymes, high biomass production, and efficient secretion and yield of target proteins.

The key to creating strains which can overproduce proteins of industrial importance lies not only in producing strains with high levels of target protein synthesis, but also in maximum reducing the degradation of these recombinant proteins in the cytosol. However, when this yeast is transferred from methanol to glucose-containing medium, most enzymes involved in methanol utilization are repressed at the transcriptional level, while the enzymes already present in the cell undergo rapid degradation and proteolysis, and the cell's carbon metabolism switches to a glycolytic pathway (Dmytruk et al., 2021).

In a eukaryotic cell, there are two types of protein degradation: proteasomal degradation occurring in the cytoplasm which typically recognizes and degrades ubiquitinated individual proteins, predominantly short-lived ones; and autophagy occurring in a special organelle, the lysosome, or vacuole. This process involves not only proteins but also intracellular organelles or other macromolecular compounds

(Mizushima & Komatsu, 2011; Sibirny, 2016). Therefore, maintaining the desired stability of heterologous synthesized proteins in the cytosol remains an unsolved problem. According to the literature reports, increased expression of recombinant proteins in *K. phaffii* is possible by inhibiting proteolytic degradation (Dmytruk et al., 2020). Therefore, to study the mechanisms of cytosolic protein autophagic degradation in *K. phaffii*, it is important to isolate mutants specifically affected in this process.

Until now, 42 ATG genes have been identified in yeast, and the products of these genes are involved in autophagy pathways (Mizushima & Komatsu, 2011). Methylophilic yeasts serve as excellent model organisms for studying the mechanisms of autophagy. Some genes involved in autophagy and pexophagy, such as *PEX3*, *PEX14*, *HXS1*, *ATG8*, *ATG26*, *ATG28*, *ATG35*, *ATG30*, and *GSSI*, have been identified in *O. polymorpha* and *K. phaffii* and most of these genes are highly conserved in eukaryote organisms (Klionsky et al., 2003; Geng & Klionsky, 2008; Farré et al., 2008; Polupanov & Sibirny, 2014; Manjithaya et al., 2010). The selective degradation of formaldehyde dehydrogenase, formate dehydrogenase and fructose-1,6-bisphosphatase has also been identified as occurring in the autophagy pathway in our previous study (Dmytruk et al., 2021; Dmytruk et al., 2020). Additionally, previous studies in *K. marxianus* have demonstrated that strains with impaired autophagy, caused by a mutation in the Mtc6p protein, exhibited significantly enhanced heterologous protein production, with protein yields increasing up to ten-fold compared to the wild type strain, suggesting that modifying autophagy pathways could be a promising strategy for improving heterologous protein production in yeast (Y. Liu et al., 2018). However, although there are some research on the relationship between autophagy deficiency and recombinant protein production, the mechanisms of degradation of these and other intrinsic cytosolic proteins, as well as recombinant heterologous proteins with cytosolic localization in methylophilic yeast (such as *K. phaffii*), remain unclear. Therefore, the investigation of the mechanisms of cytosolic protein degradation is of great fundamental and applied importance.

To explore this problem, a simple method was developed in our previous study to isolate cytosolic enzyme such as β -galactosidase activity degradation mutants. In this method, a vector for expressing the β -galactosidase gene *LAC4* from *Kluyveromyces lactis* fused with green fluorescent protein (GFP) under the control of methanol-regulated FLD1 promoter was constructed and transformed into *K. phaffii* (Dmytruk et al., 2021). The β -galactosidase was found to be inactivated due to degradation occurring in vacuoles when the transformant cells were transferred from methanol to glucose medium. The β -galactosidase of methanol-grown *K. phaffii* transformants can be assayed directly on plates using X-gal staining, thus providing an opportunity to isolate the mutants that are defective in the degradation of cytosolic proteins in *K. phaffii*. Additionally, this approach allowed isolation of insertion mutants defective in β -galactosidase inactivation due to defects in the novel gene involved in autophagy of cytosolic and peroxisomal proteins, designated ACG1 (Zazulya et al., 2023).

Hence, in this dissertation, the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used to select the mutant strains with impaired degradation of β -galactosidase. The impaired degradation of the enzyme in selected mutants was assessed by their blue color on plate with X-gal after shifting from methanol to glucose. Four mutants displaying, contrary to the parental strain, blue color after being transferred from methanol to glucose than that of the parental strain were selected. All the selected mutants showed relatively and to a different extent higher β -galactosidase activity compared to the parental strain. This result was also confirmed by fluorescence image analysis, as the enzyme is fused with GFP, which shows higher fluorescence in glucose-containing medium than that of the parental strain. Cellular viability is one of the best indicators for assessing the completion of the entire autophagic process because autophagy-deficient mutants will lose viability during incubation in starvation medium (Noda, 2008). In this study, the viability assay and phloxine B assay under nitrogen starvation conditions revealed survival defects in the mutants. Furthermore, the biomass accumulation of the mutant strains was evidently decreased compared with that of the parental strain, indicating an impairment in

autophagy. These results indicate that the selected mutants have a defect in general autophagy. AOX, a key peroxisomal matrix protein, was used as an indicator for pexophagy. Interestingly, based on the residual AOX activity after shifting the cells from methanol to glucose, the mutants MNNG-1 and MNNG-3 exhibited defects in both selective autophagy and pexophagy, while the mutants MNNG-2 and MNNG-4 were characterized solely by defects in selective autophagy of cytosolic β -galactosidase. The mutations in the selected strains will be identified through genome sequencing in the future. The identified genes will help shed light on the mechanisms of specific degradation of cytosolic proteins.

In this part of the dissertation, four mutant strains with defects in cytosolic protein degradation were identified, and subsequent verification confirmed that all these mutants exhibited autophagy deficiencies, indicating a correlation between autophagy defects and impaired protein degradation. This research provides a solid foundation for further exploration of how autophagy deficiencies can enhance protein yield. However, in this study also revealed that autophagy-deficient strains exhibit reduced cell growth, which typically correlates positively with protein yield (Dmytruk OV et al.,2020). Based on these findings, there are two potential application strategies may to enhance the production of protein in the future: first, screening and identifying autophagy-related genes to develop engineered strains capable of maintaining moderate growth while improving heterologous protein yields; second, utilizing newly discovered autophagy-related genes as modification targets through directed mutation or precise regulation to optimize the balance between growth and productivity. These strategies may provide genetic improvement approaches for enhancing recombinant protein production efficiency. Therefore, investigating the productivity of cytosolic protein synthesis in these mutants holds significant scientific interest and practical applications. Additionally, future sequencing of these mutant strains is anticipated to uncover relevant genes that elucidate this process, potentially offering insights to clarify the underlying mechanisms and providing new approaches for enhancing protein production efficiency in *K. phaffii*.

Another chapter of this dissertation was to develop new dominant selectable markers for the future application in metabolic engineering for the yeast *O. polymorpha*. Currently, the *ADE11*, *LEU2*, *MET6* and *URA3* genes have been developed as selective markers in combination with the corresponding auxotrophic *O. polymorpha* recipient strains. Based on sensitivity of *O. polymorpha* to aminoglycoside antibiotics geneticin, hygromycin B and zeocin, the dominant markers have been developed to confer resistance to these antibiotics (Cheon et al., 2009; Sohn et al., 1999). However, the most desired for commercial application appeared to be the self-cloning yeast vectors carrying no heterologous genes (Akada et al., 2002). Self-marker genes are very useful for the transformation of prototrophic industrial yeast strains (Hashida-Okado, Ogawa, et al., 1998). Such markers have not been developed yet for *O. polymorpha*, in spite of substantial development of molecular tools for this organism. Therefore, in this study the mutated *AUR1* and native *IMH3* genes from *O. polymorpha* conferring resistance to aureobasidin and mycophenolic acid, respectively, were tested.

First, two mutant forms of the *AUR1* gene were created by replacing the leucine residue with phenylalanine at position 53 along with replacement of histidine residue by tyrosine at position 72 (L53 CTT→F53 TTT; H72 CAT→ Y72 TAT) or substitution of alanine for cysteine at position 156 (A156 GCA → C156 TGT). These mutants were named pUC57/AUR1-1 and pUC57/AUR1-2, respectively. Subsequently, constructed plasmids were introduced into *O. polymorpha* wild-type strain by electroporation. As a result of transformation with plasmid pUC57/AUR1-2, there are several colonies of transformants were obtained on the third day of cultivation. While transformation with the plasmid pUC57/AUR1-1 no colonies on the medium with aureobasidin was observed. The obtained results indicate that the mutant form of the *AUR1* gene with replacement A156C can be used as a marker for selection of aureobasidin-resistant transformants in *O. polymorpha*.

Additionally, the plasmid with a gene conferring resistance to mycophenolic acid *IMH3* gene was constructed and named pUC57/IMH3. The constructed plasmid was introduced into *O. polymorpha* wild-type strain by electroporation. Transformants were

selected on YNB medium with mycophenolic acid (40 mg/L) on the fifth day of cultivation. The frequency of transformation was 20 transformants per microgram of DNA. Therefore, it can be concluded that the introduction of an additional copy of *IMH3* gene into genome of *O. polymorpha* wild-type strain was sufficient to obtain transformants resistant to mycophenolic acid. At last, *IMH3* gene was used as selectable marker for construction of a plasmid for overexpression of *TAL1*, *TKL1* and *AOX1* genes in the advanced ethanol producer BEP/ Δ cat8 strain. The obtained *O. polymorpha* recombinant strains were studied for their ethanol production during xylose alcoholic fermentation as compared to the parental strain. The results indicate that BEP/ Δ cat8/*TAL1*/*TKL1*/*AOX1* was characterized by higher ethanol production during 67 h of xylose fermentation and accumulated 39% more ethanol relative to the parental strain BEP/ Δ cat8 at 43 h. These results demonstrated that the mutated *AURI* gene and the native *IMH3* gene can be successfully applied as new dominant selectable markers in the yeast *O. polymorpha*. This study provides the first evidence that engineered *AURI* and native *IMH3* genes from *O. polymorpha* can serve as dominant selectable markers for this yeast species, offering promising potential for industrial applications. These markers could enable safer commercial development of metabolically engineered *O. polymorpha* strains by avoiding the transfer of antibiotic resistance genes to pathogens or the production of toxic or allergenic proteins.

The third part of this dissertation primarily focuses on improving the utilization of xylose for ethanol production from lignocellulosic hydrolysates in methylotrophic yeast *O. polymorpha*.

Currently, the necessity and actuality of biofuels as alternative energy sources have become increasingly evident, largely due to the limited reserves of fossil fuels and their environmental impact. As global interest in renewable energy continues to rise, the production and consumption of biofuels have emerged as critical strategies for addressing environmental challenges. Research indicates that economic considerations and environmental concerns are the main drivers prompting the global community to shift toward biofuels. Consequently, the development and utilization of biofuels not

only reduce reliance on fossil fuels but also significantly mitigate environmental pollution, thereby supporting the achievement of sustainable development goals.

The most significant and valuable attribute of biofuels lies in the renewability of their raw materials, creating opportunities for the agricultural sector to serve as both producers and consumers of various biofuels. Among these, bioethanol is considered one of the most promising renewable fuels due to its potential for production from agricultural waste, particularly lignocellulose biomass. However, the primary sugar that can be effectively utilized at present is glucose, while its hydrolysis products can contain up to 30% pentose sugars (such as xylose and arabinose). These pentoses are not efficiently fermented into ethanol by industrial microorganisms, especially the yeast *S. cerevisiae*. Consequently, researchers are primarily focused on constructing effective microorganisms capable of fermenting pentose sugars to produce ethanol.

O. polymorpha is a promising yeast for ethanol production during xylose fermentation due to its ability to ferment xylose at high temperatures (45-48 °C). This characteristic makes it an effective organism for simultaneous saccharification and fermentation (SSF). However, wild-type strains of *O. polymorpha* naturally produce very low amounts of ethanol from xylose, which is insufficient for industrial applications. The previous approaches combination of metabolic engineering and classical selection employed by our lab has successfully enhanced the ethanol yield of recombinant strains to 40 times that of the wild type, but this output still falls short of industrial requirements. Therefore, further optimization of the key fermentation parameters in *O. polymorpha* is essential to meet the demands for industrial xylose fermentation.

In the dissertation, the roles of hexose sensor *HXS1* and transcription factor with sensing properties *AZF1* on xylose and glucose fermentation in the native xylose-metabolizing yeast *O. polymorpha* were investigated. Extensive studies on glucose signaling pathways and their controls of glucose metabolism showed that efficient hexose transporters and glycolysis, which are the factors for efficient xylose metabolism, depend on activation of glucose signaling pathways (Wu et al., 2020). The

role of sugar sensors in xylose fermentation was also studied in xylose-metabolizing strains of *S. cerevisiae*. Positive effects of activation of the glucose sensing system in xylose-fermenting strains by upregulating the Snf3p/Rgt2p, Snf1/Mig1p and the cAMP/protein kinase A (PKA) pathway were found (Osiro et al., 2019). Both the *PDE1* and *PDE2* cAMP phosphodiesterase genes were deleted, increasing PKA activity in *S. cerevisiae* and consequently leading to increased xylose utilization (Wu et al., 2020). However, the role of xylose sensing/signaling in xylose metabolism and fermentation in the natural xylose-utilizing yeasts remains poorly understood and to date, no specific sensing proteins involved in xylose alcoholic fermentation have been identified (Jeffries & Van Vleet, 2009; Ribeiro et al., 2021).

In this study, the impact of sugar sensors on xylose metabolism and fermentation was investigated for the first time in the naturally xylose-metabolizing yeast *O. polymorpha*. The role of the *HXS1* sugar sensor in glucose and xylose alcoholic fermentation in *O. polymorpha* was examined through overexpression of the *HXS1* gene. The results reveal that overexpression of this gene enhanced ethanol production from xylose and glucose in both the advanced ethanol producer and wild type strain. Moreover, deletion of the *HXS1* gene strongly suppressed the utilization and fermentation of both sugars (as observed by my colleagues). These results indicate that the *HXS1* sensor is essential not only for glucose utilization, as previously reported (Stasyk et al., 2008), but also for xylose metabolism and the alcoholic fermentation of both sugars.

Furthermore, the study of transcription factors in xylose and glucose alcoholic fermentation is a relatively new research field (Alper & Stephanopoulos, 2007; Dzanaeva et al., 2021; Wei et al., 2018). In our previous research, we identified the significant role of specific transcription factors in the alcoholic fermentation of xylose and glucose in *O. polymorpha*. In particular, the *CAT8* gene has been identified as the first transcription factor specifically involved in regulating xylose alcoholic fermentation in *O. polymorpha*, without affecting ethanol production from glucose (Ruchala et al., 2017). Knocking out the *CAT8* gene was found to reduce cellular

respiration and stimulate ethanol production from xylose, while overexpression of the gene inhibited xylose fermentation (Ruchala et al., 2017). Additionally, orthologues of *S. cerevisiae* transcription activator Hap4 (Sybirna et al., 2010) and transcriptional repressors Mig1, Mig2 and Tup1 (Kurylenko et al., 2021) were also characterized. The results indicate that deleting both *MIG1* and *MIG2* genes led to a decrease in ethanol production from glucose and xylose. Conversely, deletion of the *HAP4-A* and *TUP1* genes increased ethanol production from xylose, while overexpressing *HAP4-A* and *TUP1* reduced ethanol production during xylose alcoholic fermentation. These findings demonstrate that manipulation of transcription factors represents a promising strategies for enhancing ethanol production from xylose in *O. polymorpha*, highlighting their potential for improving xylose utilization.

To gain a deeper understanding of the regulation of glucose and xylose metabolism, this dissertation focus on the *AZF1* gene in *O. polymorpha*, which is a homolog of transcription factor *AZF1* in *S. cerevisiae* and is known to be involved in regulation of carbon source utilization and glucose sensing. Most importantly, *AZF1* is required for efficient xylose fermentation in xylose-utilizing strain of *S. cerevisiae* (Myers et al., 2019; Newcomb et al., 2002; Gancedo, 2008). In this current work, the results indicated that overexpression *AZF1* elevates ethanol production from glucose and especially from xylose in both the advanced ethanol producer BEP/ Δ cat8 and wild type strain of *O. polymorpha*. Inversely, deletion leads to reduced ethanol production from both sugars (as observed by my colleagues) (Semkiv et al., 2022). Furthermore, it was observed that both strains, BEP/ Δ cat8/HXS1 and BEP/ Δ cat8/*AZF1*, exhibited increased consumption rates of glucose and xylose. The highest ethanol yield, 354 mg/g of consumed xylose, was observed in strain BEP/ Δ cat8/*AZF1* compared to the 154 mg/g in BEP/ Δ cat8. Meanwhile, strain BEP/ Δ cat8/HXS1 demonstrated the highest ethanol specific production rate (63 mg/g biomass/h, compared to 49 mg/g biomass/h in BEP/ Δ cat8) and ethanol productivity (181 mg/L/h, relative to 126 mg/L/h in BEP/ Δ cat8). Specific activities of xylitol dehydrogenase and pyruvate decarboxylase were elevated upon overexpression of *HXS1* or *AZF1* on the background of BEP/ Δ cat8,

potentially contributing to the increase ethanol production observed in these strains. However, the molecular mechanisms underlying these changes remain obscure and will be addressed in future studies.

This study found that both genes *HXS1* and *AZF1* are promising tools for increasing ethanol production from both major lignocellulosic sugars, glucose and xylose, which could be of biotechnological importance for the construction of more efficient ethanol producers using this strain.

CONCLUSIONS

In the dissertation, β -galactosidase degradation-deficient strains were isolated in the methylotrophic yeast *K. phaffii* using the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Moreover, the original approaches were used to construct overproducers of ethanol based on the methylotrophic yeast *O. polymorpha*.

The main scientific and practical results of the work are presented in the following conclusions:

1. Following MNNG mutagenesis, four mutant strains displaying higher β -galactosidase activity after cell shifting from methanol to glucose, relative to the parental strain were selected.

2. The viability assay and phloxine B assay under nitrogen starvation conditions revealed growth defects in the selected mutants. Besides, the biomass of the mutant strains was evidently decreased compared with that of the parental strain, suggesting an impairment in general autophagy. The obtained results indicate that the selected mutants have a defect in autophagy.

3. Pexophagy was studied by analyzing the residual AOX activity after shifting selected mutants from methanol to glucose. The results showed that mutants MNNG-1 and MNNG-3 exhibited defects in both selective autophagy of cytosolic β -galactosidase and selective pexophagy, while mutants MNNG-2 and MNNG-4 were characterized solely by defects in selective autophagy of cytosolic β -galactosidase.

4. The mutated *AUR1* gene and the native *IMH3* gene serve as effective new dominant selectable markers in the yeast *O. polymorpha*. Furthermore, the recombinant strain constructed using the *IMH3* gene as a selective marker, overexpressing *TAL1*, *TKL1*, and *AOX1* genes, demonstrated enhanced ethanol production compared to the parental strain. The usage of such selectable markers is the most suitable for commercial application of metabolically engineered *O. polymorpha* strains with

desired characteristics as prevents the transfer of genes conferring antibiotic resistance to pathogens.

5. Transformants of *O. polymorpha* overexpressing the *AZF1* gene, homologous to the *S. cerevisiae* *AZF1* gene that encodes a transcriptional activator involved in carbohydrate sensing, were constructed. The transformant (WT/*AZF1*) produced 10% more ethanol in glucose medium and 2.4 times more ethanol in xylose medium compared to the wild type. When overexpressed in the background of the advanced ethanol producer (BEP/ Δ cat8/*AZF1*), ethanol accumulation increased by nearly 10% in glucose medium and by more than 30% (up to 11 g/L at 72 h) in xylose medium compared to BEP/ Δ cat8.

6. Recombinant strains of *O. polymorpha* overexpressing the *HXS1* gene, encoding a hexose transporter-like sensor and a close homolog of *S. cerevisiae* sensors Snf3 and Rgt2, were constructed. In the WT/*HXS1* strain, ethanol production increased by 10% from glucose and enhanced two-fold from xylose compared to the wild-type strain. In the BEP/ Δ cat8/*HXS1* strain, ethanol production from xylose was elevated 40% (up to 13 g/L after 72 h) relative to BEP/ Δ cat8.

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LIST OF PUBLICATIONS BY DISSERTATION TOPIC:

The list of publications of the acquirer:

Seven scientific works have been published on the dissertation topic, including three manuscripts in international publications, as well as four abstracts of reports in the materials of conferences, scientific congresses, and congresses.

Articles in periodical scientific publications indexed in databases Web of Science Core Collection and/or Scopus:

1. **Zuo M**, Dmytruk OV, Dmytruk KV, Kang YQ, Sibirny AA. (2025). Isolation of mutants defective in cytosolic β -galactosidase degradation in the methylotrophic yeast *Komagataella phaffii*. *Cytology and Genetics*, Vol. 59, No. 1, pp. 71–78. doi: <https://doi.org/10.3103/S0095452725010104>. **Q4**, Scopus and WoS. (*The acquirer, together with co-authors, conducted research, analyzed and summarized the obtained data, participated in the writing and design of the publication*).

2. Bratiichuk D, Kurylenko O, Vasylyshyn RV, **Zuo M**, Kang YQ, Dmytruk K, Sibirny AA. (2020). Development of new dominant selectable markers for the nonconventional yeasts *Ogataea polymorpha* and *Candida famata*. *Yeast*, 37(9-10):505-513. doi: <https://doi.org/10.1002/yea.3467>. **Q2**, Scopus and WoS. (*The acquirer, together with co-authors, conducted research, analyzed and summarized the obtained data, participated in the writing and design of the publication*).

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