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ENGINEERING GLYCEROL UTILIZATION IN *CANDIDA FAMATA* FOR SUSTAINABLE RIBOFLAVIN PRODUCTION USING CRUDE GLYCEROL

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Abstract. *Candida famata* is a flavinogenic non-conventional yeast with a high capacity for riboflavin (vitamin B_2) production and represents a promising microbial cell factory. Glycerol, particularly crude glycerol generated as a by-product of biodiesel production, is an attractive low-cost carbon source for microbial fermentation. However, its use for riboflavin production by *C. famata* has not previously been investigated. In the present study, glycerol utilization was investigated in the riboflavin-producing *C. famata* BRPI strain by targeted overexpression of *STL1*, encoding a glycerol transporter, and *GUT2*, encoding a mitochondrial glycerol-3-phosphate dehydrogenase. Engineered strains were evaluated during cultivation on refined and crude glycerol to assess the potential of genetic modification of glycerol uptake and metabolism for improving riboflavin production and valorizing crude glycerol as a low-cost industrial by-product.
Keywords: *Candida famata*; riboflavin; glycerol utilization; crude glycerol; metabolic engineering.

Candida famata is a flavinogenic yeast capable of riboflavin (vitamin B_2) overproduction and represents a promising platform for the development of microbial cell factories. Riboflavin is an essential vitamin widely used in the food, pharmaceutical and feed industries, while microbial fermentation provides an efficient alternative to chemical synthesis. The economic feasibility of microbial production depends not only on the productivity of the production strain but also on the cost and availability of the carbon source. Therefore, the utilization of inexpensive and renewable substrates is an important direction in the development of sustainable biotechnological processes.

Glycerol is an attractive alternative carbon source due to its increasing availability as a major by-product of biodiesel production. In particular, crude glycerol represents a potentially valuable low-cost feedstock for microbial fermentation. Unlike refined glycerol, crude glycerol contains variable amounts of residual compounds originating from the biodiesel production process, including methanol, water, inorganic salts, soaps, ash, free fatty acids, residual glycerides, fatty acid methyl esters and catalyst-derived compounds. The composition of crude glycerol depends on the feedstock and processing conditions, and its impurities may affect microbial growth, metabolism and product formation. Crude glycerol has already been investigated as a substrate for the microbial production of various value-added compounds, including 1,3-propanediol, organic acids, ethanol and polyhydroxyalkanoates (Almeida et al., 2012). For example, *Ashbya gossypii* has been used for the production of orotic acid from crude glycerol (Silva et al., 2022). However, despite the use of crude glycerol in various microbial processes, its application for riboflavin production has received little attention. Although unpublished results have indicated that mutant strains of *C. famata* can overproduce riboflavin from glycerol, the production of riboflavin by *C. famata* using crude glycerol as a carbon source has not previously been investigated.

Glycerol utilization in yeasts involves several consecutive steps, including glycerol uptake, phosphorylation and subsequent oxidation of glycerol-derived metabolites. In *Saccharomyces cerevisiae*, the major glycerol utilization pathway involves *STL1*, encoding a

glycerol/H^+ symporter, *GUT1*, encoding glycerol kinase, and *GUT2*, encoding a mitochondrial glycerol-3-phosphate dehydrogenase. Glycerol is transported into the cell by Stl1p, phosphorylated by Gut1p to glycerol-3-phosphate and subsequently converted by Gut2p to dihydroxyacetone phosphate (DHAP), which can enter central carbon metabolism. The importance of these genes for growth on glycerol has been demonstrated in other yeast species. In *S. cerevisiae*, deletion of *STL1*, *GUT1* or *GUT2* resulted in the loss of growth on glycerol as the sole carbon source, confirming the essential role of this pathway (Swinnen et al., 2013). Similarly, *STL1* from *Candida albicans* has been shown to encode a functional glycerol transporter, and expression of *C. albicans STL1* restored the ability of an *S. cerevisiae stl1Δ* mutant to grow on minimal glycerol medium (Kayingo et al., 2009). These findings provided a rationale for investigating glycerol transport and metabolism as potential targets for improving glycerol utilization in *C. famata*.

In the present study, glycerol utilization was investigated in the riboflavin-producing *C. famata* BRPI strain (Dmytruk et al., 2020) through targeted genetic engineering of components involved in glycerol uptake and metabolism. The *STL1* gene, encoding a glycerol transporter, and the *GUT2* gene, encoding a mitochondrial glycerol-3-phosphate dehydrogenase, were introduced into the production background to investigate their contribution to glycerol utilization and riboflavin biosynthesis. The engineered strains were evaluated in fermentation experiments using 5 % (v/v) refined glycerol and 5 % (v/v) crude glycerol, allowing their performance to be compared on substrates differing in purity and composition.

Overexpression of *STL1* increased riboflavin production 1.48-fold on 5 % (v/v) refined glycerol and 1.36-fold on 5 % (v/v) crude glycerol compared with the parental BRPI strain. Similarly, *GUT2* overexpression increased riboflavin production 1.65-fold and 1.42-fold on refined and crude glycerol, respectively. These results indicate that targeting both glycerol uptake and intracellular glycerol metabolism can positively affect riboflavin production by *C. famata*. The differences observed between refined and crude glycerol highlight the potential influence of substrate composition on the performance of engineered strains and demonstrate that crude glycerol can support enhanced riboflavin production despite its variable composition.

Overall, this study combines metabolic engineering of glycerol utilization with the valorization of crude glycerol as an industrial by-product. The demonstrated effects of *STL1* and *GUT2* overexpression support glycerol transport and intracellular metabolism as promising targets for improving riboflavin production in *C. famata*. The use of crude glycerol as a low-cost carbon source may contribute to the development of more sustainable and economically attractive microbial processes for riboflavin production. Further studies are currently underway to investigate the effect of *GUT1* overexpression, which encodes glycerol kinase catalyzing the first enzymatic step in glycerol utilization, on glycerol metabolism and riboflavin production.

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SCREENING FOR TECHNOLOGICAL PROPERTIES OF CORYNEFORM BACTERIA PROMISING IN SMEAR-RIPENED CHEESE PRODUCTION

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Abstract. The technological properties (psychrotrophicity, halotolerance, and acid tolerance) of coryneform bacteria strains belonging to the genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, and *Brachybacterium*, isolated mainly from milk and cheese, were investigated. Based on a combination of features (ability to grow at 12–15 °C, in the presence of 3–7 % NaCl, and at pH 5.5–7.0), *Brevibacterium* and *Corynebacterium* strains promising for use in smear-ripened cheese production were selected.
Keywords: coryneform bacteria, technological properties, smear-ripened cheeses.

Coryneform bacteria play an important role in the production of smear-ripened cheeses. The characteristic taste, aroma, soft texture, and edible orange smear rind of these cheeses are formed as a result of the activity of a complex surface microbiome consisting mainly of coryneform bacteria, including *Brevibacterium*, *Corynebacterium*, *Arthrobacter*, *Glutamicibacter*, *Microbacterium* and several other genera, as well as salt-tolerant yeasts (Ritschard & Schuppler, 2024). The specific ripening conditions and the biochemical and functional potential of the aerobic smear microbiota contribute to the high nutritional and biological value of these products. The ripening process of such cheeses is controlled by regular surface treatment with a brine solution inoculated with *Brevibacterium linens*, which may be supplemented by other coryneform bacteria (particularly *Corynebacterium casei* and *Arthrobacter* spp.) as well as the yeast *Debaryomyces hansenii* (Bockelmann et al., 2003). Because smear-ripened cheeses ripen at low temperatures, high salt concentrations, and moderately acidic initial pH values, pronounced psychrotrophic capacity, halotolerance, and acid tolerance are among the principal technological characteristics required of coryneform bacteria used as adjunct starter cultures.

The production of smear-ripened cheeses is mainly concentrated at small craft creameries, which actively continue their development in Ukraine despite the military action (Peshuk et al., 2025). Given that the current ripening technology for these cheeses relies on imported commercial *B. linens* starter cultures used either as monocultures or as components of multistrain starter preparations, the selection of domestic *B. linens* strains is very promising. This will allow the creation of unique, high-quality ethnic cheeses with authentic flavour profiles that can compete on the foreign market.

The purpose of this study was to screen strains of coryneform bacteria from the Ukrainian Collection of Microorganisms (UCM) whose technological properties meet the requirements for adjunct cultures used in the production of smear-ripened cheeses.

The study included 43 collection strains of saprophytic coryneform bacteria of the genera *Brevibacterium*, *Arthrobacter*, *Corynebacterium*, and *Brachybacterium* isolated from milk and dairy products. The cultures were maintained on Corynebacterium agar medium