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Development of yeast-based integrated bioprocess for xylitol and ethanol production from xylan-rich lignocellulosic materials

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Key words: *Sugiyamaella novakii,* barley husk, nonconventional yeast, biorefinery, xylanases, lignocellulose valorisation, consolidated bioprocess

Highlights:

- *Sugiyamaella novakii* has good ability of combined xylanases and xylitol production
- Xylanases production capability of *Spencermartinsiella europaea* is demonstrated
- Optimal parameters for xylanases and xylitol production by *S. novakii* are determined
- Xylan-based integrated bioprocess producing xylitol and ethanol is demonstrated and scaled up
- Integrated bioprocess using barley husk to produce xylitol by *S. novakii* is demonstrated

Abstract

Xylan-rich agricultural residues can serve as cheap and abundant raw materials for biorefinery processes, however development of efficient and sustainable processes for their bioconversion into high-value products is still a major challenge. Integrated bioprocess, combining enzyme production, substrate hydrolysis and product formation into a single process by using a single microorganism, represents a promising strategy for overcoming this issue. In this study, selected nonconventional wild-type yeast strains were screened for their ability to produce extracellular xylanases and xylitol, aiming to identify multifunctional biocatalysts that are suitable for an integrated bioprocess of xylan-rich lignocellulosic residues.

Among the investigated strains, *Sugiyamaella novakii* NCAIM Y.00987 was found to be suitable for xylan-based integrated bioprocess applications due to its revealed capability of producing extracellular xylanases and xylitol. Process parameters affecting xylanases production and xylitol fermentation were determined and optimised, and the scalability of the optimised conditions was verified in batch processes performed in 500-mL bench-top bioreactor. The strain maintained stable enzyme production and product formation under the controlled cultivation conditions in bench-top bioreactor, forecasting its applicability in an industrial bioprocess.

Application of *Su. novakii* in integrated bioprocess for xylitol and ethanol was subsequently assessed using xylan and barley husk as model and real lignocellulosic substrates, respectively. Although a two-stage bioprocess was proposed to provide optimal conditions for both xylanases and xylitol productions, enzyme and product synthesis was simultaneously obtained, indicating the possibility of using the strain as a microorganism for consolidated bioprocessing of lignocellulosic materials. The developed bioprocess resulted in direct substrate conversions, producing 1.1 g/L xylitol and 1.8 g/L ethanol after 48 h on xylan, while 0.12 g/L xylitol was obtained from barley husk, without external enzyme supplementation. In addition, *Candida*

allociferrii NCAIM Y.02114 was confirmed experimentally for the first time, identifying it as an industrially relevant strain. The findings contribute to advancing sustainable biomass processing and support the implementation of circular bioeconomy strategies through the direct conversion of agricultural by-products into value-added biochemicals.

Overall, this study highlights the potential of nonconventional yeasts as multifunctional platforms for the integrated valorisation of hemicellulose-rich residues and provides a basis for the development of lignocellulose-based consolidated bioprocesses. The findings contribute to advancing sustainable biomass processing and support the implementation of circular bioeconomy strategies through the direct conversion of agricultural by-products into value-added biochemicals.

1. Introduction

The increasing global demand for sustainable and renewable alternatives to fossil-based products has greatly intensified research efforts in the field of biotechnological valorisation of lignocellulosic residues [1]. Integrated bioprocess using a single microbial factory represents an advanced bioengineering strategy that might integrate enzyme production, substrate hydrolysis, and fermentation into a single streamlined process in order to efficiently convert lignocellulosic raw materials into value-added products. In special case, all the above-mentioned process steps can occur simultaneously, resulting in a so-called consolidated bioprocess (CBP). This approach has considerable industrial and economic relevance because it can reduce operational costs, simplify process configuration, and improve the economic feasibility of lignocellulosic biorefineries. However, the successful implementation of single-cell integrated bioprocesses and CBPs strongly depends on the availability of robust microorganisms combining efficient hydrolytic activity with high product-forming capacity [2]. Previously reported CBP systems mostly rely on filamentous fungi, bacterial platforms, or

genetically engineered microorganisms, whereas naturally occurring yeasts capable of simultaneously hydrolysing lignocellulosic substrates and converting released sugars into value-added products have remained unexplored. In addition, recent investigations focus mainly on the valorisation of the cellulose fraction in CBP, probably due to the more complicated nature of hemicelluloses in regard of complete enzymatic decomposition and fermentative conversion into bioproducts. Thus, dedicated research to develop hemicellulose-based CBPs is of great importance to unlock the whole potential of biomass residues for sustainable biorefining [3]. Xylan is the major hemicellulosic component of the plant cell wall of many lignocellulosic residues [4], and it represents a promising substrate to produce value-added compounds. However, the efficient conversion of xylan into target products requires the synergistic action of robust microbial catalysts capable of both xylan hydrolysis and product biosynthesis [5]. In recent years, nonconventional (non-*Saccharomyces)* yeasts have emerged as promising candidates in biotechnological utilisation of lignocelluloses due to their potential for hydrolytic enzyme production, unique product-forming capabilities, and tolerance to stresses [6]. Among these, certain species have shown the ability to secrete xylanolytic enzymes, which play a crucial role in breaking down xylans into fermentable sugars [7]. In recent years, an increasing number of nonconventional yeasts are predicted to have xylanolytic activity by bioinformatic tools, however experimental confirmations of these results are still limited [8]. Some xylanase-producing nonconventional yeast strains have been isolated from different environments including soil, rotting wood, decaying sugarcane bagasse, tropical peat swamp forest, guts of insect feeding on plants, and they mainly belong to genera of *Candida*, *Cryptococcus, Scheffersomyces*, and *Sugiyamaella* [9–12]. Species of the genus *Sugiyamaella* have recently attracted increasing attention because of their ecological association with lignocellulosic habitats and their versatile carbohydrate metabolism. Among sixteen yeast isolates belonging to the genus *Sugiyamaella*, Sena et al. (2017) identified the strains of *Su.*

lignohabitans, *Su. xylanicola*, and *Su. valenteae* as good xylanases producers. However, comparative analyses regarding the integrated bioprocess-associated potential of these and other *Sugiyamaella* species have not been explored yet. Nonconventional yeasts often have great ability to utilize not only hexoses, like *Saccharomyces cerevisiae* does, but pentoses e.g. xylose [13]. One of the most valuable fermentative products from xylose is xylitol, a low-calorie sugar alcohol, that is widely used in food and pharmaceutical industries and for the production of other high-value chemicals as a platform molecule [14]. Currently, the main process of industrial xylitol production involves the chemical catalytic hydrogenation of pure xylose, that is derived from xylose-rich hemicellulose hydrolysates, using Raney-nickel catalyst. However, this method is accompanied by high costs for xylose purification and product recovery and environmental pollution issues due to the high pressure (~ 50-atm) and high temperature (80–140 °C) required. Microbial fermentation is considered as an excellent alternative to the chemical method due to its high selectivity and environmental friendliness [15]. Some members of *Candida* genus are well-known to be good xylitol-producers. Besides *Candida* species, there are other nonconventional yeasts with the capability of xylose-xylitol bioconversion, e.g. some species of *Komagataella, Kluyveromyces,* and *Scheffersomyces* [12, 16–19]. Despite the versatile properties of nonconventional yeasts in terms of xylan hydrolysis and xylose metabolism, these properties have generally been studied separately, resulting in lacking information about the xylan-based integrated bioprocess and CBP potential of these yeasts [3, 20]. Recent studies on xylan-to-xylitol bioconversion mainly focuses on the genetic modification and application of *S. cerevisiae* [8]. Although genetic engineering is a powerful tool for improving microbial performance, engineered strains may suffer from reduced robustness under industrial conditions as a consequence of metabolic burden, decreased stress tolerance, reduced growth rates, or instability of the introduced traits [21]. Thus, investigating wild type nonconventional yeasts having xylan-degrading and xylitol-producing capabilities is

of great importance to lay the groundwork of developing sustainable biorefineries including hemicellulosic CBPs [22]. Thus, this study aims to investigate the xylanases and xylitol production capabilities of selected nonconventional yeast strains, with a long-term goal of developing a consolidated bioprocessing strategy for direct conversion of xylan-rich biomass residues into xylitol and other valuable bioproducts. The findings of this work expand our knowledge about the applicability of naturally occurring nonconventional yeasts in industrial biotechnology and support the development of sustainable consolidated bioprocesses for xylitol production from lignocellulosic residues.

2. Materials and methods

2.1.Microorganisms

Sugiyamaella novakii NCAIM Y.00987, *Komagataella pseudopastoris* NCAIM Y.01541, *Spencermartinsiella ligniputridi* NCAIM Y.01993, *Spencermartinsiella europaea* NCAIM Y.01817, *Kuraishia molischiana* NCAIM Y.01736, *Candida maritima* NCAIM Y.02151, *Citeromyces hawaiiensis* NCAIM Y.00902*, Scheffersomyces stipitis* NCAM Y.00810, *Candida allociferrii* NCAIM Y.02114, *Ogatae pilisensis* NCAIM Y.01509 and *Geotrichum klebahnii* NCAIM Y. 02163 strains were obtained from the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary).The strains were maintained at 4 °C on malt agar slants: 2 w/w% malt extract and 2 w/w% agar.

2.2.Qualitative method for detecting lignocellulolytic yeasts

Potential lignocellulolytic yeasts were qualitatively identified by Congo red dye method [23]. The investigated yeasts were grown on YNBw/oAA (Yeast Nitrogen Base without Amino Acids) agar (6.7 g/L yeast nitrogen base without amino acids, 20 g/L agar) supplemented by xylan from birchwood (20 g/L), carboximethyl-cellulose (CMC, 20 g/L), or solka floc (20 g/L). The yeasts were propagated for 7 days at 30 °C in Petri-dishes. The colonies were flooded with

Congo red dye (5 g/L Congo Red, 500 mL distilled water, 500 mL absolute ethanol) for 15 min followed by destaining with 1 M NaCl solution. YNBw/oAA agar medium without carbon source was used as negative control.

2.3.Inoculum preparation for xylanase production and xylitol fermentation

Inoculum cultures were prepared in the same way for both xylanase production and xylitol fermentation and experiments. Colonies of the selected yeasts were transferred from the malt agar slant on glucose agar slant (10 g/L glucose, 10 g/L peptone, 3 g/L yeast extract, and 20 g/L agar) and propagated for 3 days at 30 °C. Then all the cells of the slant were transferred into the inoculum medium containing 2 g/L yeast extract, 3 g/L KH_2PO_4, 0.2 g/L $MgSO_4*7H_2O$, 0.6 g/L $(NH_4)_2HPO_4$, and 30 g/L xylose. The cells were propagated in the inoculum medium (150 mL medium in 750-mL Erlenmeyer flask) for 72 hours, at 220 rpm, and 30 °C.

2.4.Xylanase production by fermentation in shake flask

Inoculum preparation was performed according to the method described earlier. Sufficient volumes of the inoculum cultures were centrifuged (2800×g, 5 min) to separate appropriate number of cells, which was transferred to the fermentation medium to adjust 2 g (dry cell mass)/L initial cell concentrations. Xylanase production experiments were performed in 20 mL YNBw/oAA medium (6.7 g/L yeast nitrogen base without amino acids) or semi-defined medium (2 g/L yeast extract, 3 g/L KH_2PO_4, 0.2 g/L $MgSO_4*7H_2O$, 0.6 g/L $(NH_4)_2HPO_4$), both supplemented with 50 g/L birchwood xylan, in 100 mL shake flasks at 30 °C and 220 rpm for 72 h. Fermentations were performed in triplicates. After 72 h, the fermentation broth was centrifuged (6600×g, 5 min) to separate the solids (cells and xylan) from supernatant, which was stored at −18 °C until further use. In certain cases, the separated cells were used immediately in measurements of cell wall-associated xylanase activity.

2.5.Xylanase activity measurements

Xylanase activity of the supernatants of xylanase production fermentation (extracellular xylanase activity) was assayed in test tubes by using a birchwood xylan suspension (1 w/w%) as substrate. For this assay, 0.75 mL of appropriately diluted cell-free supernatant of xylanase production fermentation was added to 0.75 mL of substrate prepared in acetate buffer (0.05 M, pH 4). The assays were carried out at 45 °C with magnetic stirring for 30 min. The enzyme reaction was stopped by boiling the reaction mixture for 5 minutes, and the residual xylan was separated by centrifugation (6600×g, 5 minutes). One mL of clear supernatant was then analysed for reducing sugars released during the assay by the dinitrosalicylic acid (DNS) method using xylose standard curve [24].

Cell wall-associated xylanase activity was measured by using washed and resuspended cells derived from the xylanase production experiments. All the solids were separated from the 20 mL broth of xylanase production and were washed with 10 mL distilled water twice to eliminate soluble xylan residues and culture medium components. Then the cells were resuspended in 10 mL acetate buffer (0.05 M, pH 4), and 0.75 mL of this suspension was used in the xylanase activity measurement, that is performed by the following steps: 0.75 mL of the resuspended cell solution was added to 0.75 mL of substrate prepared in acetate buffer (0.05 M, pH 4). The assays were carried out at 45 °C with magnetic stirring for 30 min. The enzyme reaction was stopped by boiling the reaction mixture for 5 minutes, and the residual xylan was separated by centrifugation (6600×g, 5 minutes). One mL of clear supernatant was then analysed for reducing sugars released during the assay by the dinitrosalicylic acid (DNS) method using xylose standard curve [24].

2.6. Determination of the pH and temperature optima of the produced xylanolytic supernatant of *Su. novakii*

The pH and temperature optima of the xylanolytic supernatant produced by *Su. novakii* NCAIM Y.00987 on xylan were investigated. Regarding pH, the experiments were carried out within a pH range of 2–6, at 45 °C. At pH 3, pH 4, pH 5, and pH 6, citrate buffers (0.01 M) were used. At pH 4, and pH 5, acetate buffers (0.05 M) were used. At pH 2, HCl-KCl (0.1 M) buffer was used. The substrate suspension (1 w/w% birchwood xylan) was made using the same buffers as the reaction mixture. Regarding temperature, the experiments were investigated at 25 °C, 35 °C, 45 °C, 55 °C, and 65 °C during the enzyme activity measurements in citrate buffer (0.01 M, pH 3).

2.7.Determination of the optimal parameters for xylanase production by *Su. novakii*

The effect of different media, temperature, and pH on the xylanase production of *Su. novakii* NCAIM Y.00987 were investigated. Inoculum preparation was performed according to the method described earlier. Initial cell concentrations were set to 5 g (dry cell mass)/L. Xylanase production experiments were performed in 2 different media in 100-mL shake flasks: YNBw/oAA and semi-defined medium, both supplemented with 50 g/L birchwood xylan. Two different aeration conditions were tested with each medium: aerobic (20 mL medium, 220 rpm), and micro-aerobic (50 mL medium, 125 rpm). Three different temperatures (30, 35, and 40 °C) and three different pHs (pH 2,3, and 4) were tested. The investigated experimental settings are shown in Table 1. Xylanase productions were performed in triplicates for 72 h. After 72 h, the fermentation media were centrifuged to separate the solids (cells and xylan) from the supernatant, which was stored at −18 °C until further use. Xylanase activity of the supernatants was determined at optimal conditions (pH, temperature) of the xylanase enzyme produced by *Su. novakii*, following the xylanase activity assay described earlier.

Table 1 Experimental settings of determination of the optimal parameters for xylanase production by Su. novakii

Medium	YNBw/oAA semi-defined
Aeration	aerobic (20 mL in 100-mL Erlenmeyer flask, 220 rpm) micro-aerobic (50 mL in 100-mL Erlenmeyer flask, 125 rpm)
Temperature	30°C 35°C 40°C
pH	pH 2 (0.1 M HCl-KCl buffer) pH 3 (0.01 M citrate buffer) pH 4 (0.01 M citrate buffer)

2.8.Testing the capability of xylitol production in submerged culture

The capability of xylitol production was investigated by *Su. novakii* NCAIM Y.00987, *Candida allociferrii* NCAIM Y.02114, *Spencermartinsiella europaea* NCAIM Y.01817, *Ogataea pilisensis* NCAIM Y.01509, and *C. maritima* NCAIM Y.02151 in submerged cultivation. Xylitol fermentation experiments were performed in 100 mL-shake flasks on semi-defined medium supplemented with 30 g/L xylose in order to identify xylitol, and other metabolites that can serve as fermentation products. Inoculum preparation was performed according to the method described earlier. Xylitol fermentation was investigated under micro-aerobic conditions (50 ml medium, 125 rpm). Initial cell concentrations were set to 5 g (dry cell mass)/L. Fermentations were performed in triplicates. Daily sampling (0, 24, 48, and 72 h) was performed to monitor the concentrations of cells, xylose, xylitol and any other potential products.

2.9.Investigation of different process parameters on the xylitol production of *Su.novakii*

Xylitol fermentation experiments were performed in 100 mL-shake flasks on semi-defined medium supplemented with 30 g/L xylose in order to identify xylitol, and other metabolites that can serve as fermentation products. Inoculum preparation was performed according to the method described earlier. Xylitol fermentation was investigated under micro-aerobic conditions (50 ml medium, 125 rpm). Initial cell concentrations were set to 5 g (dry cell mass)/L. Fermentations were performed in triplicates. Daily sampling (0, 24, 48, and 72 h) was performed to monitor the concentrations of cells, xylose, xylitol and any other potential products.

The effect of two different aeration conditions on xylitol fermentation was investigated: aerobic (20 mL medium, 220 rpm), and micro-aerobic (50 mL medium, 125 rpm). The shake flasks were closed by cotton plugs. Initial cell concentrations were set to 1 g (dry cell mass)/L, or 5 g (dry cell mass)/L. Fermentations were performed in triplicates. Daily sampling (0, 24, 48, and 72 h) was performed to monitor the concentrations of cells, xylose, and potential products.

The effect of pH on xylitol fermentation by *Su. novakii* NCAIM Y.00987 was investigated. Fermentation experiments were performed in 100 mL-shake flasks on semi-defined medium supplemented with 30 g/L xylose. The pH of the medium was set to pH 2 (using 0.1 M, HCl-KCl buffer), pH 3 (using 0.01 M citrate buffer), pH 4 (using 0.01 M citrate buffer), pH 5 (using 0.01 M citrate buffer), or pH 6 (using 0.01 M citrate buffer). The initial cell concentrations were set to 5 g (dry cell mass)/L. Fermentations were carried out under micro-aerobic conditions (50 mL medium, 125 rpm) at 30 °C for 72 h. Fermentations were performed in triplicates. Daily sampling (0, 24, 48, and 72 h) was performed to monitor the concentrations of cells, xylose, xylitol, and fermentation by-products (ethanol, glycerol).

2.10. Xylitol fermentation and xylanase production by *Su. novakii* in bench-top bioreactor

Xylitol fermentation, xylanase production, and an integrated bioprocess (unifying xylanase production, hydrolysis and xylitol fermentation) were performed in 500-mL bench-top bioreactor (Solida Biotech, Germany). The bioreactor was filled with 300 mL of YNBw/oAA medium supplemented with 30 g/L xylose in the case of xylitol fermentation, or with 10 g/L beechwood xylan in the case of xylanase production, or with 50 g/L beechwood xylan in the case of consolidated bioprocess, or with 7 w/w% pretreated barley husk in the case of biomass-based consolidated bioprocess. Barley husk pretreatment was carried out autoclaving 10 w/w% barley husk suspension made in 0.01 M pH 3 citrate buffer for 1 hour (121 °C, 1 bar). The inoculum cultures of *Su. novakii* NCAIM Y.00987 was propagated in semi-defined medium supplemented with 30 g/L xylose, and then appropriate amount of separated cells were transferred into the media of xylitol fermentation, xylanase production or the consolidated bioprocess to get an initial cell concentration of 5 g (dry cell mass)/L. Xylitol fermentation, and xylanase production were accomplished at 30 °C, pH 3, for 72 hours with multiple samplings per day. Integrated bioprocess on beechwood xylan was accomplished at 30 °C, pH 3, for 96 hours with multiple samplings per day. Barley husk-based integrated bioprocess was carried out at 30 °C, pH 3, for 144 hours with daily sampling. The bioreactor was equipped with dissolved oxygen (DO) (Visiferm 120 Arc, Hamilton), and pH (Easyferm Plus 120 Arc, Hamilton) sensors. Single cell-mediated integrated bioprocesses on xylan and barley husk consisted of two stages to enhance the performance of xylanases and xylitol productions. Xylitol fermentation stage was carried out under conditions of 150 rpm agitation, and 1 vvm aeration, whereas xylanase production stage was performed at 250 rpm agitation with the same aeration rate of 2 vvm. Xylanase production and hydrolysis section of the xylan-based integrated bioprocess was carried out for 48 hours under conditions of 250 rpm agitation and 2 vvm aeration, while the xylitol production stage was proceeded for 48 hours under conditions of 150 rpm agitation and 1 vvm aeration. Xylanase production and hydrolysis section of the

barley husk-based integrated bioprocess was carried out for 65 hours under conditions of 250 rpm agitation and 2 vvm aeration, while the xylitol production part was carried out 72 hours under conditions of 150 rpm agitation and 1 vvm aeration.

2.11. Analytical methods

Concentration of xylose, xylitol, lactic acid, glycerol, and ethanol were determined by high-performance liquid chromatography (HPLC) using BioRad (Hercules, CA, USA) Aminex HPX-87H (300 x 7.8 mm) column equipped with Micro-Guard Cation H+ Refill Cartridge (30 x 4.6 mm) pre-column at 65 °C, and refractive index detector. The eluent was 5 mmol/L sulfuric acid at a flow rate of 0.5 mL/min. The injection volume was 40 µL.

The cell concentration in fermentation samples was determined by measuring the optical density at a wavelength of 600 nm by spectrophotometer (Ultrospec III, Pharmacia LKB, Uppsala, Sweden). The cell concentration was calculated by using a calibration curve based on the relationship of optical density and cell dry weigh.

2.12. Statistical analysis

For statistical evaluation, comparisons between two groups were conducted using unpaired Student's *t*-tests. Prior to *t*-tests, *F*-tests were performed to test the equality of variances. In cases involving more than two parameters, one-way analysis of variance (ANOVA) was performed to assess the potential effect of the examined parameters. Prior to performing the ANOVA, the assumptions of independence, normality, and homogeneity of variances were assessed. At 0.05 significance level of $p < 0.05$ was considered statistically significant. All analyses were conducted using Microsoft Excel software.

3. Results and Discussion

3.1. Screening for lignocellulolytic yeasts

The initial screening aimed to identify yeast strains with potential lignocellulolytic traits relevant to xylan-based integrated bioprocess applications. The selected strains originated mainly from lignocellulose-rich ecological niches such as rotten wood, fruits, and plant-derived substrates, environments that are often associated with adaptation to polysaccharide degradation. In addition, previous genomic analyses suggested the presence of xylanase-encoding genes in members of the genera *Sugiyamaella* and *Spencermartinsiella* [8, 25].

Among the eleven tested strains, *Sp. europaea*, *Su. novakii*, *Candida maritima*, *C. allociferrii*, and *Ogataea pilisensis* displayed detectable xylanolytic halos on xylan-containing agar plates (Table 2). The strongest hydrolysis zones were observed for *Su. novakii* and *Sp. europaea*, supporting previous genomic predictions regarding their xylanolytic potential. In contrast, cellulolytic activity on CMC agar was weak and no detectable hydrolysis was observed on Solka floc, indicating limited capability for degradation of recalcitrant cellulose substrates.

These findings suggest that the investigated yeasts are more specialised toward hemicellulose utilisation than cellulose decomposition, highlighting their potential role in the degradation of hemicellulose-rich plant biomass. From a biorefinery perspective, selective xylanolytic activity may be highly advantageous for valorisation of hemicellulose-rich side streams where extensive cellulase production is not required. Based on these results, the xylanolytic strains were selected for further submerged fermentation studies.

319 *Table 2 Xylanolytic and cellulolytic potential based on Congo red staining method*

Name of the yeast	Carbon source		
	Xylan	Carboxymethylcellulose (CMC)	Solka floc
Komagataella pseudopastoris NCAIM Y.01541	-	+	-
Spencermartinsiella ligniputridi NCAIM Y.01993	-	-	-
Spencermartinsiella europaea NCAIM Y.01871	+	-	-
Sugiyamaella novakii NCAIM Y.00987	+	-	-
Kuraishia molischiana NCAIM Y.01736	-	+/-	-
Candida maritima NCAIM Y.02151	+	+	-
Citeromyces hawaiiensis NCAIM Y.00902	-	-	-
Scheffersomyces stipitis NCAIM Y.00810	-	-	-
Candida allociferri NCAIM Y.02114	+	-	-
Ogataea pilisensis NCAIM Y.01509	+	+	-
Geotrichum klebahnii NCAIM 02163	-	-	-

320

321 3.2.Testing the capabilities of xylanases and xylitol production in submerged culture

322 The yeasts displayed zones of hydrolysis on xylan agar were investigated in submerged culture
323 using different media (YNBw/oAA, and semi-defined) containing xylan as carbon source to

prove their ability of efficient xylanases production. Since the complete hydrolysis of xylan results in xylose monomers, that should be valorised in a xylan-based integrated bioprocess, fermentation capabilities of the selected yeasts to convert xylose into xylitol were also examined using semi-defined medium containing xylose. The effect of the presence of organic nitrogen source on xylanases production was examined by testing the enzyme fermentation on semi-defined and YNBw/oAA media containing organic and inorganic nitrogen sources, respectively. After 72 hours of growth, extracellular and cell wall-associated xylanase activities were determined in 0.05 M pH 4 acetate buffer at 45 °C with 30 minutes reaction time.

Table 3 Extracellular and cell wall-associated xylanase production and xylitol fermentation tests of selected nonconventional yeasts. The average values of xylanase activity are given in U/mL with standard deviation in parentheses. The results of xylitol fermentation were evaluated by +/-, if the result is positive, the average value of xylitol concentration after 72 h is shown in parentheses. Xylanase production and xylitol fermentation experiments are carried out in biological triplicates. Xylanase activity was measured in duplicates from each biological replicates.

	Medium	Extracellular xylanase activity [U/mL]	Cell wall-associated xylanase activity [U/mL]	Xylitol fermentation
Sugiyamaella novakii NCAIM Y.00987	YNBw/oAA	3.85 (0.12)	0.32 (0.03)	nt
	semi-defined	1.80 (0.17)	0 (0.00)	+ (5.91 g/L)
Candida allociferrii NCAIM Y.02114	YNBw/oAA	0 (0.00)	0 (0.00)	nt
	semi-defined	0 (0.00)	0 (0.00)	+ (2.53 g/L)
Spencermartinsiella europaea NCAIM Y.01817	YNBw/oAA	2.34 (0.39)	0 (0.00)	nt
	semi-defined	0.25 (0.07)	0 (0.00)	nd
Ogataea pilisensis NCAIM Y.01509	YNBw/oAA	0 (0.00)	0 (0.00)	nt
	semi-defined	0 (0.00)	0 (0.00)	nd

Candida maritima NCAIM Y.02151	YNBw/oAA	0 (0.00)	0 (0.00)	nt
	semi-defined	0 (0.00)	0 (0.00)	nd

nt – not tested

nd – not detected

Extracellular xylanase activity was detected in the fermentation supernatants of *Su. novakii* and *Sp. europaea*. The highest xylanase activity was 3.85 U/mL, which was achieved by *Su. novakii* in YNBw/oAA medium (Table 3). *Sp. europaea* also presented high (2.34 U/mL) xylanase activity in YNBw/oAA medium, proving its suspected ability of efficient xylanases production. Although the recent genome sequence analysis proved the presence of xylanases genes in the genom of *Sp. europaea* [26], no experimental evidence of extracellular xylanases production by fermentation have been yet published. The markedly higher xylanase activities observed in YNBw/oAA medium suggest that nitrogen source composition strongly influences enzyme induction. Nitrogen limitation may promote extracellular hydrolytic enzyme production as part of a substrate-scavenging response, a phenomenon frequently described in lignocellulose-degrading microorganisms. In order to clarify which substances of the media have significant effects on the xylanase production of *Su. novakii* and *Sp. europaea*, further investigations are required. Cell wall-associated xylanase activity was obtained only in the case of *Su. novakii* by using YNBw/oAA medium (0.32 U/mL) (Table 3). Extracellular xylanase activity was not detected in the fermentation supernatants of *C. allociferrii*, *O. pilisensis*, and *C. maritima* (Table 3). Xylose fermentation, xylanases and xylitol producing capabilities of *Su. novakii* (UFMG-CM-Y2178) were also investigated by Sena et al. (2017). Xylanase production was performed on YNBw/oAA medium supplemented with xylan (1%), and the activity measurements were performed in 0.05 M pH 5.5 acetate buffer at 30 and 50 °C for 30 minutes. Xylanase activities of 0.006 and 0.072 U/mL were measured at 30 and 50 °C, respectively by Sena et al. (2017),

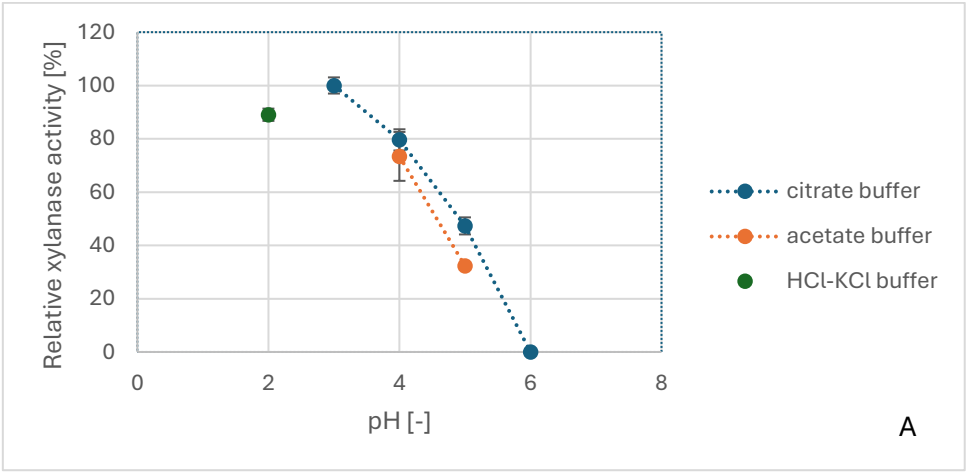
which orders of magnitude lower compared to that achieved in our study with *Su. novakii* NCAIM Y.00987.

Xylitol production of the five selected yeasts was also tested in order to examine their potential product forming attributes for xylan-based integrated bioprocesses. Based on our previous investigations of yeast-mediated xylitol production [16], fermentations were performed in semi-defined medium with xylose (30 g/L) under micro-aerobic condition at 30 °C for 72 h. Xylitol producing ability was detected in the cases of *Su. novakii* and *C. allociferrii*, resulting in 5.9 g/L and 2.5 g/L xylitol, respectively (Table 3). In the study of Sena et al. (2017), 8.77 g/L xylitol was produced by *Su. novakii* after 24 h. However, their experiment was performed in different medium (10 g/L yeast extract, 20 g/L peptone) with higher initial xylose concentration (50 g/L) [12]. No experimental evidence of xylitol production by *C. allociferrii* has been published until this study.

Among the investigated strains, *Su. novakii* was the only yeast combining significant extracellular xylanases production with detectable xylitol formation, highlighting its potential as a naturally occurring multifunctional microorganism for single cell-mediated integrated bioprocess utilizing xylan-rich raw materials. Although the achieved xylitol concentrations remain moderate compared to other xylitol-producing strains [27], the simultaneous presence of hydrolytic and product-forming capabilities in a wild-type yeast represents an important advantage for process integration. Nevertheless, the relatively low xylanase titres and incomplete understanding of regulatory mechanisms indicate that further optimisation and metabolic characterization will be necessary before industrial implementation.

3.3. Investigation of the effect of pH and temperature on xylanase activity produced by *Su. novakii*

Determining the operating range and optimal conditions of an enzyme of interest is crucial to specify its possible fields of application. Thus, the effect of pH and temperature on the xylanase activity produced by *Su. novakii* on xylan were investigated. To produce enough volume of xylanolytic fermentation broth for the measurements, new batch of xylanase production was performed by *Su. novakii*. Extracellular xylanase activity of the culture broth was measured at 45°C by changing the pH from 2 to 6, and at pH 3 by changing the temperature from 25 °C to 65 °C.



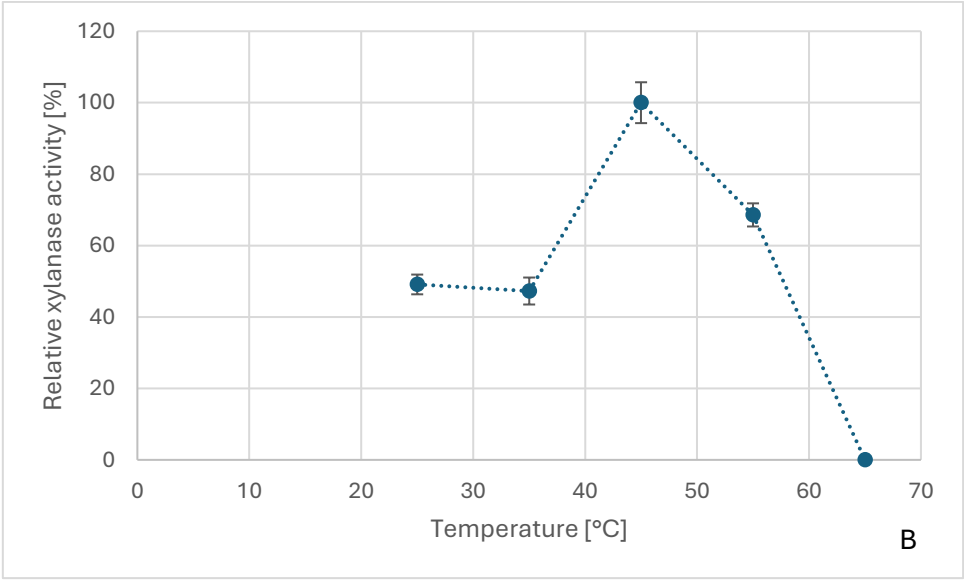


Figure 1 Relative xylanase activities at different pH values (A) and at different temperature values (B), measured in fermentation supernatant of Su. novakii cultivated in YNBw/oAA supplemented with 50 g/L birchwood xylan. The pH values were varied between 2 and 6 at 45°C, while the temperature values were changed from 25 °C to 65

°C, at pH 3. Average values of relative xylanase activity are presented with error bars representing standard deviation derived from technical duplicates of biological triplicates. Relative activities are expressed as the percentage of the maximal activity.

The xylanolytic supernatant of *Su. novakii* exhibited maximal activity under acidic conditions, with an optimum at pH 3 and 45 °C. Notably, high relative activity was retained even at pH 2, indicating pronounced acid tolerance of the produced enzyme system. Such acidic operating ranges are industrially advantageous because they reduce contamination risks and may facilitate integration with acidic biomass pretreatment processes.

The enzyme system remained active within a relatively narrow acidic range (pH 2–4), while activity rapidly declined at higher pH values. Temperature profiling similarly revealed a mesophilic-to-moderately thermotolerant character, with substantial activity maintained up to 55 °C. These properties are broadly consistent with previously reported xylanases from related *Sugiyamaella* species [25], suggesting partially conserved enzymatic characteristics within the genus.

Although the acidic activity profile is promising for industrial lignocellulose-based applications, the thermal stability and long-term operational stability of the enzyme system were not investigated in this study and require further evaluation under process-relevant conditions.

3.4.Enhancing the xylanase production of *Su. novakii*

Different fermentation conditions (aeration, medium, initial cell concentration, temperature, pH) were tested in order to find the most favourable parameters resulting in increased xylanase activity in the broth of *Su. novakii*.

Firstly, two different aeration conditions (aerobic and micro-aerobic) were tested in YNBw/oAA and semi-defined media. Aerobic and micro-aerobic conditions were set by adjusting the filling of the flasks and the rotation speed (see Materials and methods). The experiments were carried out at pH 3 with an increased initial cell concentration of 5 g/L at 30 °C for 72 hours. Xylanase activity was not measurable in the supernatants of micro-aerobic fermentations. In the case of aerobic condition, 3.21 U/mL with 0.16 standard deviation and 8.03 U/mL with 0.33 standard deviation were measured by using semi-defined and YNBw/oAA media, respectively. Statistical analysis (t-test) proved the significant effect (at significance level of 0.05) of the medium and temperature on the xylanase activity obtained (Supp. Table 1). The achieved xylanase activities are much higher than that of presented in Table 3, which can be explained by the advantage of using an increased initial cell concentration of 5 g/L instead of 2 g/L of initial cell concentration applied in previous experiments. Thus, further investigations were performed with the increased initial cell concentration under aerobic condition and by using YNBw/oAA medium.

In the second step, the effect of temperature on xylanase production was investigated under the previously selected conditions by two additional temperatures (35° C and 40° C) beside the previously tested 30 °C. These temperatures were selected because higher enzyme activity was detected above 35°C (Fig.1B), which is important in case of integrated bioprocess applications. Xylanase production at 35 °C resulted in an activity of 5.30 U/mL with 0.65 standard deviation, while at 40 °C, no xylanase activity was obtained. Since under aerobic condition and by using

YNBw/oAA medium the highest xylanase activity was reached at 30 °C (8.03 U/mL with 0.33 standard deviation) during the previous runs, this temperature was used in all the subsequent xylanase production experiments.

In the third step, xylanase production was tested by adjusting the medium pH to 2, 3, and 4 under the previously selected other conditions (aerobic condition and 30 °C). These pH range was selected because it overlaps with range of good activity of the produced xylanases (Fig.1A), which is inevitable to be used in an integrated bioprocess. Xylanase productions at pH 2, 3, and 4 resulted in xylanase activities of 7.50 U/mL (0.64 standard deviation), 7.47 U/mL (0.17 standard deviation), and 7.38 U/mL (0.20 standard deviation), respectively. Analysis of variance showed no significant difference (at significance level of 0.05) between the obtained activity values (Supp. Table 1), thus changing the pH in the tested range has no significant effect on the produced xylanase activity.

Summarizing the results of xylanases production and activity in concern of developing an integrated bioprocess based on *Su. novakii*, from the site of enzyme production and hydrolysis, the most suitable operating parameters would be aerobic conditions at 30 °C and pH 3 using YNBw/oAA medium.

3.5.Investigation of different process parameters on the xylitol production of *Su. novakii*

Su. novakii has the ability of converting xylose into xylitol (Table 3) under micro-aerobic conditions in shake flask fermentations (50 mL medium in 100-mL Erlenmeyer flask, 125 rpm) as previous tests showed. However, the production of xylanases on xylan was found to be favoured under aerobic shake flaks cultures (20 mL medium in 100-mL Erlenmeyer flask, 220 rpm). Thus, in the next step of developing a xylan-based integrated bioprocess, xylitol fermentation by *Su. novakii* was further investigated under both aerobic and micro-aerobic conditions in shake flask experiments, in addition by testing the effect of the initial cell

concentration (1 or 5 g dry cell mass /L). Other metabolites (acetic acid, lactic acid, glycerol, and ethanol) beside xylitol were also monitored during the fermentation experiments in order to identify other possible fermentative products.

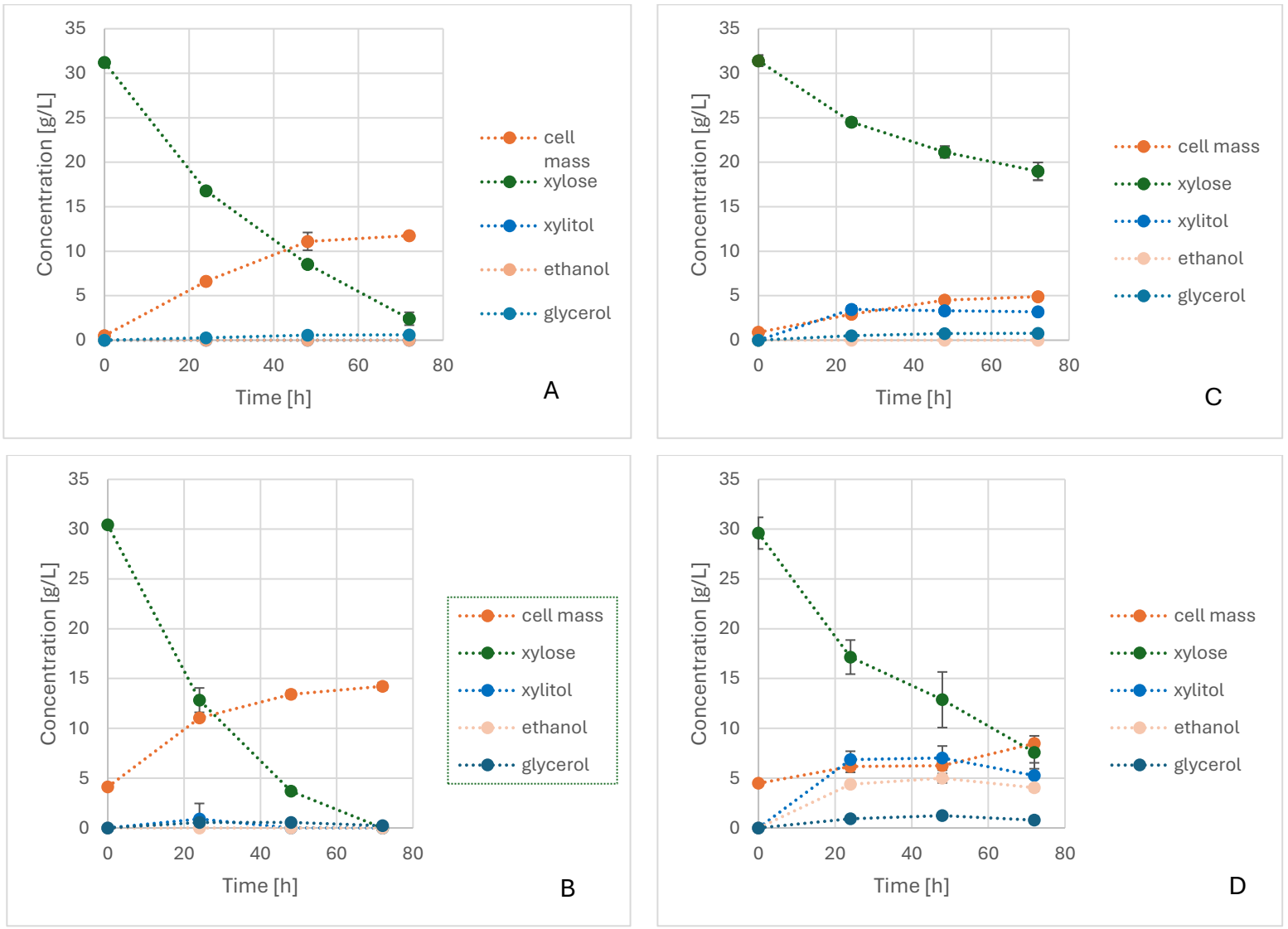


Figure 2 Concentration of cell mass, xylose, xylitol, ethanol, and glycerol of supernatants derived from fermentation of Su. novakii cultivated in semi-defined medium supplemented with 30 g/L xylose. A- fermentation under aerobic condition (20 mL medium in 100-mL Erlenmeyer flask, 220 rpm) with 1 g/L initial cell concentration B- fermentation under aerobic condition (20 mL medium in 100-mL Erlenmeyer flask, 220 rpm) with 5 g/L initial cell concentration, C- fermentation under micro-aerobic condition (50 mL medium in 100-mL Erlenmeyer flask, 125 rpm) with 1 g/L initial cell concentration, D- fermentation under micro-aerobic condition (50 mL medium in

100-mL Erlenmeyer flask, 125 rpm) with 5 g/L initial cell concentration. Average values of the concentrations are presented with error bars representing standard deviation of biological triplicates.

Aerobic fermentation resulted in intense cell mass production (10.8 g/L increase in dry cell mass) without the formation of considerable number of xylitol and by-products, and with almost complete consumption of xylose either in case of 1 g/L initial cell concentration or 5 g/L initial cell concentration. The fermentation broth contained 11.8 g/L cell mass, 0.6 g/L glycerol and 2.4 g/L residual xylose after 72 hours of fermentation (Fig. 2A) with 1 g/L initial cell concentration. With 5 g/L initial cell concentration (Fig. 2B),14.2 g/L cell mass and 0.2 g/L glycerol were measured after 72 hours of fermentation. All the xylose was consumed, and slight amount of xylitol (0.9 g/L) was detected, which were also consumed after 48 hours. In contrast with the aerobic fermentation, micro-aerobic fermentation with 1 g/L initial cell concentration (Fig. 2C) resulted in a decreased consumption of xylose (18.9 g/L residual xylose was obtained in the fermentation broth after 72 hours) and moderate propagation of cell mass (4.9 g/L) beside the production of 3.18 g/L xylitol, and negligible amount of glycerol (0.78 g/L). Micro-aerobic fermentation broth inoculated with higher (5 g/L) initial cell concentration resulted in more fermentative products: 7 g/L xylitol, 5 g/L ethanol, and 1.2 g/L glycerol were detected after 72 hours of fermentation (Fig. 2D).

Aeration strongly affected carbon flux distribution in *Su. novakii*. Under aerobic conditions, xylose was primarily channelled toward biomass formation, resulting in efficient substrate consumption but minimal xylitol accumulation. In contrast, micro-aerobic conditions promoted xylitol and ethanol production, likely due to altered intracellular redox balance. Reduced oxygen availability may limit NADH reoxidation through respiration, thereby favouring xylose reductase-mediated xylitol accumulation. Similar oxygen-dependent shifts have been described in other xylose-utilising nonconventional yeasts [28].

Increasing the initial biomass concentration further enhanced product formation under micro-aerobic conditions, suggesting that oxygen transfer limitation contributed to establishing a more reductive metabolic state. However, incomplete xylose utilisation under these conditions indicates that oxygen limitation simultaneously constrained overall metabolic activity. Therefore, precise control of aeration, e.g. through the control of oxygen transfer rate (OTR), appears to be a key parameter for balancing hydrolysis, growth, and product formation during integrated bioprocess.

The influence of pH on xylitol production by *Su. novakii* was evaluated with the aim of maximizing product concentrations. Fermentations were conducted at pH values of 2, 3, 4, 5, and 6.

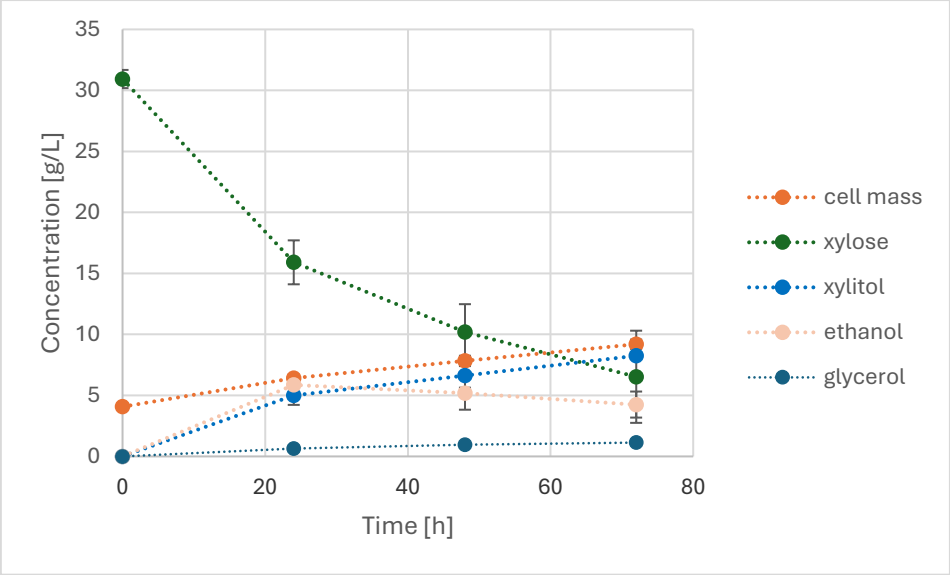


Figure 3 Concentration of xylose, xylitol, cell mass, glycerol, and ethanol of supernatants derived from fermentation of Su. novakii cultivated in semi-defined medium supplemented with 30 g/L xylose at pH 3. Average values of the concentrations are presented with error bars representing standard deviation of biological triplicates.

Figure 3 shows the fermentation profile of the xylitol production by *Su. novakii* at pH 3. The xylitol concentration was consistently increased during the fermentation, reaching its maximum at 8.3 g/L after 72 hours, indicating that xylitol was the major fermentation product. Ethanol was also produced as a notable by-product, with a maximum concentration of 4.3 g/L, while

glycerol was obtained in only minor amounts (<1.5 g/L). Similar trends were observed during the xylitol productions at other tested pHs (Suppl. Fig1A-D). The product concentrations are shown in Table 4 after 72 hours. Approximately 6 g/L of xylitol was obtained from the fermentation supernatants at each pH value, except pH 3. It means these pHs overlaps with range of good activity of the produced xylanases (Fig.1A), and with the range of optimal xylanase production (Suppl. Table 1), which is inevitable to be used in an integrated bioprocess. The ability of *Su. novakii* to maintain xylitol production under strongly acidic conditions represents a potentially valuable industrial trait. Low-pH fermentations reduce bacterial contamination risk and may decrease sterilisation and pH-control requirements, thereby improving process economics. Acid-tolerant product-forming yeasts remain relatively uncommon among nonconventional yeasts, especially in wild-type strains [29].

Although acid tolerance has previously been reported for species such as *Issatchenkia orientalis* [30, 31], industrial product formation often required metabolic engineering. In contrast, *Su. novakii* demonstrated simultaneous acid tolerance and product formation without genetic modification, which may provide advantages regarding process robustness and regulatory acceptance.

Table 4 Product concentration of supernatants derived from fermentation of Su. novakii cultivated for 72 hours in semi-defined medium, supplemented with 30 g/L xylose at pH 2,3,4,5, and 6. The average values of biological triplicates are shown with standard deviation in parentheses.

	Xylitol	Ethanol	Glycerol
pH 2	6.47 g/L (1.55)	3.82 g/L (1.02)	1.24 g/L (0.03)
pH 3	8.25 g/L (0.13)	4.25 g/L (1.06)	1.13 g/L (0.17)
pH 4	6.10 g/L (0.54)	4.41 g/L (0.38)	1.24 g/L (0.09)
pH 5	6.06 g/L (0.48)	4.14 g/L (0.08)	1.49 g/L (0.07)
pH 6	6.76 g/L (0.98)	4.33 g/L (0.88)	1.79 g/L (0.16)

Regarding the results of the statistical analysis (Suppl. Table 2) there are no significant difference between the obtained xylitol concentrations under different pH values investigated. For further investigation, pH 3 was chosen for xylitol production, which is aligned with the optimal pH conditions of xylanase production. Thus, it is conducive to implement an integrated bioprocess.

Since xylanase production by *Su. novakii* is favoured by using YNBw/oAA medium instead of semi-defined medium, xylitol fermentations were also tested and compared by using YNBw/oAA and semi-defined media. However, no significant difference was observed between the use of YNBw/oAA and semi-defined media in terms of the final xylitol concentration after 72 hours (data not shown).

D-xylose fermenting ability of *Su. novakii* UFMG-CM-Y2178 was previously evaluated by Sena et al. (2017), who reported the production of 8.8 g/L xylitol after 24 hours of cultivation. Under their experimental conditions (50 mL fermentation broth in a 125 mL Erlenmeyer flask, agitated at 200 rpm, without pH setting, in Yeast extract, Peptone, Xylose (YPX) medium), no other by-products were detected [12, 32]. Under the conditions applied in our study (50 mL fermentation broth in a 100 mL Erlenmeyer flask, agitated at 125 rpm), a similarly high xylitol concentration of 8.3 g/L was achieved in 72 hours, despite the fermentation being carried out at a lower pH (pH 3). This suggests that, *Su. novakii* maintains efficient xylitol production even under more acidic conditions, which may offer advantages for industrial processes by reducing the risk of contamination and potentially lowering the need for pH control.

3.6. Separated production of xylanases and fermentation of xylitol and ethanol; and integrated bioprocesses for xylitol and ethanol in bench-top bioreactor by using *Su. novakii*

A single cell-mediated integrated bioprocess was developed by exploiting the xylitol-producing capability and xylanase enzyme activity of *Su. novakii*. Initially, xylitol fermentation and xylanase production were independently scaled up in 500-mL bench top bioreactor in order to test their performance in fermenters under more precisely controllable environment. Following this, the two processes were merged to establish an integrated system, which was first evaluated on a xylan, and subsequently applied to barley husk as a lignocellulosic feedstock.

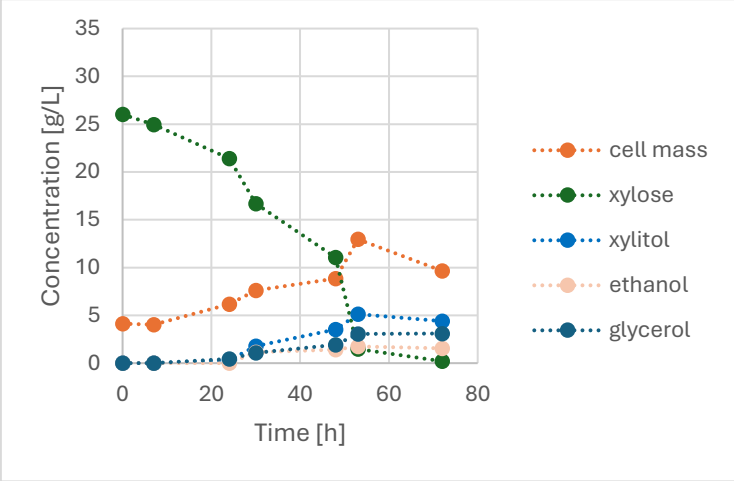


Figure 4 Concentration of xylose, xylitol, cell mass, glycerol, and ethanol of supernatants derived from fermentation of Su. novakii cultivated in 500-mL bench top bioreactor, in YNBw/oAA supplemented with 30 g/L xylose at pH 3. The fermentation was set off with 5 g/L initial cell concentration. Xylitol fermentation was carried out under conditions of 150 rpm agitation, and 1 vvm aeration.

In the separated xylitol fermentation experiment, the first day resulted in moderate cell propagation without considerable product formation. Xylitol concentration started to increase after the first day, reaching its maximum value (5.1 g/L) at 53 hours. The formation of byproducts followed the same trend, resulting in 3.0 g/L glycerol and 1.7 g/L ethanol after 53 hours of fermentation (Fig. 4). The maximal xylitol concentration obtained in the bioreactor was lower than that of achieved in shake-flask cultivation (8.3 g/L), despite the generally improved controllability of bioreactor systems. Interestingly, in contrast to the results of shake flasks experiments, more glycerol was produced than ethanol in the bioreactor. These

observations suggest that the physiological conditions established in the bioreactor were different from that occurred during shake-flask experiments. Since xylitol production in xylose-utilising yeasts is strongly influenced by oxygen availability and intracellular redox balance, differences in oxygen-transfer characteristics between shake-flask and bioreactor cultivations may have contributed to the altered metabolic behaviour.

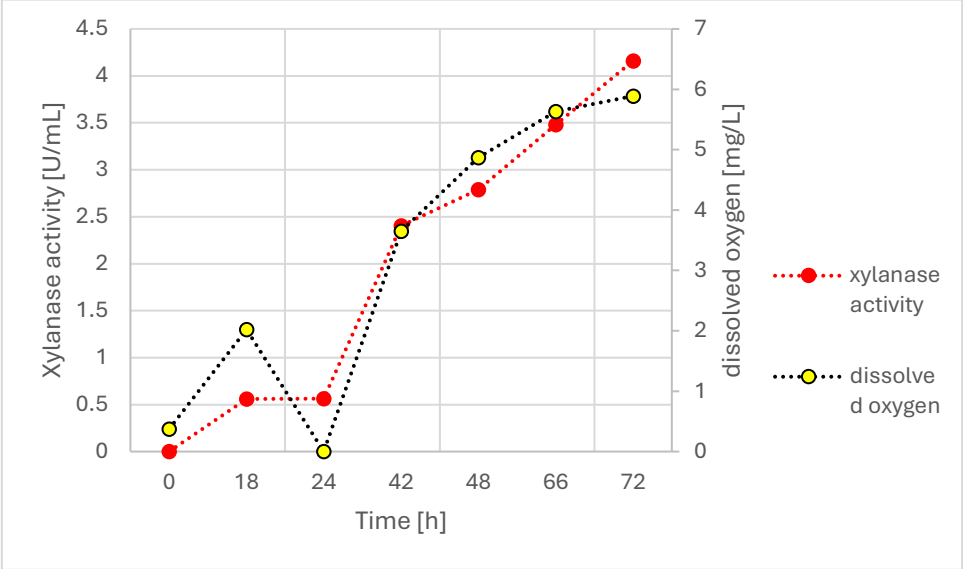


Figure 5 Xylanase activity measured from the supernatant derived from fermentation of Su. novakii cultivated in 500-mL bench top bioreactor, in YNBw/oAA supplemented with 10 g/L xylan at pH 3. The fermentation was set off with 5 g/L initial cell concentration. Xylanase production was carried out under conditions of 250 rpm agitation, and 2 vvm aeration. Dissolved oxygen concentration profile of the fermentation is shown in mg/L.

Xylanase production in bioreactor scale was carried out for 72 hours using 10 g/L xylan as carbon source in YNBw/oAA medium at 30 °C, with agitation of 250 rpm and aeration of 2 vvm. A pronounced increase in enzyme activity was observed between 24 and 42 hours, rising from 0.5 U/mL to 2.4 U/mL (Fig.5). The xylanase activity continued to increase thereafter, reaching a maximum value of 4.2 U/mL at 72 hours. This progressive rise in enzymatic activity suggests an active induction of xylanase synthesis under the applied conditions. However, the maximum xylanase activity obtained in the bioreactor was lower than that of observed in shake-flask cultivation (5.5 U/mL), which can be partly attributed to the lower initial xylan

concentration used in the bioreactor (10 g/L compared to 50 g/L in shake-flask experiments), indicating that substrate availability, in addition to aeration and agitation, could play a crucial role in maximizing xylanase enzyme production. After a small fluctuation in the first day, the DO level started to continuously increase (from zero) until the end of the enzyme production experiment, reaching a value of 6 mg/L. This change in oxygen level occurred within the same time interval as the rise observed in enzyme activity. The observed fluctuation and subsequent increase in dissolved oxygen concentration coinciding with the rise in enzyme activity may indicate a metabolic shift from the cell growth to the xylanase production phase. Oxygen availability could play a regulatory role in xylanase production, potentially through its influence on cellular respiration and energy metabolism. Although, oxygen transfer rate (OTR) of the different fermentation systems was not directly determined in the present study, our previous measurements in similar systems support the above-mentioned hypotheses about the possible differences in the aeration conditions achieved in shake flasks and bioreactor. The shake-flask cultivation condition favouring xylanases production in this study corresponds to an OTR of 6.5 mmol O_2/(L*h) [33], whereas shake-flask fermentation condition supporting xylitol accumulation corresponds to an OTR of 2.8 mmol O_2/(L*h) [34]. However, the oxygen transfer conditions achieved during bioreactor cultivations were considerably lower, possibly resulting in OTRs of 3.3 mmol O_2/(L*h) and 1.8 mmol O_2/(L*h) at 250 rpm with 2 vvm and 150 rpm with 1 vvm, respectively (not published measurements). These findings clearly highlight the importance of adequate oxygen-transfer capacity during scale-up, as differences in OTR between cultivation systems may substantially affect carbon flux, enzyme production, and reductive product formation. In addition to oxygen availability, differences in mixing, shear stress, CO_2 accumulation, and substrate gradients may also have influenced the observed metabolic response. Future studies should include quantitative characterization of oxygen-

transfer conditions and their relationship to carbon flux distribution between biomass formation, xylanases synthesis, xylitol production, and by-product formation in bioreactors.

Although the conditions applied in the bioreactor for the separate xylanases and xylitol production experiments might provide suboptimal conditions, similar settings were applied during the establishment and investigation of integrated bioprocesses for the purpose of comparability.

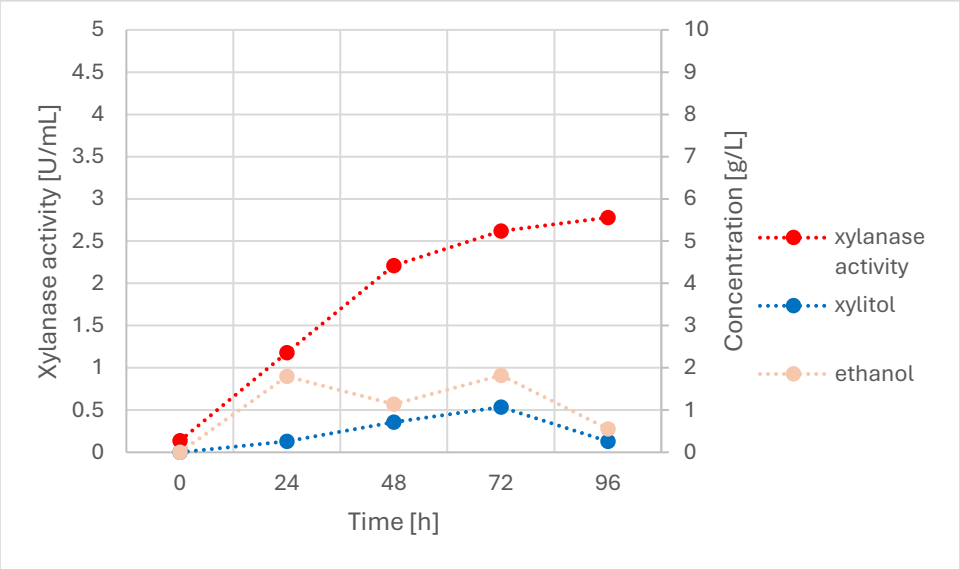


Figure 6 – Xylanase activity, and concentration of xylitol and ethanol measured from the supernatant derived from xylan-based CBP by Su. novakii carried out in 500-mL bench top bioreactor, in YNBw/oAA supplemented with 50 g/L xylan at pH 3. The fermentation was set off with 5 g/L initial cell concentration.

The established process integration was initiated with a phase dominated by enzyme production and hydrolysis, which was followed by the stage enhancing product formation. The enzyme production and hydrolysis stage were performed at 250 rpm with an aeration rate of 2 vvm for 48 hours. Subsequently, the process was continued under modified conditions of 150 rpm and 1 vvm for an additional 48 hours to promote product formation. In total, the process was maintained for 96 hours. The highest xylanase activity (2.8 U/mL) was reached after 96 hours, indicating that the enzyme producing phase was not ended by changing the conditions for xylitol production (Fig.6). Xylitol was detected in small quantities during the enzyme-forming

phase as well, but the maximal xylitol concentration (1.1 g/L) was measured at 72 hours, that corresponds to the first day of the product forming phase. Thus, in spite of the two-stage strategy aiming to enhance the enzyme synthesis and product formation separately in time, xylanases and xylitol were produced simultaneously in both stages, resulting in a real CBP and indicating the possibility of fine-tuning the fermentation conditions in order to further enhance the efficiency of this process. Ethanol production was also observed from the first day of the integrated bioprocess, reaching its maximum (1.8 g/L) also at 72 hours. In the next step, the integrated fermentation system was tested on pretreated barley husk as a real lignocellulosic biomass substrate. A mild pretreatment of barley husk was performed under conditions of 121 °C, 1 bar, and 1 hour, in pH 3 0.01 M citrate buffer. The integrated fermentation was run for 144 hours. The first stage for promoting xylanase production was carried out with 250 rpm agitation and 2 vvm aeration for 48 hours, while the second stage promoting xylitol accumulation was performed with 150 rpm agitation and 1 vvm aeration for 96 hours. After 65 hours, 0.6 U/mL xylanase activity was reached, and this value was obtained as a maximal, no further changes in its value were observed during the later stages of fermentation. After 144 hours, slight amount of xylitol (0.12 g/L) was detected from the fermentation broth. Although product concentrations obtained during fermentation remained relatively low, the study provides a proof of concept demonstrating that a wild-type *Sugiyamaella novakii* strain can simultaneously hydrolyse mildly-pretreated xylan-containing substrates and convert the released sugars into xylitol without external enzyme supplementation. The limited product titres observed with barley husk likely originated from insufficient substrate accessibility and suboptimal hydrolysis efficiency, highlighting pretreatment severity and oxygen management as major targets for future optimisation.

Although simultaneous enzyme production, hydrolysis, and product formation were successfully demonstrated in the developed integrated bioprocess system on xylan, the obtained

product concentrations remained substantially lower than those achieved during separate batch fermentations. The maximal concentrations of xylitol and ethanol obtained from xylan were only 1.1 g/L and 1.8 g/L, respectively, while process integration performed on pretreated barley husk resulted in merely 0.12 g/L xylitol. These results indicate that multiple process bottlenecks currently limit efficient substrate conversion. The primary limitation appears to be insufficient hydrolysis efficiency. During bioprocessing on xylan, the maximal extracellular xylanase activity reached only 2.8 U/mL, which was lower than the activities achieved during the separated enzyme-production experiments (4.2 U/mL). In the barley husk-based process, xylanase activity remained even lower (0.6 U/mL), suggesting restricted enzyme induction and/or reduced substrate accessibility. Consequently, the rate of xylose release from the substrate was likely insufficient to sustain high product formation. The low product formation from barley husk further highlights the importance of biomass accessibility. The mild acidic pretreatment applied in this study was likely insufficient to adequately disrupt the lignocellulosic matrix and expose hemicellulosic fractions to enzymatic attack.

In addition, the simultaneous consumption of released xylose for maintenance metabolism and biomass formation may have further restricted xylitol accumulation. The observed decrease in product concentrations after substrate depletion during separate fermentations supports the assumption that *Su. novakii* can reutilise previously formed metabolites under carbon-limited conditions.

Overall, the results suggest that hydrolysis efficiency, oxygen-transfer management, and substrate accessibility represent the major bottlenecks of the current integrated process configuration. Future optimisation should therefore focus on increasing xylanase titres, improving pretreatment severity, and implementing more precise aeration strategies to better balance enzyme production and reductive product formation.

Hoppert et al. (2025) investigated *Sugiyamaella marilandica* HYSMA006 for ethanol and xylitol production from lignocellulosic biomass. Bamboo was used after steam-explosion as the lignocellulosic feedstock. The *Su. marilandica* cells were adapted to lignocellulosic hydrolysates, and then the strain was able to utilize bamboo hydrolysate, using 20 w/w% initial concentration of the lignocellulosic hydrolysate, achieving 19.2 g/L ethanol and 3.4 g/L xylitol [35]. The harsh pre-treatment of steam explosion often results in solubilised cellulosic and hemicellulosic sugars and oligomers and a more opened structure of the substrate, that can improve the efficiency of a CBP, indicating another possible and promising way, beside adaption of the strain to the used substrate, to further improve our CBP based on *Su. novakii* in the future.

4. Conclusion

This study demonstrates that *Sugiyamaella novakii* NCAIM Y.00987 possesses a rare combination of extracellular xylanase activity and xylitol-forming capability among naturally occurring nonconventional yeasts. The strain enabled a proof-of-concept consolidated bioprocessing on xylan and a two-stage integrated bioprocess on pretreated barley husk, without external enzyme addition. The results further highlight the critical influence of oxygen availability on balancing hydrolysis and product formation. Although product titres and hydrolysis efficiency still require substantial optimisation for industrial implementation, the findings establish *Su. novakii* as a promising platform for future development of hemicellulose-based integrated bioprocesses and CBPs. In addition, *Spencermartinsiella europaea* and *Candida allociferrii* were identified as promising complementary yeasts due to their xylanase and xylitol production capabilities, respectively.

5. Declarations

Ethics statement for the use of human and animal subjects (may require consent to participate and consent to publish for human subjects)

Not applicable

Consent for publication

All authors (Kata Buda, Gábor Péter, Kaemwich Jantama and Csaba Fehér) mutually agree to submit the work and declare that the content of this manuscript has not been published or submitted to any other journal.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could appeared to influence the work reported in this paper.

Author's contribution

Kata Buda: Writing – original draft, formal analysis, data curation, investigation, methodology, and visualization. Gábor Péter: conceptualization, review and editing. Kaemwich Jantama: conceptualization, review and editing. Csaba Fehér: conceptualization, supervision, project administration, resources, validation, funding acquisition, review and editing.

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Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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