SCIENCE LETTERS

ISSN: 1675-7785
eISSN: 2682-8626

Available online at https://scilett-fsg.uitm.edu.my/

Science Letters 18(4) 2024, 133-140.

Enhancing Xylitol Production Via Recombinant Escherichia coli: A Box-Behnken Design Approach

Iffah Nazihah Mustaffa* and Zanariah Hashim

Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia (UTM), 81310 Skudai, Johor, Malaysia

ARTICLE INFO

Article history:
Received 30 April 2024
Revised 31 August 2024
Accepted 30 September 2024
Online first
Published 28 October 2024

Keywords:
Escherichia coli
Xylitol
Optimization
Response Surface Methodology

DOI:
10.24191/scl.v18i4.9722

ABSTRACT

To enhance xylitol production while addressing the inefficiencies of conventional methods, this study explores the use of genetically modified Escherichia coli BL21 (DE3). Utilizing glucose as the primary substrate, this research capitalizes on the metabolic versatility of *E. coli* to convert glucose, a readily available and cost-effective sugar, into xylitol. This process harnesses the potential of microbial synthesis for xylitol, a sugar alcohol significant for its dental health benefits and non-caloric nature. Production process optimization was conducted through Response Surface Methodology (RSM) integrated with a Box-Behnken Design (BBD). This approach facilitated the systematic evaluation of three key culture conditions: post-induction temperature (36.2°C), pH (7.2), and agitation rate (184.1 rpm). The optimization resulted in a substantial 2.7-fold increase in xylitol concentration, achieving up to 1.60 g/L. The model's effectiveness was validated by a high coefficient of determination ($R^2 = 0.9847$), emphasizing the predictive accuracy and robustness of the experimental design. This study demonstrates an economically and environmentally sustainable method for xylitol production. It advances the field of microbial engineering, underscoring the critical role of integrating advanced genetic modifications with process optimization for efficient biochemical production.

INTRODUCTION

The escalating demand for xylitol, recognized for its dental health and diabetes benefits, necessitates eco-friendly and efficient production methods. Traditional extraction from fruits and vegetables involves energy-intensive chemical processes, prompting a shift towards microbial biotechnological synthesis as a sustainable alternative. This method potentially reduces energy consumption and uses renewable resources, solving conventional processes' sustainability and cost concerns [1-3].

* Corresponding author. *E-mail address*: iffahnazihah@graduate.utm.my
https://doi.org/10.24191/scl.v18i4.9722

E. coli has emerged as a viable candidate for recombinant xylitol production among various microbial hosts. Its rapid growth rate, genetic tractability, and ability to utilize a wide range of substrates make *E. coli* an attractive option for bioengineering applications for sustainable chemical production [4,5]. Furthermore, metabolic engineering efforts have demonstrated the feasibility of enhancing *E. coli*'s native pathways to optimize xylitol production, leveraging substrates like glucose and xylose, which are abundant and cost-effective [6,7].

Utilizing BBD-RSM to optimize xylitol production in recombinant *E. coli* represents a strategic approach to optimizing bioprocess parameters efficiently. This method significantly reduces experimental runs while accurately assessing the impact of culture conditions on xylitol yield. BBD streamlines optimization by avoiding extreme factor values and merging biotechnological innovation with statistical techniques for a cost-effective enhancement of microbial production systems [8-12].

This study uses a BBD-RSM framework to optimize xylitol production in genetically engineered *E. coli* by optimizing post-induction temperature, pH, and agitation rate. It explores these parameters' interactions to find optimal conditions, enhancing efficiency and sustainability in bio-based production. The research could offer greener, cost-effective alternatives to traditional chemical synthesis, contributing to sustainable bioproduction and aligning with global sustainability objectives.

METHODOLOGY

Microbial Strain

In the recombinant *E. coli* BL21 (DE3) strain obtained from Novagen, the *phosphoglucose isomerase* gene (PGI) was excised to reroute glucose metabolism away from the glycolytic pathway toward the pentose phosphate pathway (PPP). Concurrently, the xylitol-5-phosphate dehydrogenase (*xpdh*) gene, sourced from *Clostridium difficile*, was integrated into this host using the pET21 plasmid as a vector. This strategic incorporation of the *xpdh* gene facilitates the enzymatic transformation of D-xylulose-5-phosphate into xylitol within the PPP, optimizing the metabolic flux for enhanced xylitol production.

Culture Conditions and Induction

The recombinant strains were cultured for 24 h in a 250 mL shake flask, each containing 50 mL Terrific Broth (TB) (Solarbio, Beijing, China) supplemented with 100 µg/mL chloramphenicol (Sigma-Aldrich, MO, USA). Cultures were grown until an OD600 of 0.6 was reached, at which point xylitol production was induced by the addition of 0.1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) (Merck, Darmstadt, Germany) and 20 g/L glucose (Sigma-Aldrich, MO, USA). After that, the cultures were incubated under conditions predefined by the experimental design.

Box-Behnken Design and Statistical Analysis

The impact of three factors—post-induction temperature (A: 30, 35, 40°C), pH (B: 5, 7, 9), and agitation rate (C: 160, 180, 200 rpm)—on xylitol production was investigated using a Box-Behnken Design integrated with Response Surface Methodology, employing Design Expert 13.0 software (Stat-Ease Inc., Minneapolis, MN, USA). To ensure data robustness, fifteen experiments were conducted in triplicates (n=3). Variables were set at three levels: low (-1), medium (0), and high (1), with xylitol concentration measured after 24 hours.

Subsequent analysis was performed using Analysis of Variance (ANOVA) to ascertain the model's statistical significance and elucidate the variables' interactions. This rigorous analytical approach facilitated the derivation of a second-order polynomial equation which precisely identified the optimal conditions for

https://doi.org/10.24191/scl.v18i4.9722

xylitol production. These conditions were then subjected to experimental validation, confirming the model's accuracy and the optimized parameters' efficacy in enhancing xylitol yield.

Quantifying Xylitol and Glucose Concentration via High-Performance Liquid Chromatography

The quantification of glucose and xylitol concentrations was meticulously conducted using High-Performance Liquid Chromatography (HPLC) technology provided by Waters (Milford, MA, USA), equipped with a refractive index (RI) detector. This analytical procedure was facilitated by utilizing a COSMOSIL Sugar-D Packed column (4.6ID x 250 mm, Nacalai Tesque column), carefully selected for its specificity to the compounds of interest. The chromatographic separation was achieved under precise conditions, with the column being eluted at an external temperature of 50°C utilizing a mobile phase composed of filtered acetonitrile/deionized water (85/15, v/v) at a flow rate of 1.0 ml/min over 20 minutes [13]. To ensure the fidelity of detection, the internal temperature of the detector was maintained at 30°C, with the sensitivity set at 64 mV. Each analysis entailed injecting a 10 µl sample volume, with all samples subjected to filtration through a 0.22 µm syringe filter before injection to preclude particulate interference. The analytical rigor was further underscored by the calibration process, wherein the areas of the peaks detected were meticulously correlated with a calibration curve of standards.

RESULT & DISCUSSION

Optimization for Xylitol Production through Box-Behnken Design

The study employed BBD to systematically explore the effects of post-induction temperature, initial pH, and agitation rate on xylitol production by recombinant *E. coli*. The *E. coli* BL21 (DE3) strain was chosen due to its proven effectiveness in protein stability and recombinant expression, optimizing xylitol synthesis by enhancing metabolic flux through the pentose phosphate pathway [14-15]. This strain has been previously utilized in research studies for high-yield bioproduct synthesis [16-17], making it ideal for xylitol production. The selection of the three factors for xylitol production optimization is grounded on significant findings from a fractional factorial design study from Kamarulzaman [7]. Kamarulzaman's research identified these parameters as significantly impacting xylitol yield, further supported by Abd Rahman et al.'s findings. The ranges for these factors were determined through combined insights from both studies, emphasizing their critical role in maximizing xylitol production [6-7]. Through 15 randomized trials (n=3), optimal conditions were identified, demonstrating a significant range in xylitol concentrations from 0.08 to 1.05 g/L (Table 1). This approach facilitated the development of a quadratic model to predict xylitol yield, validated by Design Expert 13.0 software (Stat-Ease Inc., Minneapolis, MN, USA), emphasizing the model's robustness without requiring transformation [18].

Table 1. Experimental design of independent variables and responses

Std	Run	A: Post-induction temperature (°C)	B: pH	C: Agitation Rate (rpm)	Response 1: Xylitol Concentration (g/L)	Response 2: Glucose Concentration (g/L)
1	1	30	5	180	0.08	1.67
10	2	35	9	160	0.46	1.13
6	3	40	7	160	0.78	0.72
14	4	35	7	180	1.05	0.41
13	5	35	7	180	1.01	0.44
5	6	30	7	160	0.6	0.85
7	7	30	7	200	0.7	0.76
11	8	35	5	200	0.32	1.31
3	9	30	9	180	0.57	0.91
12	10	35	9	200	0.5	0.96
9	11	35	5	160	0.27	1.42

https://doi.org/10.24191/scl.v18i4.9722

8	12	40	7	200	0.94	0.56
15	13	35	7	180	0.96	0.52
4	14	40	9	180	0.43	1.17
2	15	40	5	180	0.39	1.26

Statistical Validation and Analysis of the Experimental Model

The robustness of the quadratic model was rigorously evaluated using ANOVA, focusing on the significance of model terms as indicated by *p*-values. Analysis of variance data for the response surface quadratic model is shown in Table 2. A critical *p*-value of 0.0005 ($p < 0.05$) affirmed the model's significance, complemented by an insignificant lack of fit (*p*-value of 0.3079), thereby reinforcing the model's alignment with experimental data. Model terms ($p < 0.05$) of A, B, AB, A^2, B^2, and C^2 were identified as significant. The significance of the variables in descending order is as follows: pH (B), post-induction temperature (A), and agitation rate (C). This phase elucidated the intricate effects of post-induction temperature, initial pH, and their interactions on xylitol production, establishing a foundation for subsequent optimization efforts [8].

Table 2. ANOVA for response surface quadratic mode

Variables	Sum of Squares	df	Mean Square	F-value	*p*-value*
Model	1.20	9	0.1334	35.68	0.0005
A-Post-Induction Temperature	0.0435	1	0.0435	11.64	0.0190
B-pH	0.1013	1	0.1013	27.08	0.0035
C-Agitation Rate	0.0153	1	0.0153	4.10	0.0989
AB	0.0506	1	0.0506	13.54	0.0143
AC	0.0009	1	0.0009	0.2407	0.6444
BC	0.0000	1	0.0000	0.0067	0.9380
A^2	0.0681	1	0.0681	18.22	0.0079
B^2	0.9354	1	0.9354	250.23	< 0.0001
C^2	0.0495	1	0.0495	13.25	0.0149
Residual	0.0187	5	0.0037		
Lack of Fit	0.0146	3	0.0049	2.40	0.3079
Pure Error	0.0041	2	0.0020		
Cor Total	1.22	14			

*Significant $p < 0.05$ by ANOVA

The derivation of a regression equation from the ANOVA (Equation 1) results facilitated the prediction of xylitol yields under varying experimental conditions, enabling a strategic approach to optimizing production parameters.

$$\text{Xylitol production (g L-1)} = 1.00667 + 0.07375 * A + 0.1125 * B + 0.04375 * C + -0.1125 * AB + 0.015 * AC + -0.0025 * BC + -0.135833 * A^2 + -0.503333 * B^2 + -0.115833 * C^2 \quad (1)$$

The model's explanatory power (as shown in Table 3) was reflected in an R^2 value of 0.9847, indicative of its capacity to account for 98.47% of the variation in xylitol production. An R^2 value exceeding 0.8 indicates a model's adequate fit [19].

Table 3. Fit summary of the model

Std. Dev.	0.0611	R^2	0.9847
Mean	0.6040	Adjusted R^2	0.9571
C.V. %	10.12	Predicted R^2	0.8006
		Adeq Precision	18.7876

https://doi.org/10.24191/scl.v18i4.9722

This high degree of model fidelity, supported by the generation of contour and three-dimensional response surface plots, as shown in Fig. 1, allowed for precise identification of optimal production conditions.

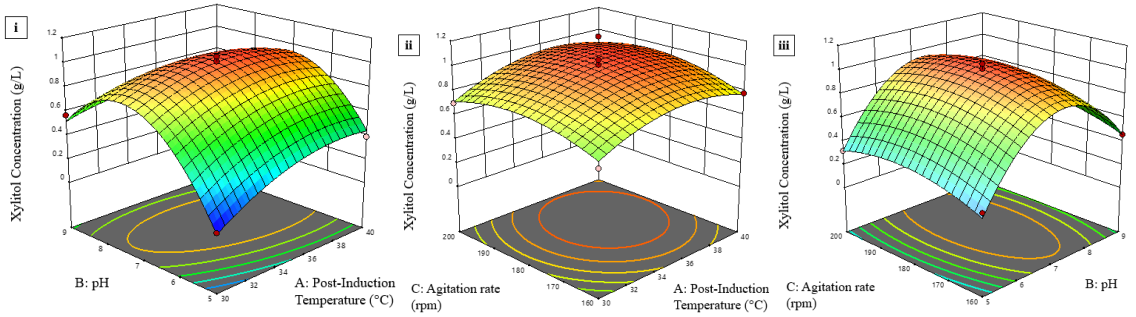

Fig. 1. Response surface plots of xylitol production: (i) Temperature vs. pH with constant agitation rate (180 rpm), (ii) Agitation vs. temperature with constant initial pH (pH 7), (iii) pH vs. agitation with constant post-induction temperature (35°C). The xylitol production of recombinant *E. coli* was measured after 24 h of post-induction time.

Contour and response surface plots, delineated via Design Expert software, facilitated the elucidation of interactions among the variables implicated in xylitol production. These graphical representations were instrumental in determining the optimal levels of responses corresponding to specific factor values. Specifically, the contour plots depicted xylitol output as a function of two independent variables while holding a third variable constant. The plots indicated that the interaction between temperature and agitation did not significantly affect the outcomes, as detailed in Table 2 and illustrated in Fig. 1(ii).

The pivotal role of initial pH in the bioconversion of glucose to xylitol was underscored by its statistical significance, evidenced by a priori *p*-value assessment as presented in Table 2, with optimal xylitol yields occurring at a moderate pH level, as seen in Fig. 1. This observation is congruent with the findings of Cheng et al. [20], who reported that a medium pH ranging from 5 to 8 markedly enhances xylitol productivity.

Temperature, a critical determinant of cellular metabolism and enzyme efficiency, was also found to influence xylitol yields substantially, as evidenced by Tamburini et al. [21], who demonstrated that over 90% of the substrate was transformed into xylitol within a temperature window of 28°C to 37°C. These results align with the current study's findings, where the highest xylitol concentrations were obtained at moderate temperatures, as shown in Fig. 1. A decline in xylitol production was noted when the temperature approached either extremity of 30°C or 37°C. Therefore, the interplay between a moderate post-induction temperature and initial pH is crucial for enhanced xylitol synthesis.

Agitation rate exhibited a parallel effect to temperature and pH on xylitol production, with the apex of production occurring at a mid-range agitation rate of 184.1 rpm. Deviations from this moderate rate resulted in a reduced xylitol yield.

The model-based response surface plots were pivotal in predicting the cultural conditions most conducive to xylitol production, pinpointing an optimum at a post-induction temperature of 36.2°C, an initial pH of 7.2, and an agitation rate of 184.1 rpm.Subsequent empirical validation of these model-proposed optimal conditions yielded a xylitol concentration of 1.6 g/L, which notably exceeded the model's prediction of 0.6 g/L, as shown in Table 4. This experimental outcome served to substantiate the model's validity.

https://doi.org/10.24191/scl.v18i4.9722

Table 4. Summary of the optimized cultural conditions for xylitol production

	Before optimization	After optimization
Cultural conditions:		
Post-induction temperature (°C)	30	36.2
Initial pH	7	7.2
Agitation rate (rpm)	160	184.1
Response:		
Xylitol production (g/L)		
Predicted		1.03
Actual	0.6	1.60

In summary, the optimization methodology augmented xylitol production by 2.7. The study validated the efficacy of RSM in dissecting the complex interdependencies among various factors influencing xylitol production. This approach not only simplifies the optimization process but also discerns the significance level of each variable within the production matrix. Collectively, RSM has proven to be an effective tool for forecasting and achieving optimal xylitol production outcomes.

Sugar Utilization Efficiency under Optimized Conditions

The optimization notably enhanced glucose utilization, as represented in Fig. 2, reducing residual glucose from 17.0 g/L under unoptimized conditions to 3.0 g/L. This improvement signifies increased process efficiency and reduced resource wastage, reflecting the potential of optimized bioprocesses for sustainable biotechnological applications. The efficient sugar utilization observed echoes the findings of Tamburini et al. [21], highlighting the adaptability and efficiency of microbial systems under tailored conditions.

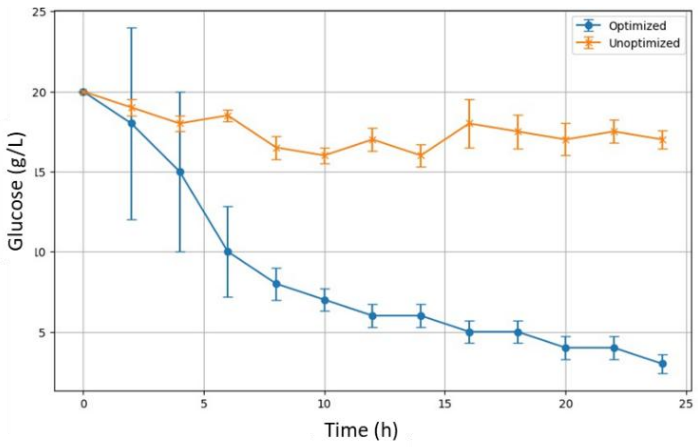

Fig. 2. The glucose concentration in culture at different cultural conditions: optimized and unoptimized conditions.

CONCLUSION

This study employed a Box-Behnken design within a Response Surface Methodology framework to optimize xylitol production in recombinant *E. coli*. The optimal conditions identified were a post-

https://doi.org/10.24191/scl.v18i4.9722

induction temperature of 36.2°C, a pH of 7.2, and an agitation rate of 184.1 rpm. Under these conditions, xylitol production was significantly enhanced, achieving a 2.7-fold increase to 1.6 g/L while reducing residual glucose from 17.0 g/L to 3.0 g/L. These results were not only experimentally validated but also demonstrated the effectiveness of the optimization strategy. The findings highlight the potential of scalable biotechnological processes for efficient xylitol production and pave the way for future research endeavors, which may include further bioreactor optimizations and genetic enhancements to increase yields. This study advances sustainable and innovative bioprocessing techniques, promoting a more efficient production of bio-based products.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of Universiti Teknologi Malaysia through the UTM Transdisciplinary Research Grant (vot. 05G43). Appreciation is extended to all research contributors, thanks to the Faculty of Chemical and Energy Engineering for their invaluable support.

AUTHOR'S CONTRIBUTION

Iffah Nazihah Binti Mustaffa conducted the research, conducted the experiments, analyzed the data, and wrote the manuscript. Dr. Zanariah Hashim supervised the research, providing critical feedback and guidance throughout the study. Both authors reviewed and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. This research was conducted without commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

[1] da Silva, S. S., & Chandel, A. K. (Eds.). (2012). *D-Xylitol: Fermentative production, application, and commercialization*. Springer. https://doi.org/10.1007/978-3-642-31887-0

[2] Dasgupta, D., Bandhu, S., & Adhikari, D. K. (2017). Challenges and prospects of xylitol production with whole cell bio-catalysis: A review. *Microbiological Research, 197*, 9–21. https://doi.org/10.1016/j.micres.2016.12.012

[3] Saha, B. C. (2003). Hemicellulose bioconversion. *Journal of Industrial Microbiology & Biotechnology, 30*(5), 279–291. https://doi.org/10.1007/s10295-003-0049-x

[4] Cirino, P. C., Chin, J. W., & Ingram, L. O. (2006). Engineering *Escherichia coli* for xylitol production from glucose-xylose mixtures. *Biotechnology and Bioengineering, 95*(6), 1167–1176. https://doi.org/10.1002/bit.21082

[5] Yu, J., Zhang, X., & Tan, T. (2011). Metabolic engineering of *Escherichia coli* for biotechnological production of high-value organic acids and alcohols. *Applied Microbiology and Biotechnology, 89*(3), 573–583. https://doi.org/10.1007/s00253-010-2970-z

[6] Abd Rahman, R., et al. (2020). Immobilization of recombinant *Escherichia coli* on multi-walled carbon nanotubes for xylitol production. *Enzyme and Microbial Technology, 135*, Article 109495. https://doi.org/10.1016/j.enzmictec.2019.109495

[7] Kamarulzaman, U. Q. (2018). *Optimization of cultural conditions for xylitol production from glucose by recombinant Escherichia coli* [Unpublished master's thesis]. Universiti Teknologi Malaysia.

[8] Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965–977. https://doi.org/10.1016/j.talanta.2008.05.019

[9] Box, G. E. P., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics, 2*(4), 455–475.

[10] Kazemian, M. E., Gandjalikhan Nassab, S. A., & Jahanshahi Javaran, E. (2021). Comparative study of Box-Behnken and central composite designs to investigate the effective parameters of ammonia-water absorption refrigerant system. *Proceedings of the Institution of Mechanical Engineers, Part C: Journal of Mechanical Engineering Science, 235*(16), 3095–3108.
[11] Zare, H., Mir Mohammad Sadeghi, H., & Akbari, V. (2019). Optimization of fermentation conditions for reteplase expression by *Escherichia coli* using response surface methodology. *Avicenna Journal of Medical Biotechnology, 11*(2), 162–168.
[12] Li, H., van den Driesche, S., Bunge, F., Yang, B., & Vellekoop, M. J. (2019). Optimization of on-chip bacterial culture conditions using the Box-Behnken design response surface methodology for faster drug susceptibility screening. *Talanta, 194*, 627–633. https://doi.org/10.1016/j.talanta.2018.10.048
[13] Zhang, X., Lai, L., Xu, G., Zhang, X., Shi, J., & Xu, Z. (2017). Effects of pyruvate kinase on the growth of *Corynebacterium glutamicum* and L-serine accumulation. *Process Biochemistry, 55*, 32–40. https://doi.org/10.1016/j.procbio.2017.01.028
[14] Studier, F. W. (2005). Protein production by auto-induction in high-density shaking cultures. *Protein Expression and Purification, 41*(1), 207–234. https://doi.org/10.1016/j.pep.2005.01.016
[15] Zhang, X., Jantama, K., Moore, J. C., Shanmugam, K. T., & Ingram, L. O. (2011). Metabolic evolution of energy-conserving pathways for succinate production in *Escherichia coli*. *Proceedings of the National Academy of Sciences, 106*(48), 20180–20185. https://doi.org/10.1073/pnas.0905396106
[16] Liu, X. X., Wang, Y., Zhang, J. H., Lu, Y. F., Dong, Z. X., Yue, C., Huang, X. Q., Zhang, S. P., Li, D. D., Yao, L. G., & Tang, C. D. (2024). Engineering *Escherichia coli* for high-yielding 2,5-dimethylpyrazine synthesis from L-threonine by reconstructing metabolic pathways and enhancing cofactors regeneration. *Biotechnology for Biofuels and Bioproducts, 17*(1), Article 44. https://doi.org/10.1186/s13068-024-02487-4
[17] Pu, W., Chen, J., Zhou, Y., Qiu, H., Shi, T., Zhou, W., Guo, X., Cai, N., Tan, Z., Liu, J., Feng, J., Wang, Y., Zheng, P., & Sun, J. (2023). Systems metabolic engineering of *Escherichia coli* for hyper-production of 5-aminolevulinic acid. *Biotechnology for Biofuels and Bioproducts, 16*(1), Article 31. https://doi.org/10.1186/s13068-023-02280-9
[18] Montgomery, D. C. (2022). *Design and analysis of experiments* (9th ed.). Wiley.
[19] Yemis, O., & Mazza, G. (2012). Optimization of furfural and 5-hydroxymethylfurfural production from wheat straw by a microwave-assisted process. *Bioresource Technology, 109*, 215–223.
[20] Cheng, K.-L., et al. (2009). Optimization of pH and acetic acid concentration for bioconversion of hemicellulose from corncobs to xylitol by *Candida tropicalis*. *Biochemical Engineering Journal, 43*(2), 203–207. https://doi.org/10.1016/j.bej.2008.09.012
[21] Tamburini, E., et al. (2015). Optimized production of xylitol from xylose using a hyper-acidophilic *Candida tropicalis*. *Biomolecules, 5*(3), 1979–1989.