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INVESTIGATION OF THE POTENTIAL OF XYLOSE ASSIMILATION BY *BRETTANOMYCES BRUXELLENSIS*

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ABSTRACT

Second-generation ethanol production is a promising but still challenging industrial process, in which the microorganism of choice is essential to achieve high efficiency. *Brettanomyces bruxellensis* has been suggested as a potential candidate to be employed in this industry, since it presents relevant physiological features, especially regarding to its fermentative capacity and resistance to inhibitors present in the industrial substrate. Previous works have suggested that Glucose Catabolite Repression (GCR) is a strain-specific trait in *B. bruxellensis*, as well as less strict than in *Saccharomyces cerevisiae*. A less stringent GCR is important since glucose often inhibits the utilization of other carbon sources, decreasing the efficiency of the fermentative process. In this sense, the present study investigated whether glucose presents any repressive effect in the assimilation of xylose, the major pentose in lignocellulosic hydrolysate, in *B. bruxellensis* JP19M strain. The results showed that the presence of glucose (5 g/L) did not inhibit xylose consumption by *B. bruxellensis* JP19M. In addition, xylose-assimilatory genes such as *XYL1*, *XYL2* and *XKS1* were not repressed by glucose. By analysing both physiological and gene expression data, it was able to confirm that JP19M does not respond to GCR. The available results might also enhance the potential interest in the application of *B. bruxellensis* in the second-generation ethanol production as well as boost the development of genetic engineering strategies aiming to overcome its poor fermentative capacity in xylose.

Keywords: Xylose metabolism. Ethanol production. Sugar co-consumption. Glucose repression. Gene expression.

1 INTRODUCTION

The yeast *Brettanomyces bruxellensis* can produce ethanol by fermentation from different carbon sources and under different oxygen availabilities, such as aerobiosis, oxygen limitation and anaerobiosis.[1,2,3,4] The adaptation to different industrial environments allows *B. bruxellensis* to compete with *Saccharomyces cerevisiae* for different substrates, as well as to use sugars not natively consumed by wild-type strains of *S. cerevisiae*, such as cellobiose, D-xylose and L-arabinose.[5,6] Besides a wide range of sugar assimilation and the ability to produce ethanol, the tolerance to fermentation inhibitors found in the lignocellulosic hydrolysate led *B. bruxellensis* to be recently suggested as a potential ethanol producer in second-generation ethanol industry.[7] Furthermore, *B. bruxellensis* JP19M strain is able to co-consume glucose and other sugars.[3] This finding is quite important, especially in the fermentation industry since this ability ultimately allows the microorganism to a rapid and simultaneous conversion of different sugars to ethanol.[8]

The co-consumption of sugars when glucose is available is a rare trait in yeasts, since even low amounts are usually enough to trigger a metabolic effect often called “Glucose Catabolite Repression” (GCR).[9,10] The GCR has a clear phenotype, which is the preference for glucose assimilation to the detriment of other carbon sources.[11,12] In *S. cerevisiae*, the presence of glucose triggers the activation of fermentation-related genes, the ones involved in the respiratory metabolism are deactivated.[13,14] This genetic programming is one of the pillars of another metabolic trait, the so-called Crabtree effect. In addition, the Crabtree effect improves cell adaptation to glucose metabolism through increased glycolytic ATP and ethanol production.[15] In *B. bruxellensis*, on the other hand, much less information is available in this regard.

Since the consumption of sugars other than glucose in the substrate might accelerate the industrial process and increase both yield and productivity, the aim of this work was to investigate the effect of glucose on the metabolism xylose in *B. bruxellensis* JP19M. Once it has been recently suggested that this strain might not be under a strict GCR control,[3] it was analysed whether glucose could trigger any repressive effect on the consumption of xylose by this strain, both in physiological and transcriptional levels. The results presented herein confirm that *B. bruxellensis* JP19M is not susceptible to glucose effect and continues to assimilate both glucose and xylose in mixed medium.

2 MATERIAL & METHODS

B. bruxellensis JP19M used in the present work was isolated from fuel-ethanol production processes and properly identified by molecular biology.[16] Fermentation assays were performed under aerobic conditions. For this, JP19M cells were precultured in YPD at 30 °C and 160 rpm for 48 h. Subsequently, cells were collected by centrifugation, washed with sterile distilled water, and suspended in sterile saline solution to a concentration corresponding to 0.1 unit of absorbance at 600 nm. Then, cells were transferred to flasks containing YNB medium supplemented with ammonium sulphate and one of the following carbon sources: D-xylose, D-glucose, L-arabinose, or a combination of these sugars (20 g/L each, mixed medium I). Cultures were maintained for 48 h at 30 °C and 160 rpm. Samples were collected at different times for absorbance determination and metabolites measurements by HPLC.

In order to investigate the influence of glucose on xylose metabolism, cells from *B. bruxellensis* JP19M were pre-cultivated in YNB medium, supplemented with ammonium sulphate and xylose at 30 ºC until reaching OD (600 nm) of 1.0. Then, cells were collected after centrifugation, washed, suspended in saline solution and added to flasks containing YNB medium, supplemented with ammonium sulphate, D-xylose plus different glucose concentrations. Samples were analyzed for sugar consumption by HPLC and RNA extraction for gene expression assays. The cultures were carried out both in biological and technical duplicates. The concentrations of ethanol, xylitol, acetate, glycerol, and sugars were determined by HPLC in a Shimadzu system. The software used for data acquisition was LC Solutions, manufactured by Shimadzu Corporation (Kyoto, Japan).

B. bruxellensis JP19M cells collected were used for RNA extraction by using Maxwell® 16 LEV simplyRNA Purification Kits (Promega, USA) and 1 µg of total RNA was converted to cDNA by using GoScript™ Reverse Transcriptase Kit (Promega, USA). The nucleotide sequences for the target genes were obtained from *D. bruxellensis* CBS 2499 database. Primers were designed using the Primer-BLAST tool and evaluated by the OligoAnalyzer IDT tool. *TEF1* and *ACT1* were used as reference genes, as previously reported.[17] RT-qPCR analyses were performed using GoTaq® qPCR Master Mix kit (Promega, USA) in 96-well plates. Data normalization, as well as the geNorm analyses and the determination of the relative quantification were carried out following the recommendations proposed by de Barros Pita and MIQE Guidelines.[17,18]

3 RESULTS & DISCUSSION

The ability to ferment D-xylose and L-arabinose by *B. bruxellensis* JP19M was evaluated under aerobic conditions. In a glucose-based medium JP19M strain presented a fermentative performance (Table 1) similar to previous studies.[4] When D-xylose was the sole carbon source, *B. bruxellensis* completely consumed this sugar, but was not able to produce ethanol in this condition. Despite previous studies reporting ethanol production by *B. bruxellensis* from secondary carbon sources, the present data indicate that carbon was preferentially directed to biomass formation and trace xylitol amounts.[5,9] In addition, JP19M was also able to metabolize L-arabinose, as previously observed for other yeasts.[20,21] However, cells did not produce ethanol from L-arabinose, with a preferential targeting for biomass formation, parallel to that observed for *Spathaspora passalidarum* CMUWF1–2.[22] Because JP19M strain was not able to efficiently ferment either D-xylose or L-arabinose individually, other experiments were carried out to test whether the presence of glucose might favor pentose consumption. Glucose was preferably consumed, followed by D-xylose, whereas L-arabinose was not significantly used. The preference for D-xylose over L-arabinose has already been described in a modified strain of *S. cerevisiae*.[23] In contrast, *Candida akabanensis* UFVJM-R131 is capable of using both sugars simultaneously, converting them to ethanol.[24] Interestingly, productivity values in the mixed medium were higher than those observed in glucose-based medium (Tables 1). One possible explanation for this observation is that D-xylose might have been diverted to biomass formation, whereas glucose was probably directed to fermentation.

Table 1 Fermentative parameters of *B. bruxellensis* JP19M in medium containing glucose, xylose, arabinose or in mixed medium under aerobic conditions

Condition	Glucose consumed (g/L)	D-Xylose consumed (g/L)	L-Arabinose consumed (g/L)	$Y_{x/s}$	$Y_{p/s}$ ethanol	Productiv. * (g.l.h)
D-Glucose	20 ± 0.00	-	-	0.55 ± 0.00	0.21 ± 0.00	0.086 ± 0.00
D-Xylose	-	19.71 ± 0.07	-	0.48 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
L-Arabinose	-	-	17.38 ± 0.11	0.59 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Mixed medium	20.00 ± 0.00	11.16 ± 0.22	0.00 ± 0.00	0.24 ± 0.01	0.22 ± 0.01	0.148 ± 0.00

Since the glucose repression effect seems to be a strain-dependent trait in *B. bruxellensis*[3], the present study aimed to investigate the real influence of glucose on the xylose metabolism in JP19M strain. The results showed that the presence of glucose did not inhibit xylose consumption by *B. bruxellensis* JP19M, in any of the concentrations tested (0 to 5 g/L), similar to *S. passalidarum*.[23,24] This profile is in accordance with the assumption that glucose repression in some strains of *B. bruxellensis* is less tightly controlled than in *S. cerevisiae*. [3,9,25] The gene expression results presented below are consistent with the physiological findings. The results show that *HXT6/7* (hexose transporter), xylose-assimilatory genes (*XYL1*, *XYL2* and *XKS1*) were not repressed by glucose in JP19M. Furthermore, the results clearly show the absence of glucose regulation for *FBP1*, *ATP1* and *SDH1* genes in this strain. This observation is different from the reports for *B. bruxellensis* GDB 248 and *S. cerevisiae*.[25,26] In addition, the glucose led to the upregulation of fermentative pathway genes (*PDC1* and *ADH*). It is interesting to note that, as a Crabtree positive yeast, *B. bruxellensis* is prone to fermentation once glucose is readily available.[27] Finally, it was observed that the gene coding for hexokinase 2 (*HXK2*) was upregulated in the mixed medium. The expression profile of *HXK2* and the other genes investigated in the present work, suggest that the absence of a clear GCR phenotype in *B. bruxellensis* JP19M.

4 CONCLUSION

The results reported in the present work show that, despite being able to use both pentoses, *B. bruxellensis* does it preferentially via biomass formation to the detriment of fermentation. On the other hand, it was observed that D-xylose and glucose might be co-consumed, which is a desired trait for a microorganism to be employed in this industry. The absence of a GCR phenotype in

B. bruxellensis JP19M might be faced as an opportunity to boost the development of genetic engineering strategies to outcome its poor fermentative performance in xylose. Once this bottleneck has been bypassed, *B. bruxellensis* JP19M might arise as potential producer microorganism in the second-generation ethanol industry.

REFERENCES

[1] DE BARROS PITA, W., CASTRO SILVA, D., SIMÕES-ARDAILLON, D., VOLKMAR, P., DE MORAIS M. A. 2013b. Physiology and gene expression profiles of *Dekkera bruxel*lensis in response to carbon and nitrogen availability. Antonie van Leeuwenhoek 104 (5). 855-68.

[2] CRAUWELS, S., VAN ASSCHE, A., JONGE, R. et al. 2015. Comparative phenomics and targeted use of genomics reveal variation in carbon and nitrogen assimilation among different *Brettanomyces bruxellensis* strains. Appl. Microbiol. Biotechnol. 99 (21). 9123-34.

[3] DA SILVA, J. M., DA SILVA, G. H. T. G., PARENTE, D. C., LEITE, F. C. B., SILVA, C. S., VALENTE, P., GANGA, A. M., SIMÕES, D. A., DE MORAIS, M. A. Jr. 2019. Biological diversity of carbon assimilation among isolates of the yeast *Dekkera bruxellensis* from wine and fuel-ethanol industrial processes. FEMS Yeast Res. 19 (3). 1-10.

[4] PEÑA-MORENO, I. C., CASTRO PARENTE, D., DA SILVA, J. M., ANDRADE MENDONÇA, A., ROJAS, L. A. V., MORAIS, M. A. JR., DE BARROS PITA, W. 2019. Nitrate boosts anaerobic ethanol production in an acetate dependent manner in the yeast *Brettanomyces bruxellensis*. J. Ind. Microbiol. Biotechnol. 46 (2). 209-220.

[5] REIS, A. L. S., DE SOUZA, R. F. R., TORRES, R. R. N. B., LEITE, F. C. B., PAIVA, P. M. G., VIDAL, E. E., DE MORAIS JR, M. A. 2014. Oxygen-limited cellobiose fermentation and the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces bruxellensis* strain. Springer Plus 3 (38). 1–9.

[6] CODATO, C. B., MARTINI, C., CECCATO-ANTONINI, S. R., BASTOS, R. G. 2018. Ethanol production from *Brettanomyces bruxellensis* in synthetic media with pentose. J. Chem. Eng. 35 (1). 11–17.

[7] DE BARROS PITA, W., TELES, G. H., PEÑA-MORENO, I. C., DA SILVA, J. M., RIBEIRO, K. C. AND DE MORAIS JUNIOR, M. A. (2019) The biotechnological potential of the yeast Brettanomyces bruxellensis. World J. Microbiol. Biotechnol. 35 (103). 1-9.

[8] HUA, Y., WANG, J., ZHU, Y., ZHANG, B., KONG, X., LI, W., WANG, D., HONG, J. 2019. Release of glucose repression on xylose utilization in *Kluyveromyces marxianus* to enhance glucose-xylose co-utilization and xylitol production from corncob hydrolysate. Microb. Cell Fact. 18 (24). 1-18.

[9] RETTORI, D., VOLPE, P. L. O. 2000. Microcalorimetria: uma técnica aplicável ao estudo do diauxismo da *Saccharomyces cerevisiae*. Quím. Nova 23 (2). 257-261.

[10] STASYK, O. G., STASYK, O. V. 2019. Glucose Sensing and Regulation in Yeasts. *In*: Non-conventional Yeasts: from Basic Research to Application. SIBIRNY, A. (ed). 1st ed. Springer, Cham. 477-519.

[11] PEETERS, K. THEVELEIN, J. M. 2014. Glucose Sensing and Signal Transduction in *Saccharomyces cerevisiae*. *In:* Molecular Mechanisms in Yeast Carbon Metabolism. PIŠKUR, J., COMPAGNO, C. (eds). 2014th ed. Springer-Verlag Berlin Heidelberg. 21-56.

[12] LANE, S., XU, H., OH, E. J., KIM, H., LESMANA, A., JEONG, D., ZHANG, G., TSAI, C.S., JIN, Y. S., KIM, S. R. 2018. Glucose repression can be alleviated by reducing glucose phosphorylation rate in *Saccharomyces cerevisiae*. Sci. Rep. 8 (1). 1-12.

[13] KAYIKCI, Ö., NIELSEN, J. 2015. Glucose repression in *Saccharomyces cerevisiae*. FEMS Yeast Res. 15 (6). 1-8.

[14] HAGMAN, A., PIŠKUR, J. 2015. A study on the fundamental mechanism and the evolutionary driving forces behind aerobic fermentation in yeast. PLoS One 10 (1). 1-24.

[15] ROTHMAN, D. L., STEARNS, S. C., SHULMAN, R. G. 2021. Gene expression regulates metabolite homeostasis during the Crabtree effect: Implications for the adaptation and evolution of Metabolism. Proc. Natl. Acad. Sci. U S A 118 (2). 1-11.

[16] DA SILVA, T. C. D., LEITE, F. C. B., DE MORAIS, M. A. 2016. Distribution of *Dekkera bruxellensis* in a sugarcane-based fuel ethanol fermentation plant. Lett. Appl. Microbiol. 62 (4). 354-358.

[17] DE BARROS PITA, W., LEITE, F. C. B., DE SOUZA LIBERAL, A. T., PEREIRA, L. F., CARAZZOLLE, M. F., PEREIRA, G. A., DE MORAIS, M. A. JR. 2012. A new set of reference genes for RT-qPCR assays in the yeast *Dekkera bruxellensis*. Can. J. Microbiol. 58 (12). 1362–1367.

[18] BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., et al. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (4). 611–622.

[19] MOKTADUZZAMAN, M. D., GALAFASSI, S., CAPUSONI, C., VIGENTINI, I., LING, Z., PISKUR, J., COMPAGNO, C. 2015. Galactose utilization sheds new light on sugar metabolism in the sequenced strain *Brettanomyces bruxellensis* CBS 2499. FEMS Yeast Res. 15 (2). 1–9.

[20] FONSECA, C., SPENCER-MARTINS, I., HAHN-HÄGERDAL, B. 2007. L-arabinose metabolism in *Candida arabinofermentans* PYCC 5603 and *Pichia guilliermondii PYCC* 3012: influence of sugar and oxygen on product formation. Appl. Microbiol. Biotechnol. 75 (2). 303–310.

[21] GRANADOS-ARVIZU, J. A., MADRIGAL-PEREZ, L. A., CANIZAL-GARCIA, M., GONZÁLEZ-HERNÁNDEZ, J. C., GARCÍA-ALMENDÁREZ, B. E., REGALADO-GONZÁLEZ, C. 2019. Effect of cytochrome bc1 complex inhibition during fermentation and growth of *Scheffersomyces stipitis* using glucose, xylose, or arabinose as carbon sources. FEMS Yeast Res. 19 (2). 1-8.

[22] RODRUSSAMEE, N., SATTAYAWAT, P., YAMADA, M. 2018. Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1 – 2. BMC Microbiol. 18 (1). 1–11.

[23] WISSELINK, H. W., TOIRKENS, M. J., WU, Q., PRONK, J. T., VAN MARIS, A. J. A. 2009. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Appl. Environ. Microbiol. 75 (4). 907–914.

[24] DE MATOS, J. P., SOUZA, K. R., SANTOS, A. S., PANTOJA, L. A. 2018. Fermentação alcoólica de hidrolisado hemicelulósico de torta de girassol por *Galactomyces geotrichum* UFVJM-R10 e *Candida akabanensis* UFVJM-R131. Quím. Nova 41 (1). 23-29.

[25] LEITE, F. C., LEITE, D. V., PEREIRA, L. F., DE BARROS PITA, W., DE MORAIS, M. A. JR. 2016. High intracellular trehalase activity prevents the storage of trehalose in the yeast *Brettanomyces bruxellensis*. Lett. Appl. Microbiol. 63 (3). 210-214

[26] STENGER, M., LE, D.T., KLECKER, T. AND WESTERMANN, B. 2020. Systematic analysis of nuclear gene function in respiratory growth and expression of the mitochondrial genome in *S. cerevisiae*. Microbial cell 7 (9). 234–249.

[27] ROZPEDOWSKA, E., HELLBORG, L., ISHCHUK, O.P., ORHAN, F., GALAFASSI, S., MERICO, A., WOOLFIT, M., COMPAGNO, C., PISKUR, J. 2011. Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera yeasts*. Nat. Commun. 2 (302). 1-7.

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