TITLE: INFLUENCE pH, TEMPERATURE AND COPPER SULFATE ON LACCASE EXPRESSION BY *Komagataella pfaffi*

AUTHORS: [1,2]OTERO, I.V.R.; [2]HALSBECK, M.; [2]SIEBER, V.; [1]SETTE, L.D.

INSTITUTIONS: [1]Sao Paulo State University and [2]Technical University of Munich

There are two main bottlenecks regarding the heterologous expression of proteins: (1) find the best chassis and the best molecular regulation for the expression; and (2) find the best media and/or condition for the expression. The Pichia toolkit (PTK) allows the assembly of libraries with different promoters, peptide signals and terminators for the expression of enzymes using *Komagataella pfaffi* (*Pichia pastoris*) as host. After a screening using the PTK system with more than 1000 variants, clone 117 was selected as the best producer of a marine-derived fungal laccase (from *Peniophora* sp. CBMAI 1063). Therefore, this variant was submitted to further investigation considering different variables in the medium composition and cultivation conditions that could positively influence the laccase expression. In this study, constitutive expression was performed in BMGY medium containing 2% glycerol. To test different pHs and buffers, BMGY was prepared with 100 mM of sodium acetate buffer (pHs 5.5 and 6) or potassium phosphate buffer (pHs 6, 6.5 and 7). Seven incubation temperatures were applied (15, 18, 20, 23, 25, 28 and 30˚C). Medium supplementation was tested with six different concentrations of $CuSO_4$ (10, 20, 40, 60, 80 and 100 µM). All experiments were performed using 24 deep-well plates with 5 mL working volume. Plates were incubated for 72h at 900 RPM. The standard condition had pH 6 with potassium phosphate buffer, 100 µM of $CuSO_4$ and incubation at 25˚C. Laccase activity was measured by the oxidation of ABTS. Cultivation in potassium phosphate buffer shown significantly ($p<0.05$) higher laccase activity (58.48 U L^{-1}) in comparison to sodium acetate buffer (15.21 U L^{-1}). Laccase activity in pH 6 was slightly higher than in pH 6.5 and 7. Among the variables tested, the temperature was the one that influenced the most. The peak of activity was reached at 18 ˚C (90.07 U L^{-1}) while the lowest activity was at 30 ˚C (7.91 U L^{-1}). Although a peak of laccase activity was found when the medium was supplemented with 60 µM of $CuSO_4$ (66.38 U L^{-1}), no significant difference ($p>0.05$) was found among all the concentrations tested. Since laccases are multicopper oxidases, $CuSO_4$ is commonly reported as laccase inducer, however, in this study lower temperature influenced more on the expression. Lower temperatures help on the folding of the heterologous enzyme and can decrease the activity of some proteases, therefore increasing the production of the heterologous laccase.

KEYWORDS: Media optimization, multicopper oxidase, *Pichia pastoris*, heterologous expression

FUNDING AGENCIES: FAPESP (2018/12098-9), CAPES (88887.467275/2019-00)