Development of a protocol for exocellulase activity direct detection in culture medium from metagenomics library clones for a high-throughput screening

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Background

Sugarcane bagasse can be used to produce second-generation ethanol. To make this possible, cellulose needs to be degraded to glucose by three classes of enzymes: endo-β-1,4-glucanase, exo-β-1,4-cellobiohydrolases and β-glucosidase. Prospecting the Brazilian microbial biodiversity for new enzymes, may lead to the identification of enzymes with high activity on plant biomass (Cunha et al, 2011). However, traditional assays for enzyme detection employs solid media, and among the limitations encountered by this approach are high cost, low sensitivity and incompatibility with quantitative high-throughput screening (HTS) systems (Ko et al, 2013). We aimed to develop a HTS method for exocellulase activity detection directly from culture media from metagenomic clones. Here we report our results using 4-nitrophenyl-β-D-glucopyranoside (pNPG) and 4-methylumbelliferyl-β-D-glucopyranoside (MUG) and bacteria grown in LB and TB media.

Methods

To develop the HTS method, bacteria presenting β-glucosidase activity (BGL11) were grown in 15 mL tubes with LB medium and 20 µg/ mL tetracycline at 180 rpm, 37 ºC for 24 hours and was used as a positive control. Bacteria with empty pCF430 vector were used as a negative control. The assay used as substrates pNPG (0, 10, 25, 50, 100, 200 µM) and MUG (0, 10, 25, 50, 100 µM). All assays were performed in 96 well plates. Each well had a total volume of 100 µL, containing the substrate, 90 µL medium LB or TB, 5 mM potassium phosphate buffer pH 6.0, 0.02% arabinose and 5 µL of overnight grown strains (methods adapted from Bergmann et al, 2014 and Ko et al, 2013). Plates were then incubated at 37 ºC for 24 hours. Cell growth was determined by measuring absorbance at OD 600 nm. The enzymatic reaction containing pNPG was stopped by addition of 100 µL sodium carbonate buffer 0.5 M pH 6.0, and that containing MUG by adding 100 µL glycine buffer 0.5M pH 10.3. Exocellulase activity was detected by measuring the release of p-nitrophenol group

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from pNPG with a spectrophotometer at 405 nm, and the release of methylumbelliferyl from MUG, with excitation at 365 nm and emission at 448 nm. All assays were performed in triplicate.

Results and Conclusions

Both strains, the β-glucosidase-activity presenting strain (i.e., BGL11) and the negative control (i.e., EPI 300 strain containing empty pCF430), grew well at the tested conditions. The results for OD 600 measurements for EPI300 pCF430 were 0.18 ± 0.02 in LB medium and 0.39 ± 0.04 in TB medium, while for EPI300 BGL11 these were 0.18 ± 0.03 in LB medium and 0.36 ± 0.07 in TB medium. There were no differences in growth between strains when the same medium was used, but they grew better in a richer nutrient medium (i.e., TB). Detection of enzyme activity in assays using pNPG occurred with 50, 100 and 200 µM pNPG when LB medium was used, whereas in TB medium detection was only possible with 100 and 200 µM pNPG. Therefore, the substrate concentrations that allowed detection of enzyme activity were lower in LB medium than in TB medium. For the MUG substrate, detection of enzyme activity occurred at 10, 25, 50 and 100 µM in LB medium, whereas in TB medium detection of enzyme activity started at 25 µM. As for pNPG, LB medium allowed detection of enzyme activity with lower concentration of MUG. It is known that assays based on fluorescent substrates are more sensitive than colorimetric assays (Bell et al, 2013). As expected, enzymatic assays with MUG were more sensitive than those with pNPG. Therefore, although the bacterial biomass was higher in TB medium than in LB medium, because the detection of p-nitrophenol and methylumbelliferyl released during enzymatic activity is easier in LB medium, this medium should be preferred. This protocol is compatible with HTS for a fast and qualitative identification of new enzymes with β-glucosidase activity, directly from culture cells of metagenomics libraries.

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