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# Selection of resistant bifidobacteria cells to hydrogen peroxide using continuous culture and immobilized cells technology

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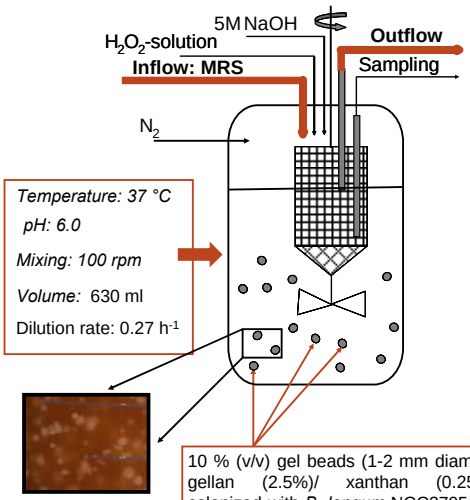
Probiotics are "live micro-organisms which, when administered in adequate amounts (as part of food), confer a health benefit on the host" (FAO/WHO 2002). A central issue in the application of probiotics as food additives is their fastidious production and sensitivity to many environmental stresses, such as acidity, heat and oxygen. *Bifidobacterium* species exhibit oxygen uptake which leads to the formation of toxic hydrogen peroxide ($H_2O_2$). The aim of this study was to test a new technological approach to select cells with enhanced tolerance to $H_2O_2$ using continuous culture combined with immobilization and selective pressure. A continuous culture inoculated with immobilized *B. longum* NCC2705 was performed in a stirred tank reactor for 23 days, with an initial dilution rate of 0.27 $h^{-1}$, and with increasing levels of $H_2O_2$ added to the MRS medium. Cell concentration in beads decreased progressively from $10^{11}$ to $10^5$ cfu/g with $H_2O_2$ concentration increasing from 0 to 100 ppm. Cells from one colony isolated from beads collected at 100 ppm $H_2O_2$ and cultivated in MRS medium showed significantly enhanced resistance for at least 70 generations to subsequent treatment at 400 ppm $H_2O_2$ for 2 hours, compared to control cells produced during 24 h batch incubation in MRS medium.

## Material and methods

### Continuous fermentation with immobilized cells




10 % (v/v) gel beads (1-2 mm diameter gellan (2.5%)/ xanthan (0.25%)) colonized with *B. longum* NCC2705 and prepared using a two-phase dispersion process (Cinquin et al., 2004)

- **Cell counts in effluent and gel beads:** Appropriate dilutions (phosphate buffer saline 1x (pH 7.3) supplemented with 0.05% cysteine) were plated on MRS agar with 0.05% cysteine and incubated anaerobically at 37°C for 48 h. For immobilized population, ca. 0.5 g of beads was mixed with 1 % EDTA and dissolved using a stomacher for 3 min prior to dilution and plating.
- **Isolation of resistant colonies:** Frozen beads (1-3 g), collected at day 18 were dissolved in 1% EDTA and centrifuged. The cell pellets were resuspended in 10 ml 40 ppm $H_2O_2$, incubated for 45, 60 and 80 min and subsequently plated on MRS agar. Colonies visible within 48 h were sub-cultured 2 times overnight in MRS broth and frozen at -80°C for further analyses.
- **Resistance to $H_2O_2$:** Frozen overnight cultures were thawed, centrifuged and cell pellets resuspended in 200 ppm $H_2O_2$ solution for 2 h and plated on MRS agar.
- **Stability over time:** Cells from successive cultures of 24 h were centrifuged and resuspended in 400 ppm $H_2O_2$ solution for 2 h and plated on MRS agar.

## Results and discussion

### Stress applied during continuous fermentation with immobilized cells

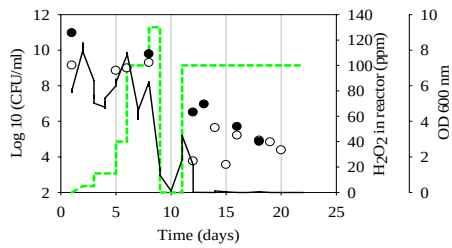



***Figure 1:*** *Optical density (—), $H_2O_2$ concentration in reactor (—) and viable cells counts in gel beads (●) and in the effluent (○) during continuous culture*

- The optical density (OD) immediately decreased after increasing the $H_2O_2$ concentration (in the range 0 to 100 ppm) followed by an increase of OD indicating that the culture adapted to the stress (Fig. 1).
- At 130 ppm the OD in the effluent dropped to 0 (day 9) indicating a lethal $H_2O_2$ concentration.
- To re-grow survivors addition of $H_2O_2$ was stopped for 2 days. The culture started to grow again (day 11).
- Concentration of $H_2O_2$ was set again at 100 ppm until day 23.

### Resistance to $H_2O_2$ of selected colonies

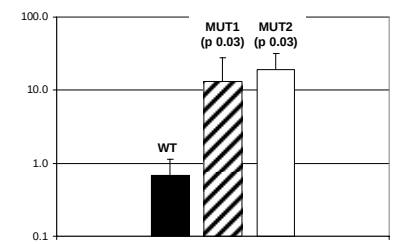



***Figure 2:*** *% survival of frozen 16 h overnight cultures after 2 h in 200 ppm $H_2O_2$, p-values refer to a t-test with the wild type as reference*

- Two colonies after day 18 (MUT1 and MUT2) showed a significant increased resistance to $H_2O_2$ compared to wild type cells produced with batch culture without selective pressure.

### Stability of $H_2O_2$ resistance of mutants

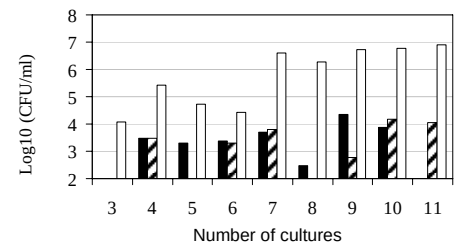



***Figure 3:*** *Resistance to $H_2O_2$ of wild type (■), MUT1 (▨) and MUT2 (□) after successive subcultures. Starting cell concentration: 9.2 ± 0.2 $log_{10}$ CFU/ml*

- MUT1 lost its acquired $H_2O_2$ resistance after three successive cultures.
- MUT2 maintained its phenotype. The $H_2O_2$ tolerance of MUT2 was stable for at least 11 subcultures corresponding to 70 generations.

## Conclusions

- Continuous culture with cell immobilization is an efficient approach to select cells adapted to environmental stresses.
- High viable cell numbers can be maintained in the bioreactor, even when high selective pressure is applied, enabling controlled application of stresses over long time periods.
- A stable variant (MUT2) which showed enhanced resistance to $H_2O_2$ stress was isolated and it is currently characterized (physiology and genomics).

## References

Cinquin, C., G. Le Blay, et al. (2004). "Immobilization of infant fecal microbiota and utilization in an in vitro colonic fermentation model." Microb Ecol **48**(1): 128-38.

FAO/WHO (2002). Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food, FAO.

## Acknowledgements

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