Physiological characterization of *Bifidobacterium longum* NCC2705 during continuous culture with immobilized cells

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Introduction

There are significant challenges for the production and application of probiotic bacteria which are generally sensitive to environmental stresses but must keep high viability during food processing, in food storage and after ingestion. (Ross, 2004) Cell immobilization has been shown to influence stress tolerance (Doleyres. 2004) and cell physiology of microorganisms (Junter, 2002)
In this work, the effects of cell immobilization (IC), growth in biofilm-like structure, and long-term continuous fermentation on cell physiology and transcriptomics were studied with *Bifidobacterium longum* NCC2705 as model probiotic strain.
A continuous IC culture was carried out for 21 days in MRS-cysteine medium in a two-stage system with a first reactor R1 inoculated with pre-colonized gel beads of *B. longum* NCC 2705, used for efficient cell production, and a second reactor R2 (1.8 l) used for stress adaptation of cells released from R1 under glucose starvation. Cell production, metabolism, transcriptional profiles (microarrays) and tolerance to gastrointestinal stresses and antibiotics were tested and data compared with free cell batch cultures.

Material and Methods

Strain and Medium
Bifidobacterium longum NCC2705 was provided by the Nestlé culture collection NCC (Nestle Research Centre, Switzerland). The growth medium for all pre-cultures and fermentations was MRS-C (MRS broth Tween 80 supplemented with 0.05 % (w/v) L-cysteine hydrochloride monohydrate.

Fermentations
A continuous IC culture was carried out for 21 days in a two stage fermenter system (figure 1).
A first reactor R1 (0.24 l) inoculated with 33% (v/v) *B. longum* NCC 2705 gel beads was continuously fed with MRS-cysteine (feed rate: 360 ml/h). The second reactor R2 (1.8 l) was connected in series with R1 and constantly inoculated with cells released from beads in R1.

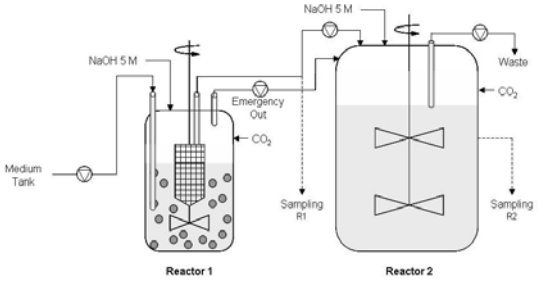

Figure 1: Diagram of the two-stage reactor setup of continuous fermentation with immobilized cells.

	Units	Reactor 1	Reactor 2
Volume	[ml]	240	1800
Temperature	[°C]	37	37
pH		6.0	6.0
Agitation	[rpm]	150	250
Flow rate	[ml h^{-1}]	360	360
Dilution rate	[h^{-1}]	1.5	0.2
residence time	[h]	0.7	5.0

Table 1 Parameters of fermentation set up in figure 1

Cell Immobilization

Cell immobilization was based on a dispersion process in a two-phase system. A mixed gel with a composition of (2.5 % (w/v) gelrite gellan gum and 0.25 % (w/v) xanthan gum (both Sigma-Aldrich, Switzerland) was used according to Cinquin *et al.* (2004).

Gel preparation: sterile gellan and xanthan polymer solution was inoculated aseptically with an active pre-culture (2% (v/v)) of *B.longum* NCC2705. Spherical bead shape was obtained by pouring the polymer-cell solution into a hydrophobic phase (sunflower oil) and afterwards hardening in a cold $CaCL_2$-solution (0.1 M). Beads (1 – 2mm) were selected by wet sieving and transferred to a 500 ml bioreactor containing MRS-C and incubated anaerobically at pH 6 for 16 h.

Cell viability

OD: biomass concentration was determined by optical density measurement at 600 nm

CFU: fermentation broth was serially diluted in PBS-C and drop plated in duplicate on MRS-C agar plates and incubated anaerobically at 37°C for 48 h.

Flow cyto. Samples were washed and diluted in PBS-C to obtain a cell density of ca. 10^7 cells/ml, stained with 7AAD as a dead- and SybrGreen I as an universal cell marker and applied to a flow cytometer (Cytomics FC 500; Beckman Coulter International SA, Switzerland).

Tolerance to gastrointestinal conditions

Cell survival to simulated gastric juice and bile salt stress was performed as follows. Washed cells were exposed to gastric juice for 30min and to bile salt solution for 10 and 20 min respectively. Cell counts were performed with stressed and not stressed (PBS-C instead of gastric/bile treatment) samples and log loss was evaluated (log loss =log CFU without – log CFU with treatment).

Tolerance to antibiotics

Antibiograms were determined using a modified antimicrobial disc susceptibility assay according to the National Committee for Clinical Laboratory Standards (1991).

Gene expression profiles

RNA isolation: Macaloïd method (Kuipers et al. 1993); Microarrays; DNA arrays (Agilent Technologies), 60mer oligonucleotides; Hybridization and analysis: RNA was labeled using the Array 900MPX kit ; (Genisphere Inc., Hatfield, U.S.A). Data were extracted and normalized using softwares ImaGene 5.6, iTap-Agilent; V1.10 and Array Pipe (Hokamp et al. 2004).;Classification of genes: Classification into clusters of orthologous groups (COGs) was based on the most recent COG classification system (66 genomes; http:// www.ncbi.nlm.nih.gov/COG)

Results

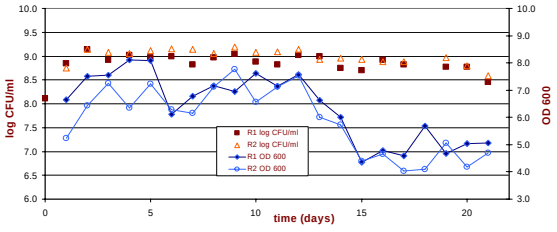

Figure 2 CFU/ml and OD600 of daily samples of Reactor 1 and Reactor 2

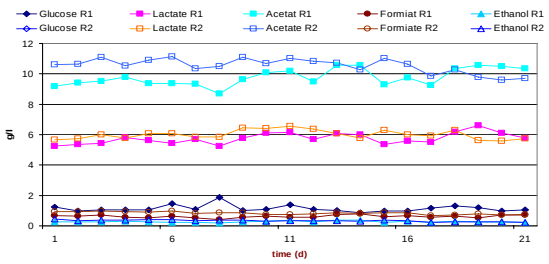

Figure 3 Concentrations of metabolites of daily samples of Reactor 1 and Reactor 2

After an equilibrium phase in the first 2 days, CFU counts are stable in both fermenters over the entire fermentation time of 21 days. OD values showed a drop after 12 days of fermentation. This drop was caused by the formation of cell aggregates in the effluent medium of both reactors. Microscopic analyses showed the formation of macroscopic cell clusters influencing OD measurement as well as the CFU counts (figure 2).
The calculated volumetric productivities ($CFU*ml^{-1}*h^{-1}$) showed more than 10x higher volumetric productivity for R1; and more than 2x for the whole system (R1 and R2) compared to batch reference cells (CFU counts not shown) (table 2).
Metabolic analyses showed stable values of culture in both reactors for 21 days. Sugar concentration in the effluent of R1 was very low (1.13 +/- 0.23 g/l) resulting in no glucose detection in R2 (figure 3).
Flow Cytometry analyses showed stable percentages of live cells in R1 and R2 distributed between 80% and 85% until day 12. Similar to the OD values, ratios of live cells in both reactors drop after 12 days of fermentation (figure 4).

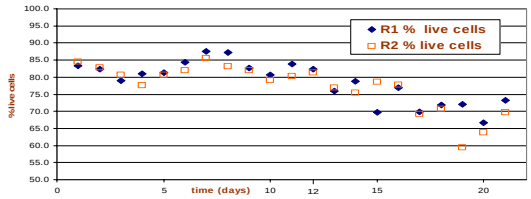

Figure 4 Percentages of live cells from daily samples from R1 and R2.

Culture	CFU counts (cfu*ml-1)	Volumetric productivity (cfu*l-1*h-1)
Batch (max.)	**$1.4*10^{9}$**	**$9.4*10^{10}$**
IC Reactor 1 (1.5 h-1)	**$7.7*10^{8}$**	**$1.2*10^{12}$**
IC R1 and R2 (total)	**$8.8*10^{8}$**	**$1.9*10^{11}$**

Table 2 Volumetric productivities of Batch reference culture*, R1 and total(R1 and R2) * CFU-data not shown

Microarray analyses were performed to check the influence of fermentation time on the gene expression pattern of cells in R2. The hybridisations were set up to compare cells from R2 from day 3 and 6 as well as from day 3 and day 9. The gene expression pattern revealed only minor changes in the transcriptome in both hybridizations. The number as well as the log2 ratio of the differently expressed genes was negligible (table 3).

Hybridization	differentially expressed genes	log2 ratio max	log2 ratio min
R2 day 6 vs. R2 day 3	44	1.81	-1.08
R2 day 9 vs. R2 day 3	37	1.71	-0.93

Table 3 Differently expressed genes of R2 of day 6 vs. R2 day 3 and R2 day 9 vs R2 day 3

Antibiotic disc (ug)	Batch Stat. phase (mm)	IC Day 3 (mm)	IC Day 12 (mm)	IC Day 21 (mm)
GM (10)	12.8 ± 3.3^{a}	16.8 ± 2.4^{a}	9.5 ± 1.9^{a}	6 ± 0^{b}
NE (10)	14.2 ± 3.0^{a}	10.5 ± 3.1^{b}	9.3 ± 2.3^{b}	6 ± 0^{c}
CTX (30)	39.6 ± 4.0^{a}	40.0 ± 1.2^{ab}	38.8 ± 1.0^{b}	33.8 ± 2.7^{c}

Table 3 Inhibition diameters of batch and IC cells in antibiotic disc assay
a, b, c = different letters =sig. different $p<0.05$, ANOVA, N=3)

Tolerance to gastrointestinal stresses was determined by CFU-counts before and after a stress test (simulated gastric juice or bile salt solution). Susceptibility to the stresses was monitored as follows: (log CFU (before) – log CFU (after) = "log loss").
Tolerance to gastric stress of IC cells did not show any significant improvements compared to batch mid stationary phase cells (figure 4). Tolerance of IC cells to bile salt stress was significantly enhanced compared to batch mid stationary phase cells (figure 5).

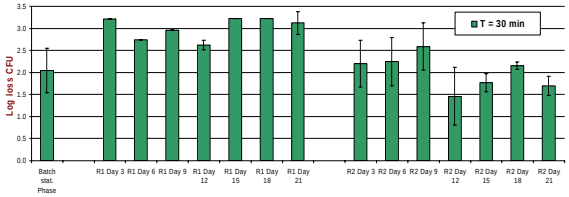

Figure 4 Susceptibility to 30 min gastric juice. Batch cultures from stationary phase compared with IC cells from R1 and R2
Bars with error bars are means and standard deviations of n=3.

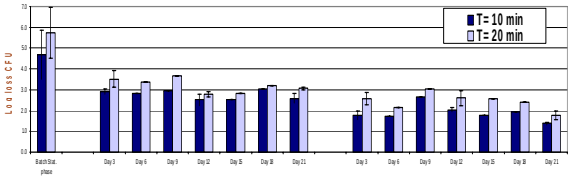

Figure 5 Susceptibility to 10 and 20 min bile salts stress. Batch cultures from stationary phase compared with IC cells from R1 and R2.
Over-all averages from day 3 – day 21 of R1 and R2 are sig. different than batch reference (n=3; $p<0.01$; ANOVA, comparison of all goups using Tukey'y test)

Antibiotic tolerance was determined by measuring inhibition parameters. The inhibition diameters of gentamycine, neomycine and cefatoxime of IC cells of day 21 are all significantly smaller than batch cells. Additionally, a time effect from day 3 to day 21 on antibiotic tolerance is detectable.

Discussion

CFU counts, OD measurement and flow cytometry data were all influenced by the formation of cell aggregates in both reactors after day 12 of fermentation. Flow cytometry data after day 12 are less reliable due to interference of cell aggregates with the cell staining process. The 2-stage immobilized cell continuous system shows more than 2 times higher volumetric productivity than batch cultures, keeping in mind that cell clusters result in an underestimation of CFU-counts.
Analyses of metabolites and transcriptome showed stable values with respect to fermentation time.
While IC cells do not show any enhanced tolerance to gastric juice, their tolerance to bile salt is improved significantly compared to batch reference cells. Changes in the membrane composition originating from immobilization of cells could explain the increased robustness towards bile salts, a detergent-like biological substance that can disorganize the lipid bilayer structure of cellular membranes (Bernstein. 1999).
IC cells are less susceptive to antibiotics than batch cells and increase the tolerance with fermentation time. This change with time could be explained with the constant adaptation of the cells growing in a challenging environment on the surface and inside the beads.

Conclusion.

A high volumetric productivity and good process stability were measured during continuous IC culture, although large bacterial clusters were formed in both reactors after 12 days. Current results show that the tolerance to bile salt and resistance to gentamycine, cefotaxime and neomycine may be enhanced for IC cells compared to stat. phase batch cells.
Our data suggest that cell immobilization and continuous culture could confer to *B. longum* cells enhanced technological and probiotic properties.

References

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