Selection of oxidative tolerant mutants from aerobic culture of *Lactobacillus plantarum* C17

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INTRODUCTION

Lactobacillus plantarum is a fermentative lactic acid bacterium involved in the production of many fermented and functional foods (Parente *et al*., 2010). Recently, several studies demonstrated that the shift to aerobic metabolism results, in this species, in the expression of a phenotype with enhanced technological and stress response properties. However, with the exception of some studies on the model strain *Lb. plantarum* WCFS1 (Brooijmans *et al*., 2009; Zotta *et al*., 2012; Watanabe *et al*., 2012), data on the aerobic pathways are relatively rare, even if this species includes strains of great technological interest.

AIM: We investigated the effect of aerobiosis on the growth and stress resistance of the promising probiotic strain *Lb. plantarum* C17 during batch, fed-batch and chemostat cultivations in a complex medium. Sixteen random mutants were also selected for their ability to tolerate H_2O_2 and menadione and further screened for their capability to growth under both aerobic and oxidative stress conditions.

MATERIALS AND METHODS

Growth conditions: Batch (10 g/L initial glucose), fed-batch and chemostat (40 g/L glucose in the feed; D=0.31 h^{-1}, 0.17 h^{-1}, 0.07 h^{-1}, 0.04 h^{-1}) cultivations were carried out in modified WMB medium (Zotta *et al*., 2012) in aerobiosis (air, 0.2 vol/vol min, with 2.5 µg/mL hemine and 1 µg/mL menaquinone) at 35°C (optimal) and at controlled pH (6.5).

Chemical and biochemical analyses: Maximum biomass yield, residual glucose, lactic and acetic acids production, H_2O_2 concentration, O_2 uptake, catalase, POX , NOX and NPR activities were measured on the late exponential (batch) and steady-state (chemostat) cells.

Stress treatments: Thermal inactivation (20 mM phosphate buffer pH 7.0, PB7, 55°C for 0, 5, 10, 15, 30 min) and tolerance to freezing and freeze-drying (7, 30 days) were evaluated by plate counts on WMA; capability to grow in WMB (pH 6.8, 16 h, 35°C) after exposure (30 min, 35°C) to different H_2O_2 concentrantions (from 0.8 to 0.001 M) was tested in microplate experiments, while menadione tolerance (from 0.3 mM to 0.018 mM) was evaluate by plating on WMA.

Selection of random mutants : For each growth condition 10 mutants were randomly selected from WMA plates containg H_2O_2 (0.8 M or 0.4 M) or menadione (a superoxide generating compound, 0.150 mM or 0.075 mM). All mutants were tested in microplates for their oxidative stress tolerance and two (C17-m19 and C17-m58) were grown in static and shaking conditions, in presence of either glucose and maltose (10 g/L).

RESULTS

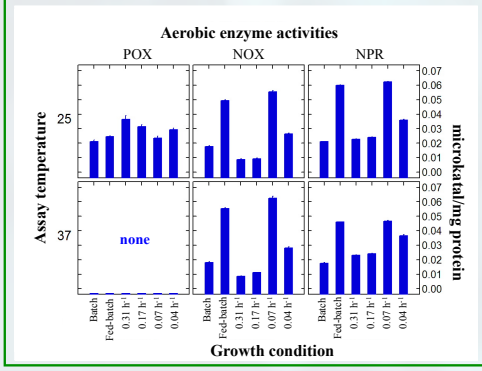
Aerobic growth

Dilution rate (D) clearly affected physiological state of cells and, generally, cultures grown at low D exhibited improved biomass production, survival to stresses, catalase activity and *in situ* O_2 uptake as compared to those obtained during batch cultivation.

However, even if the growth was carried out in aerobiosis, acetic acid concentrations were undetectable in the supernatants, probably because the inactivation of POX *in vivo* (35°C, see data below). With exception of batch cells, a significant catalase activity (19-27 mkatal/g biomass) was found in the other growth conditions (especially at low D), resulting (together with NPR contribution) in the complete degradation of H_2O_2.

Enzymes related to aerobic metabolism

Our results revealed that the activities of POX and NPR, but not NOX, were significantly affected by temperature. In particular, POX was completely inhibited at 37°C, while the activity of NPR slightly increased at 25°C. Therefore, POX is induced as expected by aerobiosis and low residual glucose, but is not necessarily active. Contrarily to the flavin dependent oxidases, POX levels were lower in slow-growing cells, and relatively high at the highest dilution rate, indicating that in *Lb. plantarum* control of aerobic metabolisms still needs to be elucidated.

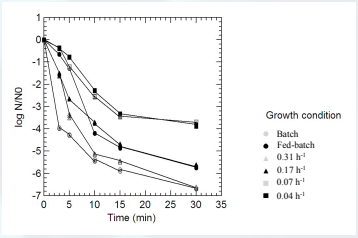


POX, pyruvate oxidase
NOX, NADH oxidase
NPR, NADH peroxidase

Implication of temperature in the regulation of oxidases and peroxidases

Tolerance to oxidative stress

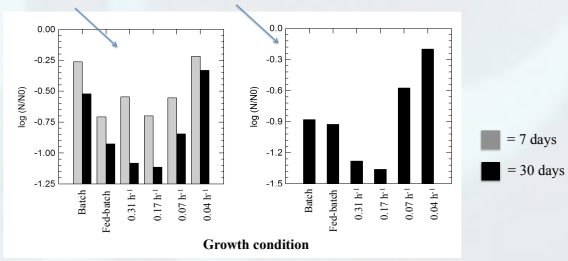
In keeping with the high levels of catalase and NPR activities measured in glucose-limited conditions, the slow-growing cells exhibited a high resistance to H_2O_2 (up to 0.8 M) and, even if they lack *sod* gene, a significant survival to menadione (up to 0.15 mM).



Thermal inactivation

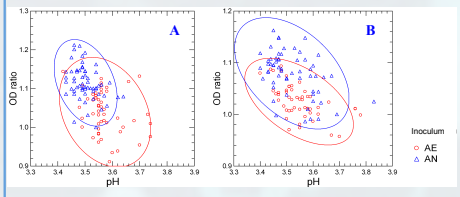
The kinetics of heat inactivation, evaluated by plate counting, showed that batch cultivation and high growth rates impaired heat tolerance with a reduction in the number of viable cells > 3 log units. These data confirmed the induction of cross-protection mechanisms under nutrient-limited growth.

Freezing and freeze-drying tolerance



Low D rates also improved the survival to freezing and freeze-drying stresses, suggesting once more that slow growth rates or depletion of nutrients lead to stationary phase-like conditions which improve general stress response.

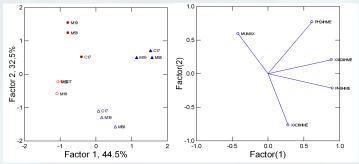
Selection of oxidative tolerant mutants



A=WMB, anaerobiosis; B=WMB with hemin and menaquinone, aerobiosis; circles=anaerobic inoculum; triangles=aerobic inoculum

Most of mutants had higher oxidative stress resistance compared to the wild type (wt) strain C17 and, additionally, they mainly expressed some typical aerobic traits (such as increasing of OD_{650nm} and pH) than the parental strain.

Growth and oxidative stress of selected mutants



Principal Component Analysis (PCA) on the Pearson's *r* correlation matrix of AN/AE growth data (μ_{max}, pH at 8 h and 24 h, biomass X g/L at 8 h and 24 h). The first two components explained the 77.0% of total variance. Empty simbols=anaerobiosis; closed symbols=aerobiosis; circles=glucose; triangles=maltose.

The two selected mutants C17-m19 and C17-m58 were more competitive in terms of biomass production and ROS degradation than wt strain, especially when cultivated on maltose (a non-PTS sugar for which the repression of *pox* genes by CcpA was not observed).

C17-m58 mutant, showing the best physiological responses, has been further used for proteomic and transcriptomic studies.

CONCLUSIONS

This work confirms that **in *Lb. plantarum* the aerobic metabolism and low growth rates confer several** physiological and metabolic advantages under unfavorable conditions. Additionally, because the construction of mutants by site directed mutagenesis requires the knowledge of gene sequence and the availability of suitable suicide vectors, the selection of random mutants with desired characteristics by selective pressure could be of practical relevance: in fact, the mutants generated in this study have improved growth in aerobiosis and inmproved tolerance of oxidative stress compared to the wt and deserve further characterization.

References

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This work was partly funded by Ministero dell'Istruzione, dell'Università e della Ricerca, Rome, Italy, PRIN 2008, n. 20088SZB9B

FoodMicro 2012, Istanbul, September 3-7, 2012