Biochemical Engineering Journal 74 (2013) 54–59

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

The simultaneous utilization of kinetic analysis and flow cytometry in the assessment of *Lactobacillus rhamnosus* ATCC 7469 physiological states produced by increasing oxygen limitation levels and lactic acid accumulation

Teresa Lopes da Silva, Susana Marques, Luis Alves, José Carlos Roseiro*

Laboratório Nacional de Energia e Geologia, I.P., Unidade de Bioenergia, Estrada do Paço do Lumiar 22, 1649-038 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 6 September 2012
Received in revised form 1 February 2013
Accepted 3 February 2013
Available online 28 February 2013

Keywords:
Lactobacillus rhamnosus
Flow cytometry
Continuous culture
Steady state
Lactic acid

ABSTRACT

Carbon limited continuous cultures of *Lactobacillus rhamnosus* ATCC 7469 were grown at dilution rates between 0.1 h^{-1} and 0.6 h^{-1}. At 0.45 h^{-1}, oxygen uptake decreases producing a deficiency in the production of cell energy, lowering the concentration of biomass and finally accumulating glucose in the broth. Under the lack of energy pressure, *L. rhamnosus* ATCC 7469 triggers the production of lactic acid from pyruvate freeing NAD^+ and stimulates glycolysis to continue, producing extra ATP from substrate-level phosphorylation. The 12-fold growing concentration of lactic acid and the 2-fold increase of succinic acid are in parallel with the steep 4-fold decrease of acetic acid production and small concentration changes of formic and propionic acids.

The way the cells balance the available energy between the growing dilution rate and detoxification produces a stress within the culture, detected and described by flow cytometry. As the dilution rate increased, the proportion of *L. rhamnosus* ATCC 7469 cells with depolarized membrane steadily increased (1% at $D = 0.20\ h^{-1}$, 8% at $D = 0.30\ h^{-1}$, 14% at $D = 0.45\ h^{-1}$ and 26% for $D = 0.62\ h^{-1}$, respectively). Only a low level of 3.7% of the population did not recover from the demanding growth rates in the acidic environment.

1. Introduction

Lactic acid is a valuable multi-function organic acid, being presently the most promising application in the manufacture of biodegradable and biocompatible polylactate polymers, an environment-friendly alternative to conventional non-biodegradable plastics derived from petrochemicals [1,2].

The production of lactic acid with *Lactobacillus rhamnosus* ATCC 7469 follows the transformation of glucose to pyruvate through the Embden–Meyerhof–Parnas pathway [3,4].

Although lactic acid can be produced by chemical synthesis, fermentation is becoming the most popular process, particularly using monosaccharides from renewable carbohydrates usually used as economic feedstock [5]. Different approaches have been used to convert lignocelullosic materials into monosaccharides and produce lactic acid, both in batch and continuous fermentation. In parallel, processes integrating the saccharification and fermentation steps for lactic acid production or as separate hydrolysis and fermentation are presently under study [1,6].

L. rhamnosus ATCC 7469 has proved to be resistant to adverse fermentation conditions [7]. However, the way the biological system adapts to environmental stress and supports both growth and product synthesis is not fully understood, being necessary to follow the metabolic fluxes of the carbon source to assess the way the cell re-direct energy under fermentation conditions. Also, the effect on process performance of culture medium composition remains to be clarified. Therefore detailed information on bacterial population dynamics within such systems becomes of extreme importance since a high number of dead or dormant cells present during any part of a process will have an obvious detrimental effect on the synthesis of a desired product or on biodegradation process efficiency. The advantages of multi-parameter flow cytometry over the more conventional microbiological techniques, such as dilution plating (cfu per mL), are well documented [8].

Using various mixtures of fluorescent dyes, it is possible to resolve the physiological state of an individual microbial cell beyond culturability. This is based on the presence or absence of an intact polarized cytoplasmic membrane and the transport mechanism across it, which enables the detection of different physiological states within a microbial cell population. Classical

* Corresponding author at: Laboratório Nacional de Energia e Geologia, Bioenergy Unit, Azinhaga dos Lameiros, 22, 1648-038 Lisboa, Portugal. Tel.: +351 210924229.
E-mail address: jose.roseiro@lneg.pt (J.C. Roseiro).

1369-703X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bej.2013.02.002

Symbols

D	dilution rate (h^{-1})
S	input glucose concentration (mmol/L)
S_r	glucose concentration in the broth (mmol/L)
X	steady-state bacterial dry cell weight (g/L)
qCO_2	specific rate of carbon dioxide production (mmol carbon dioxide/g cells/h), represents the carbon dioxide produced per biomass unit
qO_2	specific rate of oxygen utilization (mmol oxygen/g cells/h), represents the oxygen consumption per biomass unit
qOrganic acid	specific organic acid production rate (mmol/g cells/h)
$qGlucose$	specific glucose utilization rate (mmol/g cells/h)
$\%CO_2$	volumetric percentage of carbon dioxide production from the measurements in the effluent gas from the online measurement
$\%O_2$	is the volumetric percentage oxygen uptake from the measurements in the effluent gas from the online measurement
F	airflow (L/h)
V	culture volume (L)
41.3	constant for the conversion of gas volumes into mmolar unit
CR	carbon utilized for cell formation (%)
CCE	carbon conversion efficiency, which represents the carbon used in the production of cells (%)
41.7	conversion constant to obtain mmolar elemental carbon biomass composition.

microbiology states that bacterial populations are homogeneous with respect to their physiological state. However, in fermentation processes and particularly where substrate concentration heterogeneities are known to exist (both spatial and temporal) this has been shown not to be the case [9].

In this work, carbon-limited steady-state continuous cultures of *L. rhamnosus* ATCC 7469 were carried out at different dilution rates, in order to evaluate the effect of growth rate and acid accumulation on individual cell physiological responses assessed by multi-parameter flow cytometry.

2. Materials and methods

2.1. Strain

L. rhamnosus ATCC 7469 was obtained from American Type Culture Collection in lyophilized form. The strain was maintained on glass beads, supplemented with 20% (v/v) glycerol, at −72 °C.

2.2. Bioreactor experiments

The medium used in the bioreactor experiments was made up as follows (g): glucose, 5; Yeast Extract, 0.5; $(NH_4)_2SO_4$, 1.5; K_2HPO_4, 2; $MgSO_4$, 0.2; $MnSO_4$, 0.05 and 2 mL trace elements solution in 1 L distilled water [10]. All experiments were carried out in a 3 L glass vessel (Bioflo III, New Brunswick Scientific, Edison, NJ, USA) equipped with two four-bladed Rushton impellers and fitted with four equally spaced baffles. The 1.45 L working volume was kept constant by overflow using a surface dipped leveling tube. The fermentation was kept under mild aerobic conditions by a continuous air flow rate corresponding to 0.42 vvm (unit of volume of air per unit of volume of medium per minute). The temperature was maintained at 37 °C. The pH was set at 6 by adding 2 M NaOH solution. The stirrer speed was 200 rotations per minute (rpm).

Firstly, the empty bioreactor and tubing were sterilized for 240 min in an autoclave at 121 °C, 1 bar. The culture medium was sterilized inside the bioreactor for 50 min at 121 °C, 1 bar after calibrating the pH probe against buffer solutions. The NaOH solution and glucose were sterilized separately for 15 min in autoclave at 121 °C, 1 bar. The glucose solution was aseptically transferred to the reactor after cooling. A 10% liquid inoculum, grown for 24 h at 37 °C, was transferred to the vessel. After a period of batch cultivation, a variable speed peristaltic pump was used to feed sterile medium into the fermenter vessel at the required dilution rate.

Oxygen consumption and carbon dioxide production were directly monitored using, respectively, paramagnetic and infrared gas analyzers (Servomex Ltd., Sussex, United Kingdom).

2.3. Calculation of metabolic rates

Metabolic rates (substrate consumption, product formation, carbon dioxide production, oxygen utilization, carbon recovery, carbon conversion efficiency, and molar growth yields) were calculated according to Roseiro et al. [11]:

For the specific rate of glucose consumption, qS (mmol glucose/g cells/h)

$$qs = \frac{D(S - S_r)}{X} \tag{1}$$

where D is the dilution rate (h^{-1}), S is the input glucose concentration (mmol/L), S_r is the glucose residual concentration (mmol/L), and X is the steady-state bacterial dry cell weight (g/L).

For the specific rate of carbon dioxide production, qCO_2 (mmol carbon dioxide/g cells/h)

$$qCO_2 = \frac{41.3 \cdot F \cdot \%CO_2}{V \cdot X} \tag{2}$$

where F is the airflow (L/h), V the culture volume (L), $\%CO_2$ is the volumetric percentage of carbon dioxide production from the measurements in the effluent gas from the online mass spectrometer; 41.3 is a constant for the conversion of gas volumes into mmolar unit at normal temperature and pressure, defined as air at 20 °C (293.15 K, 68 °F) and 1 atm (101.325 kN/m^2).

For the specific rate of oxygen utilization, qO_2 (mmol oxygen/g cells/h) represents the oxygen consumption per biomass unit

$$qO_2 = \frac{41.3 \cdot F \cdot \%O_2}{V \cdot X} \tag{3}$$

where $\%O_2$ is the volumetric percentage oxygen uptake from the measurements in the effluent gas from the online mass spectrometer; 41.3 is a constant for the conversion of gas volumes into mmolar unit.

For carbon recovery, CR (%) which represents the total utilization of carbon by the cells

$$CR = \frac{(41.7 \cdot D) + qCO_2 + qP}{6 \cdot qS} \tag{4}$$

where qP represent the specific product production rate and qS is the specific glucose utilization rate. The constant 41.7 is the conversion constant to obtain mmolar elemental carbon composition in the biomass.

$$qP = \frac{D \cdot \text{Organic acid}}{X} \tag{5}$$

(Multiply by 4 for succinic acid, by 3 for propionic and lactic acid, by 2 for acetic acid and by 1 for formic acid).