Journal of Biotechnology 147 (2010) 46–51

Contents lists available at ScienceDirect

Journal of Biotechnology

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

High productivity of ectoines by *Halomonas boliviensis* using a combined two-step fed-batch culture and milking process

Doan Van-Thuoc[a,b], Héctor Guzmán[a,c], Jorge Quillaguamán[c], Rajni Hatti-Kaul[a,*]

[a] Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden
[b] Department of Micro-biotechnology, Faculty of Biology, Hanoi National University of Education, 136 Xuan Thuy, Cau Giay, Hanoi, Viet Nam
[c] Centro de Biotecnología, Facultad de Ciencias y Tecnología, Universidad Mayor de San Simón, Cochabamba, Bolivia

ARTICLE INFO

Article history:
Received 24 November 2009
Received in revised form 25 February 2010
Accepted 2 March 2010

Keywords:
Fed-batch cultivation
Ectoine
Hydroxyectoine
Halomonas boliviensis

ABSTRACT

A process comprising two-step fed-batch cultivation has been investigated for the production of ectoines using the halophilic bacterium *Halomonas boliviensis* DSM 15516^T. The first cultivation was performed under optimal conditions for cell growth and resulted in cell mass concentration of about 41 g l^{-1} after 24 h of cultivation. During the second cultivation at higher salt concentration, accumulation of ectoines increased while cell mass decreased with increasing salt concentration. Maximum productivity of total ectoines reached was 10 g l^{-1} d^{-1} with ectoine concentration of 6 g l^{-1} and hydroxyectoine concentration of 8 g l^{-1} after 9 h of cultivation at 18.5% NaCl, which is among the highest reported so far. *H. boliviensis* cells were further recycled for the production process after releasing the ectoines. About 75% of the accumulated ectoines were released by subjecting the cells to hypoosmotic shock. On subsequent reincubation in a medium containing higher salt concentration the cells were able to re-synthesize the ectoines resulting in a global productivity of 11.1 g l^{-1} d^{-1}, and ectoine and hydroxyectoine productivities of 9.1 g l^{-1} d^{-1} and 2.0 g l^{-1} d^{-1}, respectively.

1. Introduction

Halophilic microorganisms grow optimally at sodium chloride concentrations of 5% (w/v) or higher in the environment (Oren, 2008), and have evolved distinct metabolic strategies to withstand the high salt concentrations. Most haloarchaea accumulate cations such as K^+ in concentrations equivalent to the extracellular Na^+, while most halophilic eukaryotes and bacteria store organic compounds such as amino acids, amino acid derivatives, sugars or other polyols, that do not interfere significantly with the regular metabolism of the cells, and that help to counteract the osmotic stress caused by NaCl to the cells, thus behaving as osmolytes (Roberts, 2005; Oren, 2008). The osmolytes contribute towards maintaining turgor pressure, cell volume, and concentration of electrolytes in the cells (Roberts, 2005). Bacterial species within the family *Halomonadaceae* survive at extraordinarily high NaCl concentration (up to 32%, w/v) by synthesizing mainly ectoine and hydroxyectoine – collectively called ectoines – as osmolytes. Ectoines are even found in many other halotolerant and halophilic bacterial groups (Roberts, 2005). The ectoines protect the cells against heating, desiccation and freezing (Kuhlmann et al., 2008; Roberts, 2005; Vargas et al., 2008). The amount of osmolytes stored in the cells increases as the stress is increased, and diminishes by release of the osmolytes from the cells when the environmental conditions are optimal for cell growth (Kuhlmann et al., 2008; Roberts, 2005; Vargas et al., 2008).

The protective action of ectoines on biological compounds (e.g. enzymes, DNA, cell membranes, antibodies and whole cells) highlights their potential in fields related to molecular biology, agriculture, food processing, biotechnology, pharmacy and medicine (Lippert and Galinski, 1992; Louis et al., 1994; Göller and Galinski, 1999; Knapp et al., 1999). Currently, production of ectoine is performed by a process named "bacterial milking", involving repetitive cycles of a fed-batch fermentation of *Halomonas elongata* at 15% (w/v) NaCl to allow ectoine accumulation followed by osmotic downshock at 3% (w/v) NaCl to release the osmolytes from the cells (Sauer and Galinski, 1998). Alternative processes reported for production of osmolytes are based on fed-batch fermentation of *Brevibacterium epidermis* (Onraedt et al., 2005), and continuous synthesis and excretion of osmolytes by recombinant *Escherichia coli* strains (Schubert et al., 2007; Bestvater et al., 2008) or the wild type strain *Halomonas salina* (Zhang et al., 2009). The latter system has resulted in the highest volumetric productivity of ectoine (7.9 g l^{-1} d^{-1}). On the other hand, *Marinococcus* M52 grown in a fed-batch-microfiltration system provided elevated concentrations of hydroxyectoine from ectoine during its stationary phase of growth (Schiraldi et al., 2006).

* Corresponding author. Tel.: +46 46 222 4840; fax: +46 46 222 4713.
E-mail address: Rajni.Hatti-Kaul@biotek.lu.se (R. Hatti-Kaul).

0168-1656/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2010.03.003

Table 1
Media composition for ectoines production by *H. boliviensis* in a bioreactor.

Component	Seed culture ($g\,l^{-1}$)	Fed-batch medium ($g\,l^{-1}$)	Feed solution (first step) ($g\,l^{-1}$)	Feed solution (second step) ($g\,l^{-}$)
Glucose	10	20	500	500
NaCl	45	V	45	V
NH_4Cl	2.3	4.0	40	10
$MgSO_4{\cdot}7H_2O$	2.5	6.94	69.4	17.35
K_2HPO_4	0.55	3.54	35.4	8.85
$FeSO_4{\cdot}7H_2O$	0.005	0.01	0.1	0.025
Tris	15	–	–	–
Glutamine	3	–	–	–
Monosodium glutamate	–	20	–	–

V: component concentration was varied in different experiments.

Recent studies on members of the family *Halomonadaceae*, i.e. *Halomonas halodenitrificans*, *H. halodeneurihalina*, *H. salina*, *H. elongata* (Mothes et al., 2008) and *H. boliviensis* (Guzmán et al., 2009) have demonstrated their ability to synthesize ectoine and poly(3-hydroxybutyrate) (PHB) in the same process. The process designed for *H. boliviensis* comprises a two-step fed-batch culture in which the first fed-batch step is used to attain high cell densities, whereas the second to induce the accumulation of the products in the cells (Guzmán et al., 2009). This procedure led to high productivities of ectoine (up to $3.4\,g\,l^{-1}\,d^{-1}$), similar to those described for *H. elongata* and *B. epidermis* (Guzmán et al., 2009). Nevertheless, the entire potential of *H. boliviensis* as ectoines producer was not utilized since the synthesis of both ectoine and PHB require acetyl-CoA as intermediate, hence the anabolic routes to these products would influence each other (Guzmán et al., 2009).

The present work involves a study on increasing the production of ectoine and hydroxyectoine by *H. boliviensis* using the two-step fed-batch cultivation system coupled with bacterial milking.

2. Materials and methods

2.1. Bacterial strain, maintenance- and cultivation media

H. boliviensis LC1^T (=DSM 15516^T) was maintained on HM agar plates at 4 °C (Quillaguamán et al., 2004), containing (%, w/v): NaCl, 4.5; $MgSO_4{\bullet}7H_2O$, 0.025; $CaCl_2{\bullet}2H_2O$, 0.009; KCl, 0.05; NaBr, 0.006; peptone, 0.5; yeast extract, 1.0; glucose, 0.1; and granulated agar, 2.0. The pH of the medium was adjusted to 7.5 using 3 M NaOH.

The culture media for production of osmolytes used in this study are shown in Table 1. Glucose, KH_2PO_4 and monosodium glutamate were sterilized separately. The pH of the media was adjusted to 7.5 using 5 M HCl or 5 M NaOH solutions.

2.2. Determination of ectoines release and cell survival after osmotic downshock

H. boliviensis was grown in batch medium containing 15% (w/v) NaCl at 30 °C for 30 h (Guzmán et al., 2009). The cell pellet (corresponding to about 30 mg dry weight cells) obtained by centrifugation of 3 ml culture broth at $6000 \times g$ for 10 min, was suspended in 1 ml distilled water or saline solution (containing 1.5% and 3% (w/v) NaCl) and incubated at 25 °C with shaking at 200 rpm for 30 min for ectoines release. The suspensions were then centrifuged at $6000 \times g$ for 10 min and the ectoine content in the supernatant was analyzed. The release of ectoines was calculated as the percentage of the amount released from the cells with respect to that stored in the cells before the osmotic downshock. The cell pellet obtained after centrifugation was suspended in a sterile solution of 15% (w/v) NaCl, serially diluted in the same solution and subsequently plated on solid HM medium. Cells obtained from the culture broth without being subjected to osmotic downshock were similarly suspended, diluted and plated on HM medium. *H. boliviensis* colonies on the plates were counted after 2 days of cultivation at 30 °C. The percentage of cell survival was calculated based on the ratio of the number of colonies found after the osmotic downshock with respect to the number obtained from the cells that had not experienced the downshock.

2.3. Ectoines production by two-step fed-batch cultivation

H. boliviensis was first grown in 150 ml of seed culture medium (Table 1) in 1 l shake-flask, on a rotary shaker (New Brunswick Scientific, NJ, USA) at 30 °C and 200 rpm for 13 h ($OD_{600} = 2.5 \pm 0.1$). The medium was then used to inoculate 1.35 l of fed-batch medium with $45\,g\,l^{-1}$ NaCl (Table 1) in a 2-l bioreactor (Voyager, Luton, UK) as first fed-batch step of cultivation. Cells were cultivated for 24 h and subsequently harvested from the culture broth by centrifugation at $5500 \times g$ for 10 min at 4 °C, and used to inoculate 1.4 l of fed-batch medium with a higher NaCl concentration (12.5%, 15.5%, and 18.5%, w/v) for triggering the production of ectoines in a second fed-batch culture. The samples were taken every 3 h for biomass and ectoines analysis.

During the fed-batch cultivations, temperature was kept constant at 35 °C and the pH was maintained between 7.5 and 7.8 by adding 5 M HCl/NaOH. Stirring velocity and aeration, initially set at 700 rpm and $1\,l\,min^{-1}$, were increased during the fermentation to maintain the dissolved oxygen concentration above 20%. The highest speed for agitation and air inflow attained were 1100 rpm and $5\,l\,min^{-1}$, respectively. Glucose concentration was maintained at about $20\,g\,l^{-1}$ by adjusting the feed solution (Table 1) when a decrease in the glucose concentration was detected by off-line analysis. Monosodium glutamate (MSG) was also kept at 20 ($\pm$2) $g\,l^{-1}$ by off-line analysis of samples and adding a $500\,g\,l^{-1}$ MSG solution during the first fed-batch fermentation. For the second fed-batch step, MSG was maintained above $5\,g\,l^{-1}$. Polypropylene glycol 2025 or pure silicon oil was added as antifoam when required.

2.4. Two-step fed-batch culture coupled to bacterial milking

H. boliviensis was grown in two-step fed-batch system as described above. After 12 h of the second fed-batch in a medium with 15% (w/v) NaCl, the cells were harvested by centrifugation at $5500 \times g$ for 10 min at 4 °C and the pellet was re-suspended in 1.5 l of 1.5% (w/v) NaCl solution to induce the release of ectoines. The cell suspension was stirred at 200 rpm for 30 min at 25 °C, and subsequently centrifuged ($5500 \times g$, 10 min, 4 °C). The supernatant containing the ectoines was analyzed, the pellet was transferred to the fed-batch medium containing 15% (w/v) NaCl and incubated again for another 15 h under the conditions described for the second fed-batch step. During all cultivations, samples were taken every 3 h for biomass and ectoines analysis.