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Poly[(*R*)-3-hydroxybutyrate] production under different salinity conditions by a novel *Bacillus megaterium* strain

Research Paper

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Bacillus megaterium **uyuni S29, isolated from the Bolivian salt lake Uyuni, displays a high capability to produce poly[(*R*)-3-hydroxybutyrate] (PHB) in industrial culture media. In order to analyze the influence of salt on biomass formation and PHB production, cultivations at different NaCl concentrations were carried out according to the salinity conditions of the habitats of the strain's original isolation. In this preliminary report, the strain showed considerable adaptability to media of different salinity, obtaining the best results for both cellular growth and PHB production in media containing 45 g/L NaCl. The strain grew at 100 g/L NaCl and PHB production was observed even at high salt levels of 250 g/L without unwanted concurrent spore formation. Its tolerance to high salt concentrations together with auspicious PHB productivity makes this strain appealing not only for PHB production, but also for other biotechnological applications such as the treatment of salty wastewater; additional studies will be needed to further increase PHB productivity.**

Introduction

Halophiles constitute a versatile group of microorganisms characterized by their requirement for hypersaline environments where NaCl constitutes the predominant salt component. The adaptation to life in high salt concentrations can be accomplished in different ways. The most common strategy involves the accumulation of organic compatible osmotic solutes without the need for specialized adaptation of intracellular proteins to the high quantity of salt [1–3]. The great diversity of strategies used by halophiles to deal with high salinity in their environment, associated with the fact that halophilicity takes place throughout the entire tree of life, suggests that adaptation of living organisms to high salt concentrations is rather facile from a metabolic point of view, and probably emerged several times during evolution. A well-known example of an organism's adaptive response to changing environmental conditions is the switch in the pigment pattern of microalgae, which, besides illumination, also depends on the effective salt concentration [4].

Most authors distinguish three kinds of halophilic bacteria: halotolerant (tolerate 0–15% NaCl), moderate halophiles (require 1–15% NaCl) and extreme halophiles (require 15–30% NaCl) [5]. Representatives of the latter group of microorganisms have shown a great potential for biotechnological production of, *inter alia*, polyhydroxyalkanoates (PHAs). For example, PHA biosynthesis by the halophilic strain *Halomonas boliviensis* [6] and the extremely halophilic archaeon *Haloferax mediterranei* [7–9] are described in the literature. In addition, many *Bacillus* species have been classified as halotolerant [10] and constitute potential candidates for PHA production. As Gram-positive organisms, *Bacilli* are of increasing importance for production of endotoxin-free PHA which displays considerable advantages for *in vivo* applications of biopolymers, for example, as implants or suture materials [11]. In this context, *Bacillus megaterium* strain uyuni S29 was recently isolated from Bolivian saline water and mud samples from Uyuni salt lake

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and studied for poly[(*R*)-3-hydroxybutyrate] (PHB) production in conventional industrial media [12,13]. Diverse beneficial, but also negative features arise from cultivation of microbes in saline media, such as a minimized energy requirement by reduced sterility precautions and facilitated downstream processing, but also special requirements for the bioreactor equipment [14,15]. A particularly high impact on growth and product formation kinetics can be expected with dependence on the salinity.

Regarding *B. megaterium*, there is little information available in the literature dealing with the influence of salt concentration on growth and biopolymer production. Hence, the objective of this study was to analyze the influence of salt concentration both on cell growth and production of PHB as an intracellular product of secondary metabolism in the novel strain *B. megaterium* uyuni S29; PHB production was induced by limitation of exogenous nitrogen source. The intention was to get deeper insights into the strain's metabolic versatility and to assess its biotechnological potential as a biocatalyst for biopolyester production: it aimed to provide new data related to the influence of salt on PHB production by this strain and to reveal the function of salt in halophilic microorganisms. It was demonstrated that, since the strain accumulates PHB both at high concentrations of NaCl and its absence, it can be regarded a promising candidate for biotechnological applications under fluctuating environmental conditions.

Materials and methods

Microorganism and culture medium

The eubacterial wild type strain *Bacillus megaterium* uyuni S29 isolated from mud with saturated brine of the hypersaline Uyuni Lake (Bolivia) was used in this study [12,13]. The strain was deposited at the Spanish Type Culture Collection (CECT number 7922).

Cultivation conditions

Pre-cultures were prepared by inoculation of single colonies from solid M medium and cultivation for 24 h at 35°C in 100 mL of liquid M medium as described previously [14]. A pre-culture with an optical density at λ = 420 nm (OD_{420}) of 10.9 and a pH-value of 7.0 was selected to inoculate four cultivation set-ups, each one supplemented with different salt concentration: 5, 45, 100 and 250 g/L NaCl. The fermentations were carried out in two parallel set-ups per salt concentration in baffled 1 L flasks, containing 250 mL of M medium with its corresponding salt content. The incubation was carried out at pH-7 at 35°C and 130 rpm. Glucose as sole carbon source was supplied by adding a concentrated solution of monohydrated glucose (50%, w/v).

Analytical methods

Five samples of 5 mL of culture medium were taken throughout the incubation at t = 0, 11, 15, 17, and 21 h of cultivation from every flask. Analyses for the determination of cell dry mass (CDM) (g/L), PHB concentration (g/L), PHB content in cell dry mass (CDM) (wt%), residual biomass (RB) (g/L), and salt content (g/L) were carried out following standardized procedures as described previously [14,15]. In addition, the FTIR of the extracted polymer from the different cultivations was recorded and analyzed according to [14].

Determination of substrates

Glucose and salt content were determined by means of HPLC equipment, consisting of a thermostated Aminex HPX 87H column (thermostated at 75°C, Biorad, Hercules, USA), a LC-20AD pump, a SIC-20 AC autosampler, a RID-10A refractive index detector and a CTO-20 AC column oven. LC solution software was used for registration and evaluation of the data. Quantities of 1.5 mL of the cell-free cultivation supernatant were transferred into vials, and water was used as an eluent at a flow rate of 0.6 mL/min. The standards were prepared with different concentrations of glucose and NaCl. For determination of nitrogen source, 2 mL of supernatant was mixed with 50 μL alkaline ISAB solution containing 5 M NaOH, 10% methanol, 0.05 M Na_2–EDTA and a colour indicator. The mixture was immediately analyzed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring different ammonium sulphate standards solutions of defined concentrations.

Microscopic monitoring of cells

After each sampling, shape and physiological state of bacterial cells were examined microscopically by an Olympus BH-2 phase contrast microscope.

Results

The main results of the cultivations are shown in Table 1 and Fig. 1. In each set-up, independent of the salinity of the cultivation medium, the PHA produced was identified as the homopolyester of (*R*)-3-hydroxybutyrate (PHB). The growth curves of the strain in media of different salinity showed two distinct tendencies depending on the salt concentrations. The first obvious trend was associated with lower concentrations of NaCl (5 and 45 g/L); the other related to the highest salt concentrations (100 and 250 g/L), respectively. As a major outcome, the optimal salt concentration for cellular growth was 45 g NaCl/L, although significant growth was also observed at 5 and 100 g/L NaCl, hence within a broad range of salinity. Cultures containing 250 g NaCl/L showed only a very modest increase of OD_{420} until 11 h of cultivation, followed by a decrease of OD_{420} practically to zero. Therefore, these salt concentrations were not further considered. Observed by light microscopy, none of the investigated *B. megaterium* uyuni S29 cultures displayed spore formation, a well-known problem in cultivation of *Bacilli* [16], throughout the fermentation in M medium.

Fig. 1a shows the growth curve of cultures with 5 g/L NaCl in the medium. Although the nitrogen source (ammonium) was limited, no changes in PHB production were observed under these conditions, since the maximal PHB accumulation was already achieved before this time; values for PHB, RB, and CDM remained constant from t = 11 h until the end of the cultivation. Growth curves of the cultures with 45 g/L NaCl are shown in Fig. 1b. PHB, RB, and CDM increased during the entire fermentation. As soon as the nitrogen source became limited, an increase in PHB accumulation was observed, resulting in a maximal PHB content of 2.09 g/L PHB (or 41 wt%, respectively) after 21 h of cultivation. Fig. 1c shows growth curves of the cultures with 100 g/L NaCl. The strain shows a distinct lag phase (until the sampling at t = 11 h) with lower final values for PHB compared to the other cultivation set-ups. Here too, an increase in PHB concentration can be observed, when the