

Short communication

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

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Abstract

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.

Key words: *Bacillus*; *Bacillus megaterium* uyuni S29; biopolyesters; halophilic microorganisms; polyhydroxyalkanoate

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, e.g. 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 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 hours at 35 °C in 100 ml of liquid M medium as described previously [14]. A pre-culture with an optical density at $\lambda=420$ 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 Na₂-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 OD₄₂₀ until 11 h of cultivation, followed by a decrease of OD₄₂₀ 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 = 11h 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 nitrogen source (ammonium) was limited after 17 h of cultivation. In this case, RB concentration was lower compared to the set-ups containing 45 g/L NaCl, but higher than for the cultures with 5 g/L NaCl.

Glucose consumption was constant in all cultures and was maintained in excess by adding a concentrated stock-solution. The salt concentration in the media changed during the cultivation time; the value was constant at the beginning, followed by a decrease in all set-ups.

The FTIR of the extracted polymer from the different fermentations always showed the same spectrum corresponding to PHB homopolyester. An example of such a spectrum is shown in Fig. 2.

Discussion

Some recent articles provide new methods [7] and results [17] on the influence of salt concentration on growth and biopolymer production by *B. megaterium*. In this sense, the present study provides new data related to the influence of salt on PHB production by *Bacillus megaterium* uyuni S29 and broadens knowledge about the metabolic behaviour of this new strain.

Bacterial growth and PHB production

The results of the study show that the strain behaves differently depending on the quantity of salt present in the medium and, similar to findings reported by Salgaonkar [17], shows longer lag phases in higher salt concentration. In this case, the bacterium is confronted with salt stress conditions that cause a physiological reaction to overcome this unusual situation, implying a decreased growth rate and lower PHB production. Conversely, studies carried out with *Cupriavidus necator* and *Rhizobium* DDSS-69 show that NaCl stress results in unaffected biomass formation and enhanced PHB accumulation [18]. *B. megaterium* uyuni S29 grew and accumulated PHB in fermentations with different NaCl concentrations. However, better results for CDM and PHB concentration were achieved in cultures with 45 g/L NaCl (2.09 g/L PHB, or 41 wt-%, respectively) after 21 h cultivation. This higher salt concentration not only enhanced the growth rate of the culture, but the polymer formation rate as well. Therefore, around 45 g/L NaCl can be considered the optimal salt concentration range for *B. megaterium* uyuni S29 to grow and to synthesize biopolymer in response to the high osmotic pressure of the medium; detailed further studies for an exact medium optimization should be performed to estimate this value more precisely. This salt concentration is among the highest found in the literature associated with PHB production. However, it will be necessary to develop new strategies to further increase PHB productivity by the strain, because the results are not yet sufficiently competitive in comparison with established bacteria [5,7].

The strain shows an increase in maximum specific growth rate (μ_{\max}) for the set-ups with higher salt content (100 g/L NaCl). This result could be due to its ability to adapt

to “stressful” media after a longer lag phase. Taking into account the fact that the strain was originally isolated from Uyuni salt lake with salt concentration varying over the year and extremely high values in the dry season, it is very likely that the organism could have developed special strategies to survive [12,13]. In this sense and similar to other extremely halophilic, halophilic and halotolerant bacteria [18], the synthesis of PHB most likely stimulates the synthesis of compatible solutes acting as osmolytes. This “salt-out-cytoplasm” strategy is especially important to balance the osmotic stress when the salt concentration is extremely high [19,20].

Cultivation in media with 100 g/L NaCl led to a decrease in PHB content. This result can be explained by inhibition of the PHB biosynthetic pathway and activation of other metabolic processes related to osmotic stress response [21]. *B. megaterium* uyuni S29 did not grow in cultures with 250 g/L NaCl during the 21 h period of cultivation. This high concentration of salt inhibits its growth and could be the maximum salt content tolerated for survival by the strain.

A decrease of salt concentration of around 6% after 21 h of fermentation was observed at all set-ups, independently of the salt concentration in the culture media. It appears that *B. megaterium* uyuni S29) has the ability to balance the osmotic pressure by using the “salt-in-cytoplasm”-strategy [15]. In this case, import of inorganic ions, acting as charge stabilizers into the cytoplasm occurs until the internal and extracellular salt concentrations are equal.

Halophilic classification of B. megaterium uyuni S29

Oren reports that halophiles grow optimally at NaCl concentrations of 5% (w/v) (50 g/L) or higher, and tolerate at least 10% (w/v) (100 g/L) salt [17]. *B. megaterium* uyuni S29 fulfills this condition and therefore can be classified as a typical halophile.

Potential application of the findings

The use of halophilic microorganisms as the auspicious candidates for various biotechnological applications has been suggested based on their versatility in the choice of a wide range of substrates and their simple requirements during discontinuous or continuous cultivation processes [22,23]. Haloarchaea display some advantages for the overall economic efficiency of PHA production. For example, sterilization cost is decreased, since at such a high concentration of salt, the growth of non-halophilic microbial competitors” is strongly suppressed, allowing a process without strict sterility precautions [6]. The fact that *B. megaterium* uyuni S29 is classified as halophilic makes it possible to apply the advantages of haloarchaea related to PHB production, but with the additional benefit of Gram-positives to produce endotoxin-free PHA [11,24]. In addition, several halotolerant *Bacillus* species have been used in the treatment of wastewater as denitrifiers [25], as phosphate removers [26], and as a biological solution for environmental problems associated with considerable salt concentrations [27]. In this case, the enrichment of the media with competent microorganisms that tolerate higher salt concentrations might constitute a biological and economical solution. Therefore, *B. megaterium* uyuni S29 could be an expedient strain to be applied in treatment of highly saline wastewater and at the same time to produce a biopolymer of added value. Moreover, the fact that *B. megaterium* uyuni S29 can grow in salt concentrations very similar to that of sea water (around 3.5% w/v salts, 98% of which is

NaCl) makes the strain very appealing because of the potential to use this inexpensive and abundant source as a culture medium [28].

Conclusions

Salt concentration influences both cellular growth and PHB production by the novel *Bacillus megaterium* uyuni S29. This strain behaves differently depending on the quantity of salt in the medium, requiring adaptation of the intracellular enzymatic machinery to be functional and equilibrate the osmotic pressure in the presence of high salt concentrations. The optimal salt concentration improves cellular growth as well as PHB production. The fact that, according to previous studies, the strain also displays satisfactory growth rates and PHA productivity even in absence of salt, suggests that it constitutes an auspicious candidate for biotechnological applications in additional areas apart from the PHA field. In order to finally assess the organism's competitiveness in comparison with currently applied microbial PHA producers, further studies are required to assess and optimize the strain's PHA production potential in terms of quantity (productivity, optimized downstream processing) and quality (copolyester production).

Declaration of interest

The authors report no declarations of interest.

Table legend

Table 1 Values of the main parameters of the cultivations

Figure legends

Fig. 1 CDM, RB and PHB content during the cultivation of *B. megaterium* uyuni S29 at four different salt concentrations in medium: 5 g/L NaCl (**a**), 45 g/L NaCl (**b**) and 100 g/L NaCl (**c**). The error bars refer to deviations between two parallel experimental set-ups. Black arrow indicates the limitation of the nitrogen source

Fig. 2 FTIR spectrum of PHB extracted from *B. megaterium* uyuni S29

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Figure 1

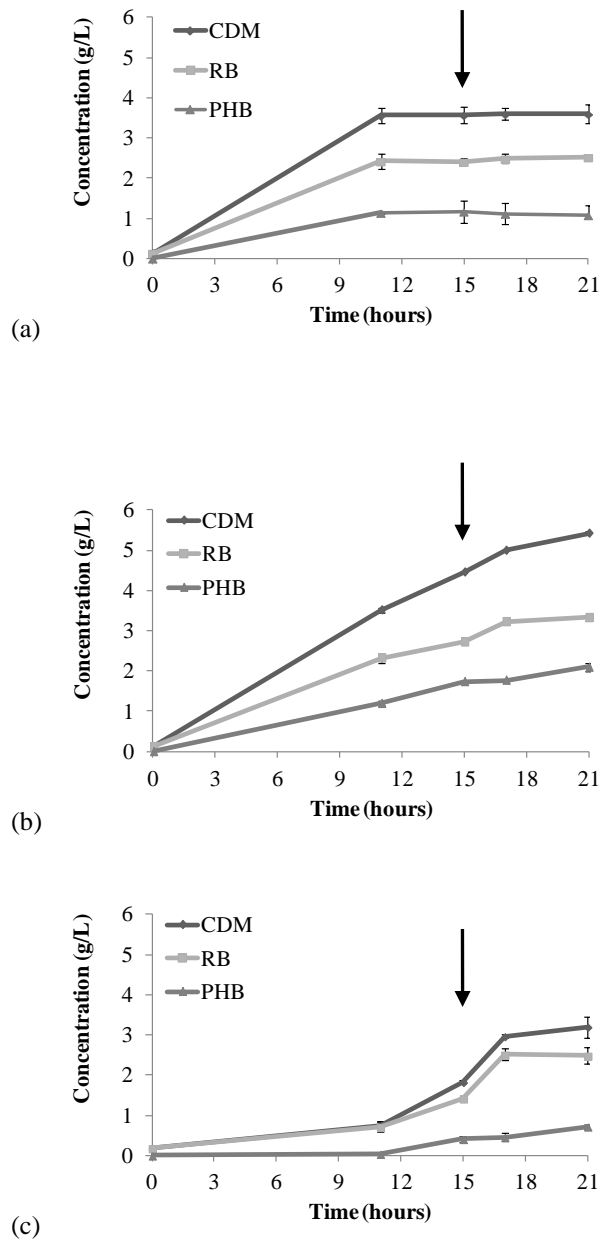


Fig. 1 CDM, RB and PHB content during the cultivation of *B. megaterium* uyuni S29 at four different salt concentrations in medium: 5 g/L NaCl (a), 45 g/L NaCl (b) and 100 g/L NaCl (c). The error bars refer to deviations between two parallel experimental set-ups. Black arrow indicates the limitation of the nitrogen source

Figure 2

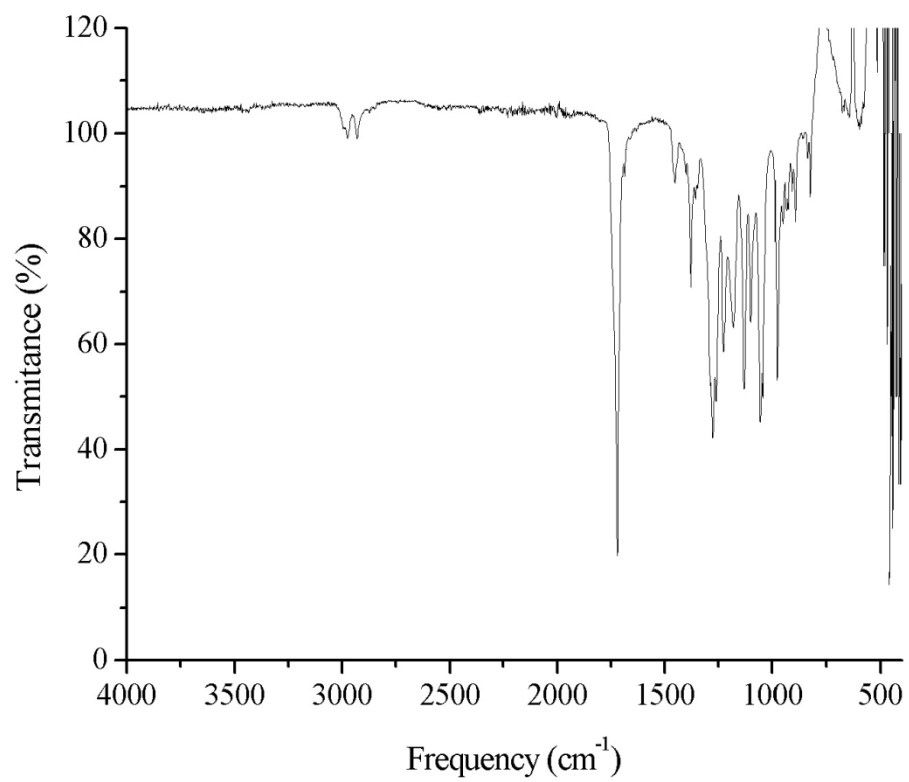


Fig. 2 FTIR spectrum of PHB extracted from *B. megaterium uyuni* S29

Table 1 Values of the main parameters of the cultivations

Salt (NaCl) in each medium [%]	5 g/L	45 g/L	100 g/L
Final CDM [g/L]	2.60±0.23	5.42±0.04	3.20±0.26
Maximum PHB [g/L]	1.17±0.28	2.22±0.11	0.72±0.06
Final PHB [g/L]	1.07±0.25	2.09±0.11	0.72±0.06
Maximum PHB content [g/g]	0.33±0.06	0.41±0.01	0.22±0.00
Maximum specific growth rate μ_{\max} [1/h]	0.22±0.08 ¹	0.21±0.05 ¹	0.30±0.06 ²
Volumetric Productivity PHB [g/Lh]	0.06±0.01	0.10±0.03	0.03±0.00
Total consumption of glucose from t = 0 to 21 hours [g/L]	9.67	15.44	3.32
Yield _(PHB/Sugars) from t = 0 to 21 hours [g/g]	0.11±0.03	0.13±0.04	0.22±0.01
Yield _(CDM/Sugars) from t = 0 to 21 hours [g/g]	0.27±0.02	0.35±0.00	0.96±0.07

¹ Calculated from the beginning of the cultivation until 11h

² Calculated from 11h until 17h