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Study on the Production and Re-use of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and Extracellular Polysaccharide by the Archaeon *Haloferax mediterranei* Strain DSM 1411

M. Koller,[a,b,*] E. Chiellini,[c] and G. Braunegg[b]
[a]University of Graz, Institute of Chemistry, Division of Physical and Theoretical Chemistry, Research group Interfaces, NAWI Graz, Heinrichstrasse 28, A-8010 Graz, Austria
[b]ARENA – Association for Resource Efficient and Sustainable Technologies; Inffeldgasse 23, 8010 Graz, Austria
[c]Department of Chemistry & Industrial Chemistry, University of Pisa, Via Risorgimento, 35 – 56126 – Pisa, Italy

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The halobacterium *Haloferax mediterranei* was used to study the production of two types of biopolymers: The biopolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) was accumulated as intracellular granules, whereas an extracelluar polysaccharide was excreted in parallel to biopolyester synthesis.

After production, microbial re-use and degradation of these polymers under different conditions were investigated to assess the requirements for handling the product-rich fermentation broth prior to the downstream processing for product recovery. Degradation kinetics of the polymers and the impact of different storage conditions on molar mass of PHBV were studied.

It turned out that the biotechnological fermentation process can be run without any sterility precautions. No major product losses were observed without pasteurization of fermentation broth after the stop of fermentation. In addition, neither PHBV nor EPS are re-utilized by the cells for biomass formation even if the culture is maintained under conditions of carbon starvation for an extended time.

Key words:
Archaea, copolyester, degradation, extracellular polysaccharide (EPS), *Haloferax mediterranei*, poly(hydroxyalkanoate) (PHA), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV)

Introduction

Archaea for "White Biotechnology"

Implementation of extremophile archaea in "*White Biotechnology*" becomes increasingly intriguing due to the fact that such organisms display high metabolic versatility that is by far insufficiently studied by the scientific community. Apart from the utilization of archaea for bioleaching to mine valued metals from diverse ores, these ancient cellular factories have also the potential to provide a broad number of marketable bio-products by conversion of various raw materials. The following groups of compounds are anticipating their market launch: Bacteriocins to be used as antibiotics[1,2,3,4,5], bacteriorhodopsin pigments for biosensors[6,7], enzymes resistant under extreme conditions[8], and, most of all, biopolymers to be used in the food industry (extracellular polysaccharides, EPS) or for production of "bioplastics", namely poly(hydroxyalkanoates) (PHA)[8].

*Corresponding author: martin.koller@uni-graz.at; 0043-316-380-5463

Biotechnological potential of the strain *Haloferax mediterranei*

For the present study, *Haloferax mediterranei* was selected due to the fact that this representative of the extremely halophilic branch of the archaea domain displays a vast spectrum of easily accessible carbon sources for the heterotrophic production of diverse intra- and extracellular bio-products with various vital functions for the strain; *inter alia*, the production of the subsequent compounds is described in the literature:

– Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) as carbon and energy reserve[8,9,10,11]

– EPS as protecting biofilms[12,13]

– C50- (bisanhydrobacterioruberin, monoanhydrobacterioruberin and bacterioruberin) and C45 (2-isopentenyl-3,4-dehydrorhodopin) carotenoide

pigments for fluidity of the membrane, adaptation to salinity and temperature, and as scavengers of free radicals[14,15], and

– Bacteriocins (in this case: *halocins*) against microbial attack[1,4].

Production of these valued compounds was described starting from carbon sources like glucose[16], hydrolyzed lactose from dairy waste[9,10], starchy residues[17,18], vinasse[19], rice-based ethanol stillage[20], or glycerol from biodiesel production[21]. Already in 1995, the high potential of this organism to be implemented in "*White Biotechnology"*, especially for bioplastic production, was discussed[22], whereas only a few years ago, the application of the strain for PHA production explicitly from different agro-industrial waste streams was suggested by several authors[9,10,17–21]. A comprehensive overview of the biotechnological potential of *H. mediterranei* and related halophilic species was provided by Quillaguamán and colleagues[23].

PHA and EPS from *H. mediterranei*

PHA are future materials of choice for several potential applications, e.g. in food packaging, or in the biomedical and pharmaceutical sector. However, their broad implementation is currently hampered due to high production costs and, sometimes, rather modest physical and mechanical properties[24]. PHA production costs depend on several factors like substrates, selected strain, cultivation strategy, and downstream processing. The utilization of cheap substrates[24–28], kinetic modeling of the processes[29], and tailored experimental design[30–31] are considered as outdoors to make PHA more competitive.

As reviewed by Koller and Muhr[32], PHA production by *H. mediterranei* is investigated both in discontinuous and continuous mode. The outcomes of published studies evidence the high stability of this organism during long-term continuous cultivation and the convenient recovery of accumulated PHA-granules without the need of organic extraction solvents[33]. In addition, similar to *Cupriavidus necator*, the best investigated PHA producer[34], the entire genome sequence of *H. mediterranei* is deciphered and reported[35].

As an outstanding property, the organism accumulates PHBV copolyesters (structure see Fig. 1) from simple carbon sources structurally unrelated to 3-hydroxyvalerate (3HV), such as carbohydrates[9,10,11,36,37] or glycerol[21]; microbes contemporarily applied for large-scale PHA production convert such substrates to poly(3-[*R*]-hydroxybutyrate) (PHB) homopolyester. This homopolyester is of modest quality, predominantly due to high crystallinity and high melting point. Independent from the carbon source (carbohydrates or glycerol) and the

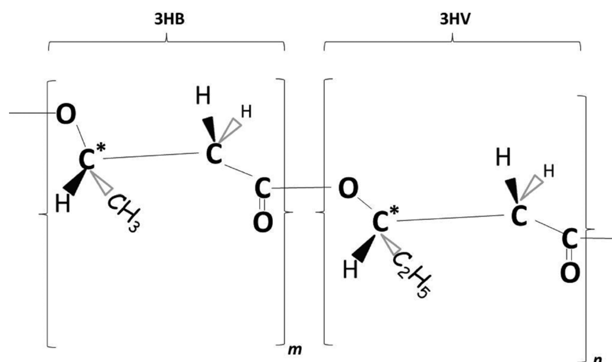

Fig. 1 *Detail from the chemical structure of PHBV produced by H. mediterranei.* ***m*** *indicates the molar fraction of 3HB in PHBV copolyesters produced by the strain from simple carbon sources with* ***m*** *~ 0.9, and* ***n*** *the molar fraction of 3HV with* ***m*** *~ 0.1.* ***m*** *+* ***n*** *amount to 1. Asterisks (*) indicates the chiral centers of the PHBV constituents.*

cultivation phase, the molar fraction of 3HV in *H. mediterranei* PHBV copolyesters always amounts to around n = 0.1 mol mol^{-1}. This is an important feature for the large-scale production of easily processible PHBV copolyesters without the need to supply 3HV-related precursors, which are costly, and often negatively impact growth and product formation kinetics. The background of this metabolic particularity was only recently elucidated and understood on the enzymatic and genetic level[37–39].

Regarding EPS biosynthesis, the strain is known to excrete an anionic EPS of high molar mass[8,16,40], resulting in a typical mucous character of colonies grown on solid medium (see also Fig. 2).

Even low concentrations of this EPS in aqueous phase result in a highly viscous solution with rheological properties comparable to those of xanthan solutions; hence, this EPS evidences its potential application in food technology as a thickening and gelling agent or stabilizer[13]. On the other hand, this change of rheological properties complicates the biotechnological cultivation process of *H. mediterranei*. Higher viscosity causes difficulties in oxygen input during the aerobic process, and higher performance and energy demands for the stirring system of the bioreactor. As elucidated by Parolis *et al.*[41] who used a smart combination of glycolysis, methylation, sulfate analysis, periodate oxidation, and NMR analysis, this EPS constitutes a sulfated (hence anionic) polysaccharide, consisting of a regular trisaccharide repeating unit of one mannose (*Man*) and two 2-acetamido-2-deoxyglucuronic acid (*GlcNAcA*) moieties; one sulfate ester bond is present per trisaccharide unit on carbon number 3 of the second *GlcNAcA* unit. Structure and linkage between the building blocks are illustrated in Fig. 3. The potential application of similar sulfated EPS of

Fig. 2 – *Haloferax mediterranei DSM 1411 after 48 h of cultivation on solid media containing yeast extract plus defined carbon source (a-d) or only yeast extract (e). Defined carbon source: starch (a); sucrose (b); fructose (c); glucose (d). The mucous character and the light pink color of samples a-d in comparison with the non-mucous reddish colonies in e are clearly visible.*

algal origin in cosmetics (as inhibitor of hyaluronidase, anti-allergic and anti-inflammatory agents and, due to antioxidant activity as nutraceuticals), as well as in therapeutic agents (due to antibacterial, antiviral, anti-inflammatory and anti-tumor activities) were only recently reviewed[42].

Aim of the study

Regarding the polymeric products of *H. mediterranei* (PHA and EPS), ample information is already available on singular aspects such as the structure of these compounds, enzymatic background, and formation kinetics of one or another of

Fig. 3 – *Structure and sequence of the regular repeating unit of the sulfated EPS produced by Haloferax mediterranei (based on findings of [41])*

these two types of polymers[8] (Poli *et al.*, 2010). What was missing until now is the investigation of the correlation between PHA and EPS formation, and the establishment of viable strategies to save energy costs on the one hand during the fermentative cultivation of *H. mediterranei* in bioreactors, and, on the other hand, during downstream processing for PHBV and EPS recovery.

In the case of the fermentation process, it was intriguing to investigate whether the classically high costs for sterilization of the production-scale bioreactor vessel could be omitted due the high salinity of the cultivation medium. *H. mediterranei* requires an impressive NaCl concentration of up to 200 g L^{-1} for optimum growth; concentrations below 100 g L^{-1} even result in cell lysis. This extremely saline medium composition should prevent microbial contamination; hence running PHA production processes without sterility precautions should be viable. This approach might be superior to contemporary attempts using mixed bacterial consortia on non-sterile nutrient-rich industrial effluent streams that necessarily result in PHA of minor to mediocre quality and restricted processibility[43]. At this point, it has to be emphasized that the application of highly saline fermentation media on a larger scale challenges the choice of material for the bioreactor. Beside high quality steel, composite materials of polyetherether ketone (PEEK), ceramics, and tech glass can be used, as previously demonstrated for two halophilic biopolymer-producing microbial isolates by Hezayen *et al.*[44] Regarding downstream processing, the task of the study at hand was to investigate what means are necessary to protect the two polymers (PHA and EPS) against degradation and catabolism before product recovery *via* cell disintegration or extraction (PHA), or precipitation (EPS). In more detail, the subsequent questions had to be clarified:

– Can the process be run without sterility precautions?

– To avoid degradation and drop in the molar mass of the accumulated PHBV: Is pasteurization of the fermentation broth necessary prior to its storage, if there is a time delay between harvest and separation of cells from supernatant e.g. by centrifugation?

– Is PHA and/or EPS re-used by the strain as reserve material under carbon-limited conditions, when the fermentation broth is still aerated and supplemented with nitrogen and phosphate source as growth-essential components?

The clarification of these questions is vital in order to design a cost-effective industrial process for PHA-production at reduced energy consumption.

Materials and methods

Microbial strain, pre-culture and inoculum preparation

H. mediterranei, obtained from DSMZ culture collection, Germany, as lyophilized sample of strain DSM1411, was maintained on agar-agar slants containing the following medium ingredients (g L^{-1}): NaCl 150; $MgCl_2 \cdot 6H_2O$ 13; $CaCl_2 \cdot 2H_2O$ 0.69; KCl 4; $NH_4Fe(III)citrate$ 0.063; SL6 1.25; $MgSO_4 \cdot 7H_2O$ 20; $NaHCO_3$ 0.25; NaBr 0.5; yeast extract 6.25; glucose 10; and agar-agar 15. From the agar slants, single colonies were transferred into 100 mL of the same liquid medium (without agar-agar) adjusted to a pH-value of 7.0, and agitated on a reciprocal laboratory shaker at 130 rpm (in baffled shaking flasks; *pre-cultures*) at 37 °C. When the cells had reached the late exponential phase (monitored by the achievement of an optical density at λ = 420 nm of app. 20), 5 mL of a selected pre-culture were transferred to 250 mL fresh culture medium baffled shaking flasks (*inoculum cultures*).

Bioreactor cultivation

The *inoculum cultures* were cultivated at 37 ºC on a reciprocal laboratory shaker at 130 rpm. After 48 h of growth, the cells had reached the late exponential phase and were added as inoculum to 7 liters of non-sterilized growth medium (same composition as inocula preparation) in a non-sterilized 10-Liter bioreactor (L 1523, Bioengineering, Wald, Switzerland). The bioreactor was equipped with a two-axial propeller stirrer. Temperature was controlled with a bioengineering temperature controller SPC, the pH-value with a Hamilton electrode and the oxygen partial pressure (pO_2) using an Ingold electrode. The pO_2 was controlled and maintained at about 20 % of air saturation by varying both agitation speed (~300–650 rpm) and aeration rate. Temperature was kept constant at 41 ºC and the pH-value in a narrow range between 6.8 and 7.4. 10 % (w/v) *Structol*™ was used as antifoam agent, 10 % (w/v) H_2SO_4 as acid, and 10 % (w/v) NaOH as hydroxide for correction of the pH-value. The fermentation was designed as a fed-batch process; re-feeding of glucose was accomplished by pulse feeding of a glucose solution (50 % w/w) according to the conversion of the substrate by the cells. The fermentation was stopped after 62 h, when the extracellular carbon source glucose was depleted. Samples were taken at regular time intervals to analyze optical density, cellular protein, concentration of glucose, concentration and composition of intracellular PHA, and concentration of extracellular EPS, as described further herein.

Surveillance of microbial culture

After each sampling, the culture was surveyed by observation using an Olympus™ BH2 light microscope at a magnification of 1/1000.

Storage experiments

After termination of the fermentation of *H. mediterranei* on glucose, the fermentation broth was distributed as follows:

– *Aerobic depolymerization experiment*: Twice 100 mL of the fermentation broth was transferred into baffled shaking flasks and supplemented with 2 g L^{-1} ammonium sulfate and app. 3 g L^{-1} phosphate (added as a mixture of Na_2HPO_4 and KH_2PO_4). The flasks were cultivated at 37 °C at a rcf of 2,600 g for 100 h. Samples were taken daily and analyzed for EPS and PHA.

– *Storage at RT without pasteurization*: One liter of fermentation broth was transferred into a glass bottle, closed, and stored at room temperature for 262.5 h. The broth was analyzed after this incubation for EPS (after the end of the experiment) and PHA (determined daily).

– *Storage at RT after pasteurization:* One liter was transferred into a glass bottle, pasteurized (80 °C, 30 min), closed and stored at room temperature for 262.5 h. The broth was analyzed after this incubation for EPS, NH_4^+ (after the end of the experiment) and PHA (determined daily).

– *Storage at 4* °C *after pasteurization*: One liter was transferred into a glass bottle, pasteurized (80 °C, 30 min), closed and stored at cooling room temperature (4 °C) for 262.5 h. The broth was analyzed after this incubation for EPS (after the end of the experiment) and PHA (determined daily).

– *Storage at 4* °C *after pasteurization*: One liter was transferred into a glass bottle, closed and stored at cooling room temperature (4 °C) for 262.5 h. The broth was analyzed after this incubation for EPS (after the end of the experiment) and PHA (determined daily).

Separation of cells for determination of PHA and cellular protein

Twice 5 mL of fermentation broth was centrifuged (Megafuge 1.0R Heraeus Sepatech, 4000 rpm, 4 °C) in glass tubes (for PHA determination), together with twice 5 mL fermentation broth in plastic tubes (for cellular protein determination). The supernatants were united, filtered, and used for substrate and EPS determination. The remaining pellets in the glass tubes were frozen and vacuum-dried overnight for subsequent PHA determination, whereas the pellets in plastic tubes were suspended in 5 mL of distilled water and directly used for determination of cellular protein.

Determination of cellular protein

Protein determination was done, after ultrasonic disruption (20 min at 35 kHz; Sonorex Digitec, Bandelin) of the cells in the pellets in the plastic tubes, according to Lowry's method[45].

Total carbohydrate analysis and EPS determination

Total carbohydrate concentration (glucose plus EPS) in supernatant was measured by a method based on the acidic anthrone method[46] and adjusted to the conditions in a *H. mediterranei* medium. EPS concentration was defined as the difference between total carbohydrate and glucose concentration.

Preparation of the anthrone reagent: 5 mL of absolute ethanol and 200 mg of anthrone were put into a 100 mL volumetric flask and dissolved with 75 % (v/v) sulphuric acid. For EPS determination, 1 mL diluted standard- or sample solution was transferred into glass tubes. The tubes were put in an ice-water bath and 5 mL of ice-cold anthrone reagent was added. Immediately after addition of reagent, the sample was vortexed until complete homogenisation and thereafter put back into the ice-water bath. The tubes were kept in the ice-water bath for about 5 minutes after adding of the reagent. All samples and standards were stirred once or twice during the cooling. Thereafter, all tubes were put in a hot water bath (96 ºC) for exactly 10 minutes, after which they were returned to the ice-water bath. After cooling, all samples and standards were measured with a spectrophotometer (Genesys 10S UV-VIS Spectrophotometer, Thermo Scientific) at a wavelength of λ = 625 nm.

Glucose analysis

Glucose was determined with HPLC equipment consisting of a thermostated Aminex HPX 87H column, an HP 7673 Controller, a JASCO 880-PU intelligent HPLC pump, and a BISCHOFF RI-Detector 8110. The substrates to be analyzed were eluted with 5 mmol L^{-1} H_2SO_4 at 0.60 mL min^{-1} flux. Glucose monohydrate standards of defined concentrations were used for external calibration.

PHA analysis

The PHA detection method was based on the simultaneous extraction and transesterification of PHA. The frozen and lyophilized biomass pellets in glass tubes were used for measuring the PHA concentration and composition in microbial biomass according to the method of Braunegg *et al.*[47] A HP 6890 chromatograph equipped with an HP 6890 injector, an autosampler, and a 15 m DB-WAX column, protected by a 5 m *HP1* capillary pre-column,

was used for polymer analysis. As an adaptation, all samples were neutralized at pH 7 after the acidic methanolysis by addition of 10 % (w/v) $NaHCO_3$ in order to prevent the column from damage. A flame ionization detector (FID) was used for detecting the methyl esters of the monomer compounds of the PHA. Helium (*Linde*, purity 4.6) was used as carrier gas, H_2 (*Linde*, purity 5.0), and synthetic air (*Linde*, purity "free of hydrocarbons") as detector gases, and N_2 (*Linde*, purity 5.0) was used as auxiliary gas. A split-ratio of 1/10 was applied.

Pure PHB and PHBV copolyester with a 3HV content of 19.1 % from Biopol™ were used for calibration; hence, the content of 3-hydroxybutyrate (3HB) and 3HV was measured; PHA was defined as the sum of 3HB and 3HV.

The PHA content in cells (% w/w) was defined as the ratio of PHA concentration to the sum of PHA and cellular protein.

PHA extraction

From the centrifuged (Sorvall RC-5B Refrigerated Superspeed centrifuge), frozen and lyophilized (Lyophilisator Christ Alpha 1–4 B) biomass, PHA was recovered by Soxhlet extraction. The dried biomass was stirred in a tenfold mass of ethanol overnight in order to remove the fatty acids from the biomass. Thereafter, the biomass was vacuum-filtrated and dried at 70 ºC to constant mass. Subsequently, the biomass was flushed with a 35-fold amount of chloroform for 20 h, resulting in the overall dissolution of the polymer. The polymer was precipitated from the chloroform solution reduced to small volume phase by adding a ten-fold surplus of iced ethanol, followed by vacuum-assisted filtration of the polymer.

Molar mass determination of PHA

Molar mass data were obtained from gel permeation chromatography (GPC) measurements on a Jasco PU-1580 HPLC connected to Jasco 830-RI detector and equipped with two PLgel 5 µm mixed-C columns. Chloroform was used as solvent at a flow rate of 1.0 mL min^{-1}. Monodisperse polystyrene standards were used for calibration.

Results and discussion

PHA production

Fig. 4 illustrates the time curves of glucose, cellular protein and PHA. Re-feedings of glucose (50 % w/w) and yeast extract (20 % w/w) solution are indicated by arrows. After a lag phase of almost 20 h, a typical value for this organism, an increase in biomass can be observed from the curve of cellular protein. It is well visible that *H. mediterranei* synthesizes PHA already during the phase of balanced growth. As shown in Fig. 5, the investigated organism possesses the rare and precious property to produce 3HV units directly from hexoses like glucose in the present case. Fig. 5 shows the percentage of PHA in the sum of PHA (here: PHBV) and protein, and the percentage of 3HV units of the entire PHBV. From the curve PHA/(PHA+protein) *vs*. time, it is obvious that PHA synthesis begins simultaneously with biomass production ("growth-associated PHA production"[48]). During the entire fermentation, the curve PHA/(PHA+protein) *vs*. time resembles a linear function. 3HV production was detectable already after the sampling at t = 4 h; the share of 3HV remains quite constant during the entire process, amounting to a mass fraction of m (3HV/PHA) = about 0.1 (10 wt.-%, respectively). For most applications, a 3HV mass fraction in PHA share of about 0.2 is necessary in order to sufficiently lower the crystallinity of the product[49]. Because approximately half of this percentage is di-

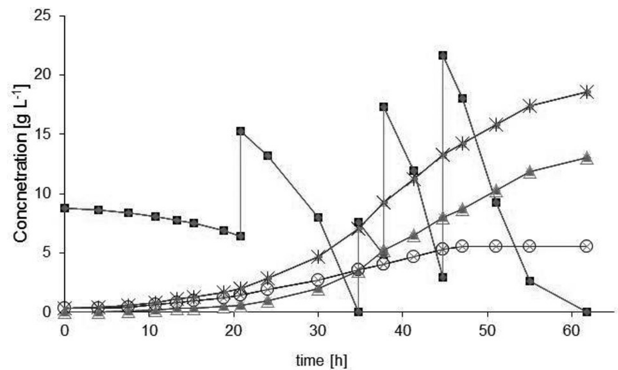

Fig. 4 – *Fermentation pattern. Basic fermentation Haloferax mediterranei on glucose and yeast extract. Filled squares: Glucose; open circles: Protein; open triangles: PHA; asterisks: sum (PHA + protein). Arrows indicate the re-feeding of glucose and yeast extract; glucose concentration was determined before and after the sampling.*

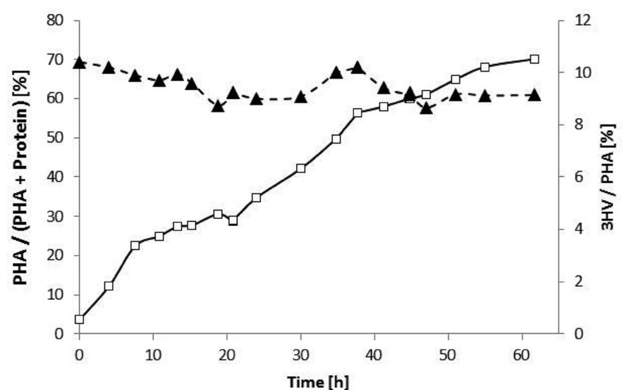

Fig. 5 – *Percentage of PHA of the cell dry mass and 3HV of the total polymer. Open squares: PHA/(PHA + protein) [% w/w], black triangles: 3HV/PHA [% w/w].*

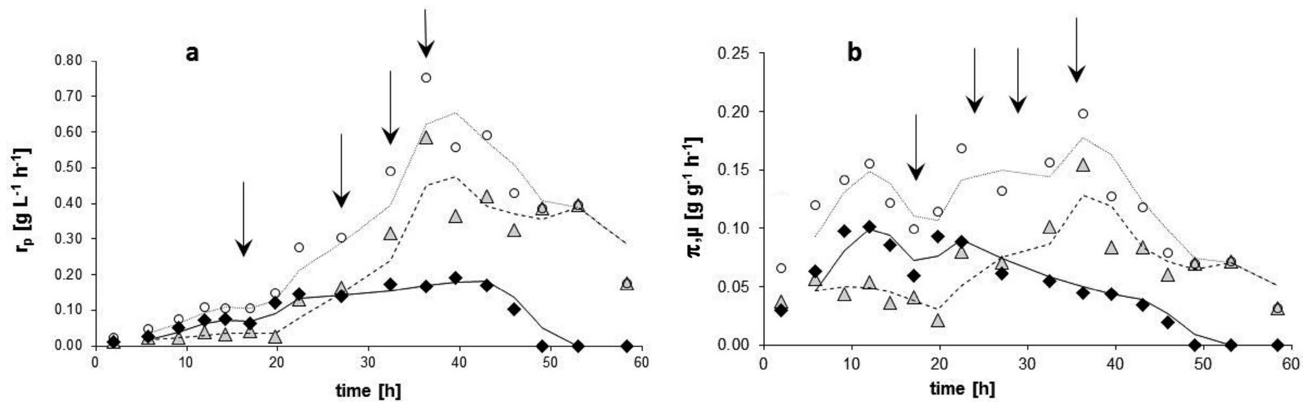

Fig. 6 – *(a) Growth rates and production rates r_p and (b) specific growth rates μ and specific production rates π of formation of protein (black diamonds), PHA (grey triangles), and sum protein + PHA (open circles). Arrows indicate the re-feeding of glucose and yeast extract; glucose concentration was determined before and after the sampling.*

rectly produced from the unrelated carbon source glucose, the necessary amount of 3HV-related precursors decreases to only 50 %.

As an important issue, the culture remained monoseptic during the entire period, although no sterility precautions were taken during this process.

From Figs. 4 and 6a, it can clearly be seen that the lag phase of the bacteria lasted about 20 h, a typical value for this strain[9,10]. Thereafter, glucose utilization rates increased as the exponential phase started (data not shown). Figs. 6a and 6b show that production of polymer rapidly increased after about 30 h. The last supplementation with yeast extract (used as nitrogen and phosphate source) was performed at hour 35. Even though growth would have been possible, as phosphate and nitrogen were abundant (data not shown), the production rates (r_p) for protein remained constant and finally even decreased after about 40 h. After 50 h, both phosphate and nitrogen were limited, and consequently the rates (r_p) and specific rates (μ) for protein production amounted to zero after 50 h (Figs. 6a and 6b). After about 49 h, r_p for protein already started to decrease, which correlated to the limitation of phosphate and nitrogen. The final concentration of protein at $t = 74$ h amounted to 5.54 g L^{-1} (Fig. 4). The final concentration and volumetric productivity for PHA for the entire process amounted to 13.02 g L^{-1} (Fig. 4) and 0.21 g L^{-1} h^{-1} (Table 1), respectively. Maximum specific PHA production [π_{max} (PHA)] was observed at sampling at $t = 36$ h with 0.15 g g^{-1} h^{-1} (see Table 1 and Fig. 6b). Table 1 lists the yields, productivities, and maximum specific rates. For conversion of glucose towards PHA, a yield $Y_{(PHA/Glu)}$ of 0.23 g g^{-1} was estimated for the entire process; regarding the sum of PHA and EPS ($Y_{[(PHA+EPS)/Glucose]}$), the yield amounted to 0.25 g g^{-1}. For formation of active biomass ($Y_{(Cellular\ protein/Yeast\ extract)}$), 1 g of added yeast extract yields 0.33 g cellular protein.

Table 1 – *Total yields, PHA productivity, and maximal specific rates of the fermentation*

Yields	
$Y_{(PHA/Glucose)}$ [g g^{-1}]	0.23
$Y_{[(PHA+EPS)/Glucose]}$ [g g^{-1}]	0.25
$Y_{(Cellular\ protein/Yeast\ extract)}$ [g g^{-1}]	0.33
Volumetric productivities	
Protein (t = 0 – 47 h) [g L^{-1} h^{-1}]	0.12
PHA (t = 0 – 61.75 h) [g L^{-1} h^{-1}]	0.21
EPS (t = 0 – 61.75 h) [g L^{-1} h^{-1}]	0.02
Maximal specific rates	
μ_{max} (protein) [h^{-1}]	0.10
π_{max} (PHA) [g g^{-1} h^{-1}]	0.13
π_{max} (EPS) [g g^{-1} h^{-1}]	0.0105

Extracellular polysaccharides (EPS) production

It was of interest to investigate the amount and formation rate of EPS during the bioreactor fermentation in direct comparison with the formation of PHA, because the deviation of the carbon flux towards EPS formation lowers the yields for PHA accumulation (see Table 1). In the described fermentation, all glucose utilized after 44 h was used for respiration towards CO_2, PHA or other non-growth associated metabolites like EPS.

It is well visible that the production of EPS occurs in parallel to PHA; this is illustrated by the production rates r_p (Fig. 7). Up to approximately 30 h, concentrations of both PHA and EPS remained at a rather low level. At approx. 50 h of cultivation, high rates of productivity are determined for both products (during this phase, formation rates for protein are highest also, see Fig. 6a). This is followed

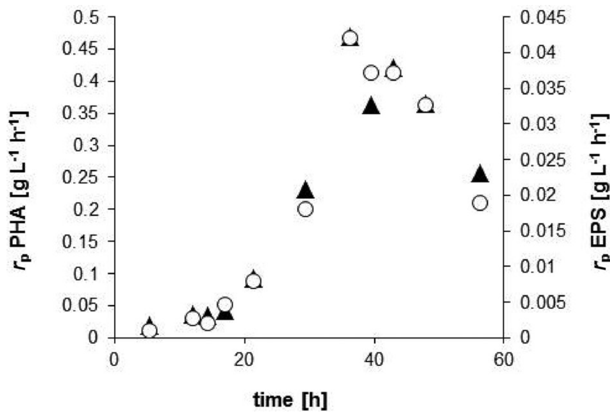

Fig. 7 – *Comparison of formation rates of EPS (open circles) and PHA (black triangles). Dashed trend line: PHA; full trend line: EPS.*

by a slowing down of the formation rates (see Fig. 7). At the end of the fermentation (t = 61.75 h), a final EPS concentration of 1.31 g L^{-1} was obtained, corresponding to a volumetric productivity for EPS of 0.02 g L^{-1} for the entire process; maximum specific productivity was observed at the sampling at t = 36 h with 0.0105 g g^{-1} h^{-1} (see Table 1). This clearly indicates that for *H. mediterranei* formation of EPS and production of PHA are competing for the available exogenous carbon source. Hence, EPS production negatively impacts the conversion yields of PHA biosynthesis due to the partial shift of carbon towards EPS instead of biopolyester production. On the one hand, this results in a loss of carbon yielding in lower end concentrations of PHA. In addition, EPS production is a process that requires a noticeable energy cost for the cells of up to 70 % of total energy reserve, hence a significant carbon investment for *H. mediterranei*[8]. Recently, it was demonstrated how to overcome this problem by deleting the region of the gene cluster responsible for EPS biosynthesis in *H. mediterranei*; in comparison with the wild type strain, a genetically engineered strain displayed PHA productivity enhanced by about 20 %[50]. On the other hand, one must not forget that the produced EPS might be of interest for different applications such as in food technology and higher sophisticated applications (see *Introduction* section).

Additionally, the formation of EPS opens the path to a simple and rapid method to scrutinize if a carbon source is converted by *H. mediterranei* or not. If the strain is grown on solid medium containing nitrogen- and phosphate source in a complex form (e.g. yeast extract; media composition see *Materials and Methods* section) plus a defined carbon source of interest, it can be distinguished in a short time by the appearance of the colonies if their growth occurs merely by conversion of the complex source (yeast extract), or if the cells also convert the defined carbon source. Colonies utilizing both the complex source and the defined carbon source are light pink and show the typical mucous character due to the EPS excretion that goes in parallel with PHA accumulation already during the early stages of cultivation. If the cells only utilize the complex compounds, the colonies do not appear mucous and display stronger reddish staining than those producing EPS. Fig. 2 presents examples for these findings.

Depolymerization and storage experiments

Polyhydroxyalkanoate degradation

Fig. 8a shows a significant decrease in PHA concentration during the first day of aerobic cultivation under conditions of limitation of exogenous carbon source, but with supplementation of nitrogen and phosphate source. This decrease slows down until the end of the second day of cultivation. After that, the concentration of polyester remains constant. In combination with the fact that no more biomass and no more EPS was generated at this stage (data not shown), it can be assumed that the consumed PHA was merely utilized for generation of maintenance energy.

If the time curve of the decrease of polymer concentration is mathematically converted into a function describing a 1st-order reaction kinetic, the decrease in polymer concentration can be calculated with a degradation constant of k = 0.0138 h^{-1} (see Eq. 1). For the case described in Fig. 8, this is valid for the first two days, because with longer cultivation, no further decrease in PHA concentration was monitored. From this point, degradation of PHA slowed down to the absolute minimum needed to maintain the basic metabolism for the culture´s survival. The nutrients necessary for optimum maintenance of the enzymatic PHA degradation system, especially nitrogen source, were already depleted at this point (data not shown).

$$PHA = PHA_0 \cdot e^{-kt} \tag{1}$$

PHA PHA concentration [g L^{-1}]
PHA_0 PHA concentration at beginning of the storage experiment [g L^{-1}]
t time [h]
k degradation constant [h^{-1}]

Polymer data from the storage bottles (Fig. 8b) show a slight loss of PHA within the range of measuring accuracy (see error bars), when fermentation broth is stored at room temperature without prior pasteurization, but no significant decline in concentration at all other investigated conditions (RT after pasteurization, storage at 4 °C with and without pasteurization).

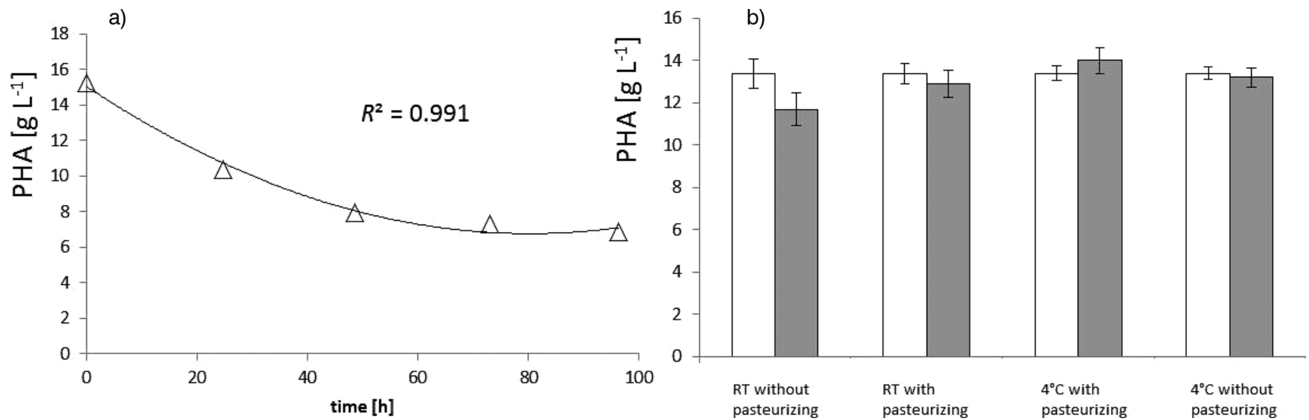

Fig. 8 – *Time courses of intracellular PHA concentration under starvation conditions: aerobic cultivation in shaking flasks (a; data from two parallel cultivations) and aerobic storing experiments (b) at two different temperatures (RT and 4 °C) with and without prior pasteurization. Fig. 8b shows the start concentrations of PHA (white bars) and the final concentrations after 262.5 h of storage (gray bars).*

EPS degradation

Fig. 9a shows that, under carbon limiting conditions, no degradation of EPS occurs, even if the cells are aerated and supplemented with nitrogen source and phosphates. This means that EPS cannot be utilized as a reserve carbon source if no external carbon source is available. This finding underlines published assumptions for the role of EPS excreted by other extremophile archaea, various Gram-negative eubacteria, or algae; available literature suggests that such EPS act as a kind of "biofilm" to protect the cells against microbial predators, mechanical damage or desiccation (reviewed by Poli *et al.*[8]). The slight increase of EPS in Fig. 9a can be considered as a result from evaporation of water during the long-term experiment.

Also in the anaerobically stored glass bottles (Fig. 9b), the final EPS concentrations did not significantly change when fermentation broth was not pasteurized prior to storage. After pasteurization, slightly higher values were obtained both after storage at room temperature and cooling room temperature. This might be due to the fact that EPS attached to the cell´s surface was released during the thermal treatment, resulting in higher EPS concentrations in the final samples than at the beginning.

PHA molar mass distribution

The polymer produced in the described fermentation trial had a weight average molar mass of approximately 650 kDa (Table 2). The polymer appeared to be of high quality and might be used as an alternative to thermoplastics from the fossil fuel industry in specialty applications by considering their much higher production and processing costs.

It is visible from Table 2 that the two samples from storage at RT show a molar mass distribution consisting of two maxima ("melting endotherms"). One of the peaks corresponds to very high molar masses (1134 and 1480 kDa), combined with ex-

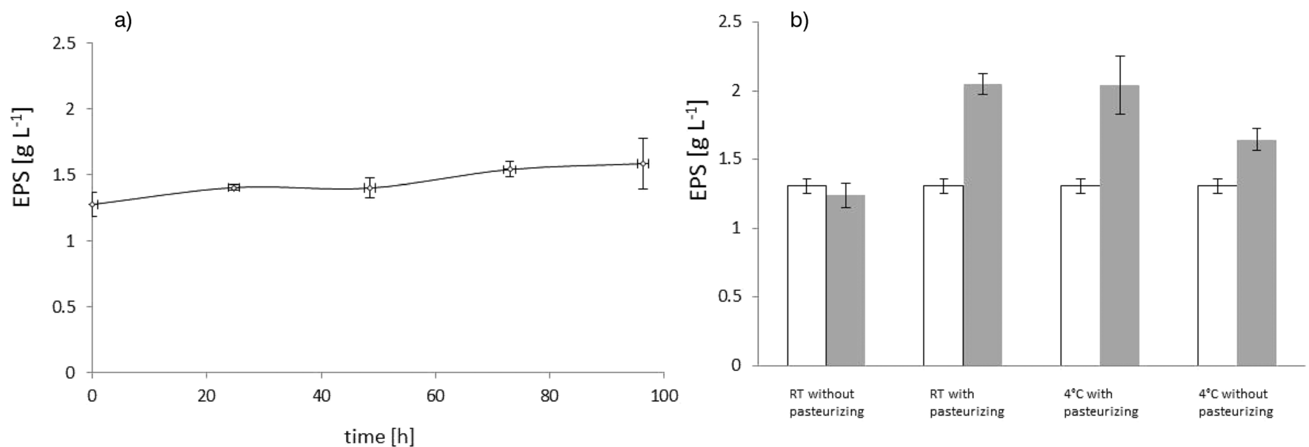

Fig. 9 – *Time courses of EPS concentration under starvation conditions: aerobic cultivation in shaking flasks (a; data from two parallel cultivations) and aerobic storing experiments (b) at two different temperatures (RT and 4 °C) with and without prior pasteurization. Fig. 9b shows the start concentrations of EPS (white bars) and the final concentrations after 262.5 h of storage (gray bars).*

Table 2 *Molecular mass distribution of PHBV samples*

Polymer isolated after	$M_{w\,1}$ [kDa][b)]	$P_{i\,1}$[b)]	$M_{w\,2}$ [kDa][c)]	$P_{i\,2}$[c)]
Cell harvest	649	3.0	–	–
100 h of aerobic cultivation and addition of nitrogen and phosphates	714	2.9	–	–
262.5 h of storage at room temperature without prior pasteurization[a)]	1134	1.3	499	13.2
262.5 h of storage at room temperature with prior pasteurization[a)]	1480	1.5	486	8.5
262.5 h of storage at 4 °C with prior pasteurization	705	3.1	–	–
262.5 h of storage at 4 °C without prior pasteurization	649	2.9	–	–

[a)]Bimodal distribution of M_n in the quoted samples
[b)]Values for the first melting endotherm
[c)]Values for the second melting endotherm

tremely low polydispersity indices. The second maximum refers to smaller chain lengths (ca. 500 kDa) with a very wide distribution (P_i = 13.2 and 8.5, respectively). The occurrence of bimodal molar mass distribution indicates the intracellular formation of a PHA blend, hence, there is evidence for two distinguished PHA-production stages performed by the microorganisms. All other samples show a similar molar mass distribution according to the weight average molar mass and the polydispersity indices. Only one peak is detectable in the GPC traces of these polyesters (data not shown). Interestingly, these samples did not result in a clear solution,when they were tried to be dissolved in chloroform for GPC analysis: a suspension of white particles occurred. Only the samples originating from storage at room temperature were easily soluble. The direct comparison of molar masses of PHA isolates after cell harvest from the bioreactor and PHA from cells cultivated a further 100 h under aeration show that the latter have higher weight average molar masses. Together with the results depicted in Fig. 8a (decrease in PHA concentration), it can be assumed that smaller PHA chains were preferentially metabolized by *H. mediterranei*.

Conclusions and outlook

The following main perceptions can be deduced from the reported experiments related to product-rich *H. mediterranei* fermentation broth handled at different operating conditions:

– Sterilization of the production bioreactor and the fermentation medium is not needed to maintain a monoseptic culture.

– According to the data, pasteurization of fermentation broth is not necessary when the broth is subsequently stored at 4 °C. When pasteurization is done, the cells can be stored for a long time without significant loss in PHA concentration (in these experiments: 262.5 h). Even storage at RT without additional treatment only causes a negligible loss of PHA.

– Under aerobic conditions with supplements of nitrogen source and phosphates, *H. mediterranei* does not re-utilize intracellular PHA for biomass formation, as would be the case for those microbial strains contemporarily used for large-scale PHA production. Even after one day of cultivation under conditions of aeration and supply of nitrogen and phosphate, together with depletion of exogenous carbon source, the major part (about 70 % w/w) of the polymer remains intact. This value is much higher than is the case for contemporarily applied PHA producers such as *Cupriavidus necator*. This indicates that a fermentation batch does not have to be interrupted immediately after the depletion of exogenous carbon source; hence, a higher degree of flexibility for the scheduling of large-scale fermentation batches is possible. The conversion of the low-molecular fractions during the first day (Fig. 8a) might even be an advantage for obtaining a more homogenous material, hence high quality at the expense of productivity.

– Excreted EPS is not converted by *H. mediterranei* under carbon-limited conditions, even when the cells are cultivated under aerobic conditions and supplemented with exogenous nitrogen and phosphate. Hence, this type of polymer does not act as a carbon reserve material for the investigated production strain.

– Combining these findings, one can argue that it is possible to design a PHA-production process that operates at low energy requirements. Together with the fact that also for downstreaming, simple alternatives can be applied by disrupting the osmophilic cells of *H. mediterranei* in hypotonic medium to release PHA granules, one can argue that a step towards an economically feasible biopolymer production is definitely accomplished.

– The potential of the excreted anionic (sulfated) EPS for various applications, especially in the neutraceutical, pharmaceutical, and therapeutic fields, needs further research efforts. Pros and cons of this material in comparison with concurring algal EPS should be elucidated, and target fields of application should be determined.

– The impact of the media composition on the diverting of carbon flux to PHA and EPS, respectively, demands detailed investigation. E.g., restricted supply of sulfate might have a significant impact on fostering PHA biosynthesis at the expense of EPS formation.

– Zero emission concepts, based on the principles of Cleaner Production, are the pre-requisite for truly sustainable biopolyesters production on an industrially relevant scale[51,52,53]. Therefore, one has to consider the ecological drawback of applying such extreme halophilic production strains. Strategies to close the saline wastewater cycle, e.g. the recyclability of the spent fermentation broth, and to convert the salt-rich fraction of biomass remaining after product recovery, need to be assessed.

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List of abbreviations and symbols

EPS – Extracellular polysaccharide
GlcNAcA – 2-Acetamido-2-deoxyglucuronic acid
GPC – Gel permeation chromatography
h – Hour
Man – Mannose
M_n – Number average molar mass
M_w – Weight average molar mass
n – Molar fraction
PHA – Poly(hydroxyalkanoate)
PHB – Poly(3-[*R*]-hydroxybutyrate)
PHBV – Poly(3-[*R*]-hydroxybutyrate-*co*-3-[*R*]-hydroxyvalerate)
P_i – Polydispersity index
pO_2 – Oxygen partial pressure
q_p – Specific production rate [g g^{-1} h^{-1}]
rcf – Relative centrifugal force
r_p – Production rate [g L^{-1} h^{-1}]
RT – Room temperature
3HB – 3-Hydroxybutyrate
3HV – 3-Hydroxyvalerate

References

1. *Cheung, J., Danna, K. J., O'Connor, E. M., Price, L. B., Shand, R. F.*, J Bacteriol **179** (1997) 548.
2. *Kis-Papo, T., Oren, A.*, Extremophiles **4** (2000) 35.
doi: http://dx.doi.org/10.1007/s007920050005
3. *Li, Y., Xiang, H., Liu, J., Zhou, M., Tan, H.*, Extremophiles **7** (2003) 401.
doi: http://dx.doi.org/10.1007/s00792-003-0335-6
4. *Platas, G., Meseguer, I., Amils, R.*, Int Microbiol **5** (2002) 15.
doi: http://dx.doi.org/10.1007/s10123-002-0053-4
5. *Torreblanca, M., Meseguer, I., Ventosa, A.*, Lett Appl Microbiol **19** (1994) 201.
doi: http://dx.doi.org/10.1111/j.1472-765X.1994.tb00943.x
6. *Stoeckenius, W., Bogomolni, R. A.,* Ann Rev Biochem **51** (1982).
7. *Oesterhelt, D., Bräuchle, C., Hampp, N.*, Quart Rev Biophys **24** (1991) 425
doi: http://dx.doi.org/10.1017/S0033583500003863
8. *Poli, A., Di Donato, P., Abbamondi, G. R., Nicolaus, B.*, Archaea **2011** (2011)
9. *Koller, M., Atlić, A., Gonzalez-Garcia, Y., Kutschera, C., Braunegg, G.*, Macromol Symp **272** (2008) 87.
doi: http://dx.doi.org/10.1002/masy.200851212
10. *Koller, M., Hesse, P., Bona, R., Kutschera, C., Atlić, A., Braunegg, G.,* Macromol Biosci **7** (2007) 218.
doi: http://dx.doi.org/10.1002/mabi.200600211
11. *Rodriguez-Valera, F., Lillo, J. G.* (1990). Halobacteria as Producers of Poly-ß-Hydroxyalkanoates. in *E.A. Dawes* (Ed.), Novel Biodegradable Microbial Polymers. Kluyver Dordrecht 1990, pp 425–426.
doi: http://dx.doi.org/10.1007/978-94-009-2129-0_35
12. *Antón, J., Meseguer, I., Rodriguez-Valera, F.*, Appl Environ Microbiol **54** (1988) 2381.
13. *Oren, A.*, J Ind Microbiol Biotechnol **28** (2002) 56.
doi: http://dx.doi.org/10.1038/sj/jim/7000176
14. *D'Souza, S. E., Altekar, W., D'Souza, S. F.*, Arch Microbiol **168** (1997) 68.
doi: http://dx.doi.org/10.1007/s002030050471
15. *Fang, C. J., Ku, K. L., Lee, M. H., Su, N. W.*, Bioresour Technol **101** (2010) 6487.
doi: http://dx.doi.org/10.1016/j.biortech.2010.03.044
16. *Rodriguez-Valera, F., Lillo, J. G.*, FEMS Microbiol Lett **103** (1992) 181.
doi: http://dx.doi.org/10.1111/j.1574-6968.1992.tb05836.x
17. *Chen, C. W., Don, T. M., Yen, H. F.*, Proc Biochem **41** (2006) 2289.
doi: http://dx.doi.org/10.1016/j.procbio.2006.05.026
18. *Huang, T. Y., Duan, K. J., Huang, S. Y., Chen, C. W.*, J Ind Microbiol Biotechnol **33** (2006) 701.
doi: http://dx.doi.org/10.1007/s10295-006-0098-z
19. *Bhattacharyya, A., Pramanik, A., Maji, S. K., Haldar, S., Mukhopadhyay, U. K., Mukherjee, J.*, AMB Express **2** (2012) 1.
doi: http://dx.doi.org/10.1186/2191-0855-2-34
20. *Bhattacharyya, A., Saha, J., Haldar, S., Bhowmic, A., Mukhopadhyay, U. K., Mukherjee, J.*, Extremophiles (2014) 1.
21. *Hermann-Krauss, C., Koller, M., Muhr, A., Fasl, H., Stelzer, F., Braunegg, G.,* Archaea (2013).
22. *Ventosa, A., Nieto, J. J.*, World J Microbiol Biotechnol **11** (1995) 85.
doi: http://dx.doi.org/10.1007/BF00339138
23. *Quillaguamán, J., Guzmán, H., Van-Thuoc, D., Hatti-Kaul, R.*, Appl Microbiol Biotechnol **85** (2010) 1687.
doi: http://dx.doi.org/10.1007/s00253-009-2397-6
24. *Khosravi-Darani, K., Mokhtari, Z. B., Amai, T., Tanaka, K.*, Biotechnol **97** (2013) 56.
25. *Khosravi-Darani, K., Vasheghani-Farahani, E, Tanaka, K.*, Iran J Biotechnol **4** (2006) 193.
26. *Mokhtari-Hosseini, Z. B., Vasheghani-Farahani, E., Heidarzadeh-Vazifekhoran, A., Shojaosadati, S. A., Karimzadeh, R., Khosravi-Darani, K.*, Bioresour Technol **100** (2009) 2436.
doi: http://dx.doi.org/10.1016/j.biortech.2008.11.024

27. *Mokhtari-Hosseini, Z. B., Vasheghani-Farahani, E., Shojaosadati, S. A., Karimzadeh, R., Heidarzadeh-Vazifekhoran, A.*, Chem Technol Biotechnol **84** (2009) 1136.
doi: http://dx.doi.org/10.1002/jctb.2145
28. *Shah-Hosseini, Sh., Sadeghi, M. T., Khosravi-Darani, K.*, Iran J Chem Chemical Eng **22** (2003) 35.
29. *Khosravi-Darani, K., Vasheghani-Farahani, E, Shojaosadati, S. A.,* Iran J Biotechnol **1** (2003) 155.
30. *Khosravi-Darani, K., Vasheghani-Farahani, E., Shojaosadati, S. A.,* Iran J Chem Chemical Eng **23** (2004) 131.
31. *Khosravi-Darani, K., Vasheghani-Farahani, E.*, Crit Rev Biotechnol **25** (2005) 1.
doi: http://dx.doi.org/10.1080/07388550500354841
32. *Koller, M., Muhr, A.*, Chem Biochem Eng Q **28** (2014) 65.
33. *Lillo, J. G., Rodriguez-Valera, F.*, Appl Environ Microbiol **56** (1990), 2517.
34. *Pohlmann, A., Fricke, W. F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Pötter, M., Schwartz, E., Strittmatter, A., Voß, I., Gottschalk, G., Steinbüchel, A., Friedrich, B., Bowien, B.*, Nat Biotechnol **24** (2006) 1257.
doi: http://dx.doi.org/10.1038/nbt1244
35. *Han, J., Zhang, F., Hou, J., Liu, X., Li, M., Liu, H., Cai, L., Zhang, B., Chen, Y., Zhou, J., Hu, S., Xiang, H.,* J Bacteriol **194** (2012) 4463.
doi: http://dx.doi.org/10.1128/JB.00880-12
36. *Han, J., Hou, J., Liu, H., Cai, S., Feng, B., Zhou, J., Xiang, H.*, Appl Environ Microbiol **76** (2010) 7811–7819.
doi: http://dx.doi.org/10.1128/AEM.01117-10
37. *Han, J., Hou, J., Zhang, F., Ai, G., Li, M., Cai, S., Xiang, H.*, Appl Environ Microbiol **79** (2013) 2922.
doi: http://dx.doi.org/10.1128/AEM.03915-12
38. *Feng, B., Cai, S., Han, J., Liu, H., Zhou, J., Xiang, H.*, Acta Microbiologica Sinica **50** (2010) 1305.
39. *Hou, J., Feng, B., Han, J., Liu, H., Zhao, D., Zhou, J., Xiang, H.,* Appl Environment Microbiol **79** (2013) 5104.
doi: http://dx.doi.org/10.1128/AEM.01370-13
40. *Fernandez-Castillo, R., Rodriguez-Valera, F., Gonzalez-Ramos, J., Ruiz-Berraquero, F.*, Appl Environ Microbiol **51** (1986) 214.
41. *Parolis, H., Parolis, L. A., Boán, I. F., Rodríguez-Valera, F., Widmalm, G., Manca, M. C., .Jansson, P.-E., Sutherland, I. W.*, Carbohydr Res **295** (1996) 147.
doi: http://dx.doi.org/10.1016/S0008-6215(96)90134-2
42. *Raposo, M. F. D. J., de Morais, A. M. M. B., de Morais, R. M. S. C.*, Life Sci **101** (2014) 56.
doi: http://dx.doi.org/10.1016/j.lfs.2014.02.013
43. *Salehizadeh, H., Van Loosdrecht, M. C. M.*, Biotechnol Adv **22** (2004) 261.
doi: http://dx.doi.org/10.1016/j.biotechadv.2003.09.003
44. *Hezayen, F. F., Rehm, B. H. A., Eberhardt, R., Steinbüchel, A.*, Appl Microbiol Biotechnol **54** (2000) 319.
doi: http://dx.doi.org/10.1007/s002530000394
45. *Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.*, J Biol Chem **193** (1951) 265.
46. *Pons, A., Roca, P., Aguiló, C., Garcia, F. J., Alemany, M., Palou, A.*, J Biochem Bioph Methods **4** (1981) 227.
doi: http://dx.doi.org/10.1016/0165-022X(81)90060-9
47. *Braunegg, G., Sonnleitner, B. Y., Lafferty, R. M.*, Europ J Appl Microbiol Biotechnol **6** (1978) 29.
doi: http://dx.doi.org/10.1007/BF00500854
48. *Horvat, P., Špoljarić, I. V., Lopar, M., Atlić, A., Koller, M., Braunegg, G.*, Bioprocess Biosyst Eng **36** (2013) 1235.
doi: http://dx.doi.org/10.1007/s00449-012-0852-8
49. *Kunioka, M., Tamaki, A., Doi, Y.*, Macromolecules **22** (1989) 694.
doi: http://dx.doi.org/10.1021/ma00192a031
50. *Zhao, D., Cai, L., Wu, J., Li, M., Liu, H., Han, J., Zhou, J., Xiang, H.*, Appl Microbiol Biotechnol **97** (2013) 3027.
doi: http://dx.doi.org/10.1007/s00253-012-4415-3
51. *Schnitzer, H., Ulgiati, S.*, J Clean Prod **15** (2007) 1185.
doi: http://dx.doi.org/10.1016/j.jclepro.2006.08.001
52. *Koller, M., Sandholzer, D., Salerno, A., Braunegg, G., Narodoslawsky, M.*, Res Cons Rec **73** (2013) 64.
doi: http://dx.doi.org/10.1016/j.resconrec.2013.01.017
53. *Titz, M., Kettl, K. H., Shahzad, K., Koller, M., Schnitzer, H., Narodoslawsky, M.,* Clean Technol Environ Pol. **14** (2012).