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Evaluation of the production of exopolysaccharides by two strains of the thermophilic bacterium *Rhodothermus marinus*

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ABSTRACT

The thermophile *Rhodothermus marinus* produces extracellular polysaccharides (EPSs) that forms a distinct cellular capsule. Here, the first data on EPS production in strains DSM4252^T and MAT493 are reported and compared. Cultures of both strains, supplemented with either glucose, sucrose, lactose or maltose showed that the EPS were produced both in the exponential and stationary growth phase and that production in the exponential phase was boosted by maltose supplementation, while stationary phase production was boosted by lactose. The latter was higher, resulting in 8.8 (DSM4252^T) and 13.7 mg EPS/g cell dry weight (MAT493) in cultures in marine broth supplemented with 10 g/L lactose. The EPSs were heteropolymeric with an average molecular weight of 8×10^4 Da and different monosaccharides, including arabinose and xylose. FT-IR spectroscopy revealed presence of hydroxyl, carboxyl, N-acetyl, amine, and sulfate ester groups, showing that *R. marinus* produces unusual sulfated EPS with high arabinose and xylose content.

1. Introduction

Extracellular polysaccharides (exopolysaccharides, EPSs) are the major part of extracellular polymeric substances produced by microorganisms (Jindal, Singh, & Khattar, 2011). They exist in two main forms; as a capsule associated with the cell surface or secreted out of the cell, either to the surroundings or remain loosely attached to the cell surface (Tallon, Bressollier, & Urdaci, 2003). Exopolysaccharides have important ecological and physiological functions and play special roles in protecting the microorganisms that produce them. EPSs are believed to protect cells against antimicrobial substances, desiccation, bacteriophages, osmotic stress, and antibodies (Mata et al., 2006; Tallon et al., 2003).

EPSs can be found as homopolysaccharides or heteropolysaccharides and can be decorated with other residues such as phosphates, sulfates, N-acetyl-aminosugars, and acetyl groups (Laws, Gu, & Marshall, 2001). The properties of the EPSs are influenced by their composition which is affected by nutrient availability as well as by other factors such as their molecular mass and the location of functional groups.

The unique and complex chemical structures of EPSs, which are natural polymers with different functional properties, make them interesting for various industrial applications in food, pharmaceutical, petroleum and other industries (Castellane, Lemos, & deMacedo Lemos, 2014), for example by affecting the fluidity of active compounds. Both prokaryotes (Gram-positive and Gram-negative bacteria) and eukaryotes (fungi, some algea, and phytoplankton) are known to produce EPSs and new species are currently being targeted in the search for EPSs with novel properties. As a result of these screening efforts, marine bacteria are now widely accepted as the source of EPSs with unique properties that can be exploited for novel biotechnological processes (Chi & Fang, 2005).

Rhodothermus marinus is a thermophilic, reddish colored aerobic, and Gram-negative bacterium that was first isolated from shallow marine hot springs in Iceland (Alfredsson, Kristjansson, Hjörleifsdotter, & Stetter, 1988). The bacterium grows heterotrophically and is known to produce highly thermostable enzymes (Nordberg Karlsson, Bartonek-Roxå, & Holst, 1998; Blücher, Nordberg Karlsson, & Holst, 2000). It is of interest as repre-

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senting the deepest lineage in the phylum *Bacteroidetes* (Nolan, Tindall, Pomrenke, Lapidus, & Copeland, 2009). The complete genome sequence is available for the type strain and tools have been developed for genetic manipulation of a second strain, MAT493 (Bjornsdottir, Fridjonsson, Hreggvidsson, & Eggertsson, 2011; Bjornsdottir, Thorbjarnardottir, & Eggertsson, 2005). The cells of *R. marinus* have been shown to form a distinct capsule when grown on carbohydrate-rich medium (Alfredsson et al., 1988). To our knowledge, however, no information is available on the production of EPSs by *R.marinus,* or on their physico-chemical properties. The aim of this study was the evaluation of EPS production by the *R.marinus* type strain (DSM 4252^T) as well as by a strain that is amenable to engineering (MAT 493). The work involved the examination of the kinetics of EPS production in batch cultures using shake flasks under varied nutritional conditions. Also, the isolation and characterization of novel EPS from the two *R.marinus* strains has opened possibilities for applying EPSs from *R.marinus* for industrial purposes.

2. Materials and methods

2.1. Materials

All materials and reagents were purchased from Sigma-Aldrich unless otherwise specified.

2.2. Bacterial strains

The *R. marinus* strains DSM 4252^T and MAT 493 obtained from the Matis culture collection, were used in the present study.

2.3. Culture conditions

R.marinus DSM 4252^T was taken from the stock culture and inoculated in ATCC medium 1599: Thermus Enhanced Medium (ATCC medium 1598) containing agar and 1% NaCl. The plate was incubated at 65 °C for 24 h, after which the cells were transferred from the plate into Marine broth (Difco 2216, USA) (10 ml) in a 50 ml falcon tube and incubated in a rotary shaker incubator at 65 °C and 200 rpm for 24 h.

R.marinus MAT 493 was inoculated directly from a stock culture into Marine broth (10 ml) in a 50 ml falcon tube and incubated in a rotary shaker incubator at 65 °C and 200 rpm for 24 h.

After the first incubation, 0.25 ml (10%) of the produced cell cultures of both strains were separately inoculated into Marine broth (2.5 ml) in 50 ml falcon tubes, and were grown for 8 h. The resulting cultures were subsequently used as inoculum for the shake flask cultivations where EPS production was studied.

The cell cultures used for analysis of EPS production were grown in marine broth (25 ml in 250 ml baffled shake flasks) containing the sugars glucose, maltose, lactose, and sucrose, respectively, as additional carbon source at the concentrations 1 and 10 g/l. The cells were inoculated with 2.5 ml (10%) of the inoculum and grown at 65 °C and 200 rpm for 48 h in a shaking incubator (IKA, KS 4000 i control). Samples were taken after 0, 6, 15, 24, and 48 h and were analysed for residual carbon source and total produced EPSs. A parallel control experiment was carried out using Marine broth without carbon source supplementation.

2.4. Determination of cell biomass

Bacterial growth was quantitatively determined by measuring cell dry weight. After centrifugation, cells were washed once in water and then resuspended in 2 ml of 0.05 M EDTA sodium salt solution (Horn et al., 2013). The mixture was shaken gently on a rocking table at 4 °C for 4 h to remove any capsular EPS. After this, cells were harvested by centrifugation, washed with water, transferred to aluminum weighing pans and dried in an oven set at 100 °C. The cell dry weight was measured periodically until a constant weight was reached.

2.5. Isolation of exopolysaccharides

Strains DSM 4252^T and MAT 493 were grown as described in section 2.3, and the capsular EPS (in 0.5 ml samples) were separated as described in section 2.4. Subsequently, the samples taken at different times from the cultivations were centrifuged at 4000 rpm for 30 min at 4 °C (SigmaPK). The EPSs were precipitated by adding the threefold volume of ethanol (99.5%) to the cell free supernatants. After mixing and storing overnight at 4 °C, precipitates were harvested by centrifugation at 4000 rpm for 30 min at 4 °C (Sigma 3–16PK) and put in a fume hood to evaporate the remaining ethanol. The precipitates then were dissolved in milliQ water and lyophilized (Labconco freeze dry system) to obtain the crude EPSs.

2.6. Purification and exopolysaccharide fractionation

The purity and size fractions of the crude EPS was examined by size exclusion chromatography using a column of HiPrep Sephacryl S-200 HR (16 mm × 600 mm) (HiPrep, GE healthcare life sciences). Each crude EPS was dissolved in milliQ water (10 g/l) and after filtration (13 mm syringe filter w/0.2 μm PTFE membrane) was loaded on the Sephacryl S-200 column. and eluted with milliQ water at the flow rate of 0.3 ml/min. The flow rate was controlled by a FPLC pumping system (Pharmacia LKB, Pump-500) and fractions were detected using refractive-index monitoring (ERC-7510, ERMA INC) and collected every 10 min using a fraction collector (LKB Bromma, 2212 HELIRAC).

2.7. Determination of molecular weight

For determination of molecular weight of the EPS fractions, standard dextrans (1.27, 5, 12, 50, and 80 kDa) (Sigma) were passed through a Sephacryl S-200 column (16 mm × 600 mm) and the retention time of each dextran was obtained from the chromatographic data. The retention times of the dextrans were plotted against the logarithms of their respective molecular weights. The molecular weights of the produced EPSs were then determined using their retention time.

2.8. Monosaccharide analysis

The analysis of EPSs monosaccharide composition, produced at different times during cultivation, and after fractionation by size exclusion chromatography (Section 2.6) was done according the hydrolysis method described by Sluiter, Hames, Ruiz, and Scarlata (2008) with modifications.

Briefly, sulfuric acid (72%) was added to the isolated EPSs and water was added after 30 min. The samples were heated at 100 °C for 3 h and neutralized after hydrolysis with 0.1 M $Ba(OH)_2{\cdot}H_2O$. After centrifugation, the monosaccharide content of the EPSs in the supernatants was analysed by High Performance Anion-Exchange Chromatography (HPAEC) (ThermoFisher Scientific, Waltham, USA) using a Dionex CarboPac PA-20 analytical column which was coupled to a Dionex CarboPac PA-20 guard column. The sugars were separated using NaOH (0.75 mM) as a mobile phase with the flow rate of 0.5 ml/min and the column was regenerated with 200 mM NaOH for 4 min with the same flow rate at the end of each cycle. Detection was performed with an ED40 electrochemical detector.

2.9. Analysis of functional groups

Fourier transformed infrared (FT-IR) spectroscopy was used to determine the functional groups of the purified EPSs. Infrared spectra of the purified EPSs fractions were recorded in the 4000–400 cm^{-1} region using a FT-IR system (Nicolet is5, ThermoFisher Scientific).

The determinations were performed in two independent replicates and are reported as the mean with standard deviations.

3. Results and discussion

3.1. Growth and EPS production by the two R. marinus strains

The two *R. marinus* strains exhibited a distinct difference in growth behaviour, with the strain DSM 4252^T forming aggregates while strain MAT 493 did not. Aggregation has been observed for many *R. marinus* strains (Bjornsdottir et al., 2005), and it is now known that bacteria predominantly live within surface-attached biofilm structures in their natural habitats (Davey & O'Toole, 2000). Biofilms offer several advantages, including protection from different stress factors (Jagmann, Henke, & Philipp, 2015; Monier & Lindow, 2003). Bacteria within these structures are encased in an extracellular matrix composed of secreted proteins, polysaccharides, DNA and other substances. The reason for the observed difference in strains DSM 4252^T and MAT 493 is unknown, as is the mechanism of aggregation in *R. marinus.* In fact, the lack of aggregation was one of the reasons for choosing strain MAT 493 for the development of methods for genetic manipulation, which require the use of single colonies. The difference in aggregation, could however not be shown to be immediately related to the EPS production level (Tables 1 and 2), which for both strains was initiated in the exponential growth phase, but with some differences.

As initial trials with the type strain (DSM 4252^T) showed that use of disaccharides as carbon source supplementation generally resulted in higher EPS production than the use of monosaccharides (data not shown), lactose, maltose and sucrose were selected as the supplementation of the Marine broth and was used for both strains. In addition, glucose was chosen as a respresentative of monosaccharides, and a parallel growth experiment without additional added sugars was run to monitor the background production of EPS (Figs. 1–4). The added carbon sources were supplied at two concentrations, 1 g/L (Figs. 1 and 3) and 10 g/L (Figs. 2 and 4), respectively.

Consumption of the monosaccharide glucose, and the disaccharides lactose and maltose was verified for the type strain (Fig. 1). In all cases, production of EPS was initiated in the exponential growth phase and was shown to continue in the stationary phase.

At the 1 g/L supplementation level (Fig. 1), the consumption rate of glucose was determined to 0.13 g/l,h during the first 6 h and after 15h all glucose was consumed and the cell mass was 1.2 ± 0.14 g/l. After glucose depletion, the change in cell concentration was marginal during 9 h (reaching 1.25 ± 0.35 g/l after 24 h) but then increased to 1.68 ± 0.16 g/l at 48 h. The late increase in cell concentration might be due to consumption of produced EPS, as it was accompanied by a decrease in the ratio of produced EPS per cell dry weight in the same period of time (from 7.4×10^{-5} to 6.2×10^{-5}), reaching a final EPS concentration of 1.04 ± 0.15 μg/ml (at 48 h). The experiments using marine broth without added sugar, showed a slightly lower maximum cell mass (0.87 ± 0.03 g/l, after 24 h, in principle maintained at the end of the cultivation, 0.85 ± 0.07 g/l after 48 h) and the final EPS concentration was 0.75 ± 0.24 μg/ml (with a production rate of 0.014 μg/ml,h after 48 h). This indicates that the 1 g/L glucose-addition had a small boosting effect on both cell mass and EPS production.

Addition of lactose did not stimulate cell growth, but resulted in increased EPS production. The consumption rate of lactose during the first 6 h was 0.03 g/l,h but increased to 0.095 g/l,h (6–15 h) until all lactose was consumed (reaching a cell mass of 0.85 ± 0.21 g/l at 24 h). After 48 h the cell mass finally reached 0.95 ± 0.35 g/l, while production of EPS continued (0.057 μg/ml,h during the whole 48 h of cultivation), reaching a final concentration of 2.75 ± 0.13 μg/ml.

The cell mass obtained in maltose supplemented cultivations resembled that of the glucose supplementation (1.3 ± 0.28 g/l after 15 h, maintained at 24 h as 1.4 ± 0.14 g/l, with an overall maltose consumption rate of 0.036 g/l,h during 24 h) (Fig. 1). The EPS concentration in this case reached 3.3 ± 0.73 μg/ml (after 48 h) which was the highest observed at 1 g/L supplementation level, reaching a production rate of 0.22 μg/ml,h between 6–15 h, which decreased to 0.031 μg/ml,h in the stationary phase (24–48 h).

Sucrose supplemented cultivations were more difficult to interpret, as this substrate was not consumed by *R.marinus* DSM 4252^T (sucrose concentration was 0.87 ± 0.03 g/l after 48 h of cultivation). The maximum cell concentration was 1.15 ± 0.21 g/l and the concentration of produced EPS was 1.98 ± 0.85 μg/ml after 48 h. The above results showed that although the effects on cell mass were rather small, production of EPS increased upon addition of the disaccharides lactose (stationary phase) and maltose (primarily in the exponential but also in the stationary phase).

A further increase in the concentration of added sugars to 10 g/l resulted in a significant increase in the production of EPS (except for sucrose added cultures), which was most pronounced for cultures with added lactose (Fig. 2, Table 1). No significant increase in cell mass or growth rate was observed, and cell growth ceased after 15 h at all conditions tested, which might be the consequence of either oxygen limitation (which is difficult to control in shake flasks) or more likely the decrease in pH observed (decreasing from 7.2 to 5.03, 5.18, and 4.84 in glucose, lactose, and maltose medium, respectively). At 10 g/L supplementation level it was also observed that maltose was gradually degraded extracellularly to glucose (from 6 h of cultivation).

Strain MAT 493 grew without visible aggregation and consumed glucose, lactose and maltose with similar rates (0.14, 0.14, and 0.135 g/l.h, respectively) in media with 1 g/l supplementation of the respective carbon source. In addition, strain MAT 493 could consume sucrose (which was not the case for the type strain) with a consumption rate of 0.065 g/l.h. All sugars were consumed within 15 h of cultivation.

In accordance with the type strain, production of EPS started at the beginning of the cultivation and the concentration of EPS increased during the exponential growth phase. After 24 h, the concentration of EPSs was 0.282 ± 0.03, 0.29 ± 0.08, and 0.3 ± 0.18 μg/ml with corresponding cell concentrations of 0.95 ± 0.07, 1 ± 0.28, and 0.8 ± 0.00 g/l in the presence of glucose, maltose and sucrose, respectively. This shows that in this strain, the production of EPS in the exponential growth phase in principle was independent of the carbon source supplementation.

In the stationary phase, the levels of EPS were, however, affected differently and depended on carbon source supplementation. In the media supplemented with 1 g/L sucrose and lactose, respectively, the EPS concentration increased with time in the stationary phase, in line with the pattern observed in lactose supplemented cultivations of the type strain. For example, the concentration of produced EPS in the medium containing lactose was 0.2 ± 0.15 μg/ml after 24 h (at a cell mass of 0.5 ± 0.14 g/l) and then increased 0.46 ± 0.05 μg/ml after 48 h. In cultivations supplemented with maltose, the amount of EPS was almost constant after 24 h, also in line with the general production pattern observed for the type strain.

In both glucose supplemented and unsupplemented cultivations, there was an apparent decrease in cell mass in the stationary

Table. 1
Evaluation of exopolysaccharide production and cell dry weight for *R.marinus DSM 4252*^{T} grown in the presence of different sugars.

Type of sugar	Sugar concentration (g/l)					
	1			10		
	EPS[a] (μg/ml)	CDW[b] (μg/ml)	EPS/CDW	EPS (μg/ml)	CDW (μg/ml)	EPS/CDW
Glucose	1.04	1680	6.0×10^{-4}	2.56	700	3.6×10^{-3}
Lactose	2.75	950	2.8×10^{-3}	8.4	950	8.8×10^{-3}
Maltose	3.3	920	3.5×10^{-3}	5.41	1100	4.9×10^{-3}
Sucrose	1.98	1150	1.7×10^{-3}	1.04	1050	9.0×10^{-4}

[a] Exopolysaccharide.
[b] Cell dry weight.

Table 2
Evaluation of exopolysaccharide production and cell dry weight for *R. marinus* MAT 493 grown in the presence of different sugars.

Type of sugar	Sugar concentration (g/l)					
	1			10		
	EPS[a] (μg/ml)	CDW[b] (μg/ml)	EPS/CDW	EPS (μg/ml)	CDW (μg/ml)	EPS/CDW
Glucose	0.14	650	2.0×10^{-4}	0.58	400	1.4×10^{-3}
Lactose	0.46	600	7.0×10^{-4}	8.21	600	1.37×10^{-2}
Maltose	0.34	1050	3.0×10^{-4}	7.68	750	1.02×10^{-2}
Sucrose	0.44	550	8.0×10^{-4}	0.24	650	3.0×10^{-4}

[a] Exopolysaccharide.
[b] Cell dry weight.

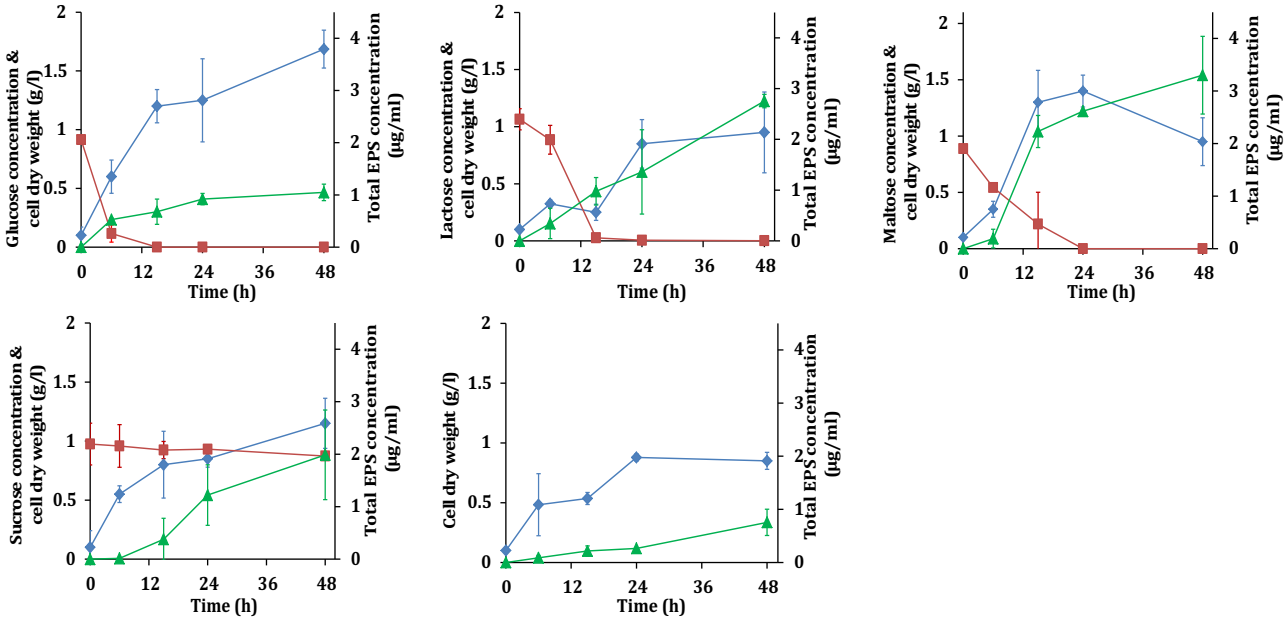

Fig. 1. Growth profile and EPS production of *R.marinus DSM 4252*^{T} cultivated in marine broth containing (1 g/l) glucose, sucrose, lactose and maltose, separately and marine broth without additional sugars as a control. Symbols indicate (♦) for cell dry weight, (▲) for total EPS concentration, and (■) for sugar concentration in the media. Results are the mean of duplicate measurements.

phase. In the glucose supplemented medium this coincided with a decrease in EPS concentration, indicating that there might be a degradation of the EPS by active enzymes produced by the cells, which may be released upon cell lysis (Mata et al., 2006). No corresponding decrease was however observed in the nonsupplemented cultures (in that case the monitored EPS was 0.36 ± 0.029 μg/ml at 24 h and 0.6 ± 0.12 μg/ml at 48 h).

An increase in the amount of added sugar to 10 g/l resulted in a higher relative production of EPS by strain MAT 493, for all the tested carbon sources except sucrose (Fig. 4; Table 2), which is in accordance with the pattern obtained for DSM 4252^T (Table 1). Degradation of the disaccharide to its monosaccharide components (after 6 h of cultivation) was observed for maltose (in accordance with the data for the type strain) but also for sucrose (resulting in detection of both glucose and fructose). The increase in EPS productionin lactose and maltose supplemented cultures was also more pronounced for MAT 493 (Table 2) than for DSM 4252^T, while cell mass production was approximately in the same range. The pH value in the cultures containing glucose, lactose, sucrose and maltose was also shown to decrease significantly (to 4.65, 4.39, 4.47, and 4.54, respectively after 48 h) and may be a reason for stopped growth in shake flasks.

In conclusion, the relative efficiency of EPS production which is the ratio of the total EPS to cell dry weight after 48 h, was evaluated and showed that marine broth supplemented with 10 g/l lactose resulted in the highest EPS production efficiency in both *R.marinus*

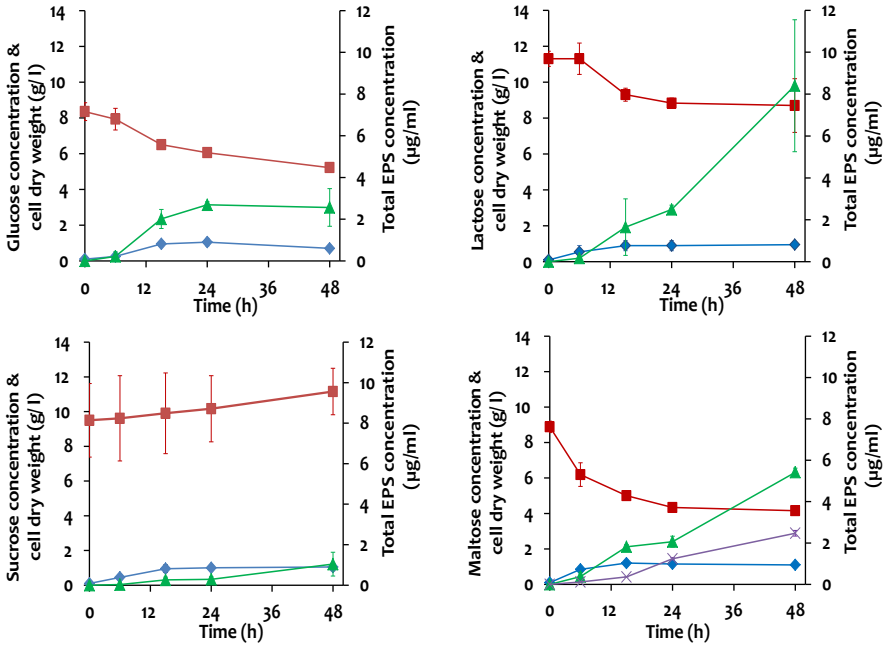

Fig. 2. Growth profile and EPS production by *R.marinus DSM 4252*^{T} cultivated in marine broth containing (10 g/l) glucose, sucrose, lactose and maltose, separately and marine broth without additional sugars as a control. Symbols indicate (♦) for cell dry weight, (▲) for total EPS concentration, (■) for sugar concentration in the medium, and (×) for glucose concentration. Results are the mean of duplicate measurements.

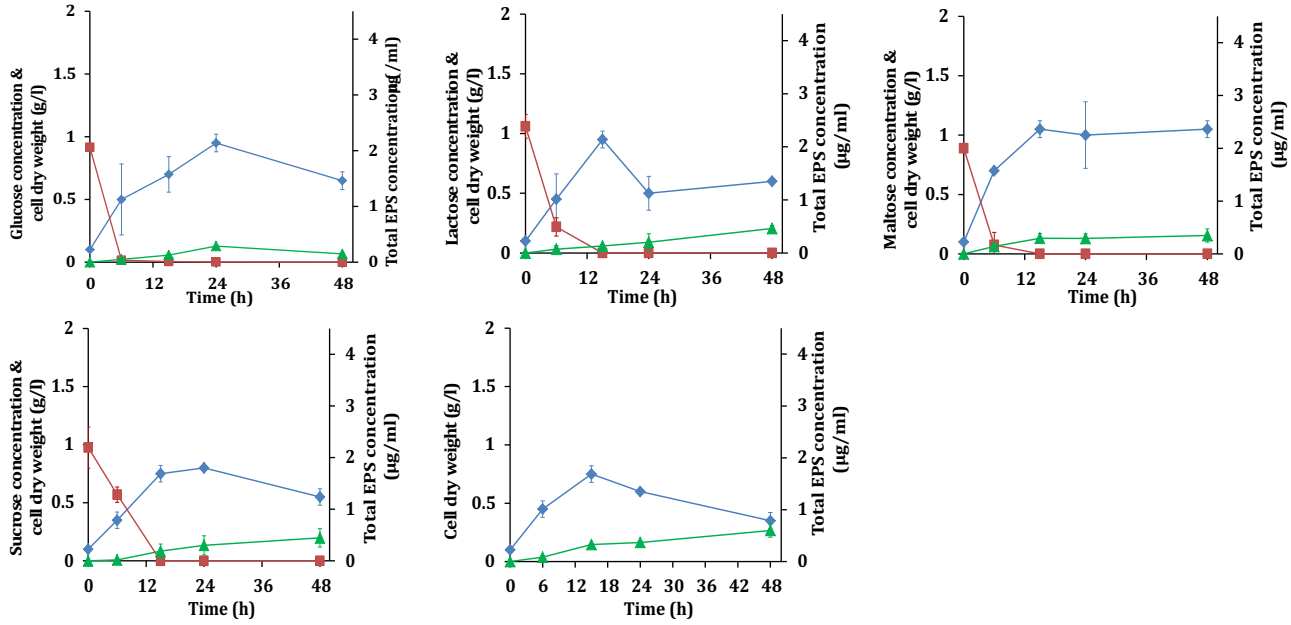

Fig. 3. Growth profile and EPS production by *R.marinus* MAT 493 cultivated in marine broth containing (1 g/l) glucose, sucrose, lactose and maltose, separately and marine broth without additional sugars as a control. Symbols indicate (♦) for cell dry weight, (▲) for total produced monosaccharide concentration, and (■) for sugar concentration in the media. Results are the mean of duplicate measurements.

DSM 4252^T and MAT 493 followed by marine broth containing 10 g/l maltose (Tables 1 and 2) Using these two carbon sources DSM 4252^T was shown to produce a higher amount of EPS/CDW at lower concentration of the carbon source, while strain MAT 493 appeared to be more dependent on the amount of carbon source supplied for its EPS production.

3.2. Purification and fractionation of the exopolysaccharide

The crude exopolysaccharides obtained from the different supplemented cultures of *R.marinus* DSM 4252^T and MAT 493 were fractionated using size exclusion chromatography as described in section 2.6. Results showed one major peak for each sample, which corresponded to a high molecular weight fraction. The retention time of the major peak in the crude EPSs from *R.marinus* DSM 4252^T in the media containing glucose, lactose, maltose, sucrose, and the medium without additional sugars was 138.35, 138.56, 134.14, 138, and 135.41 min, which corresponded to molecular weights of 73.8, 73.5, 80.8, 74.4, and 78.6 kDa, respectively. Also, the retention times of the major peak in the crude EPSs from *R.marinus* MAT 493 in the media containing glucose, lactose, maltose, sucrose, and the medium without additional sugars was 130.07, 133.33, 131.49,

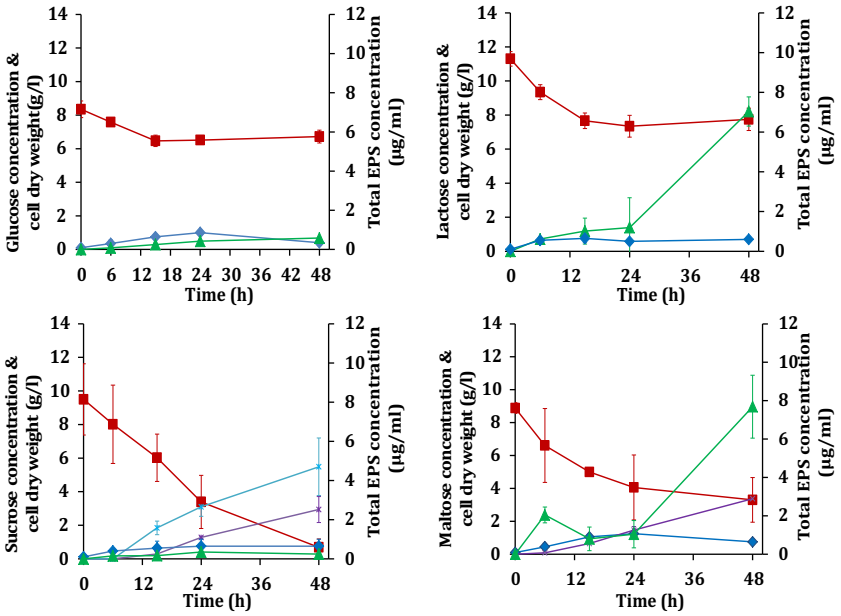

Fig. 4. Growth profile and EPS production by *R.marinus* MAT 493 cultivated in marine broth containing (10 g/l) glucose, sucrose, lactose and maltose, separately and marine broth without additional sugars as a control. Symbols indicate (♦) for cell dry weight, (▲) for total produced monosaccharide concentration, (■) for sugar concentration in the media, (×) for glucose concentration, and (∗) for fructose concentration. Results are the mean of duplicate measurements.

130.01, and 128.81 min corresponding to a molecular weight of 88.1, 82.2, 85.5, 88.2, and 90.5 kDa, respectively (Supplementary data). Generally, the EPSs produced by marine bacteria are often linear with an average molecular weight ranging from 1×10^5 to 3×10^5 Da (Poli, Anzelmo, & Nicolaus, 2010) which is compatible with the molecular weight of our produced EPSs.

3.3. Characterization of EPS monosaccharide content

After hydrolysis, the monosaccharide composition of the purified EPSs was analysed by HPAEC-PAD and the analysis showed that all the EPSs were heteropolysaccharides (Table 3). The main components of the pure EPSs from *R.marinus* DSM 4252^T were xylose, arabinose, and glucose. Also, there was a mixture of galactose with glucosamine, and a mixture of mannose with an amino sugar which might be N-acetyl-glucosamine or N-acetyl-galactosamine (data not shown). Quantification of those components was however not possible due to overlapping peaks.

Analysis of the pure EPSs from MAT 493 allowed quantification of glucose, arabinose, xylose, and mannose (Table 3). Also in this strain, there was a small quantity of galactose and galactosamine.

In all EPSs chromatograms there were three unidentified peaks which needs to be further investigated since the identification of them with the known monosaccharide standards was not successful (Supplementary data).

Arabinose and xylose are not common sugars in bacterial EPSs (Ahmed et al., 2013; Nichols et al., 2005). Thus, it can be claimed that the EPSs produced by the *R. marinus* strains are unique bacterial EPSs. Interestingly, the ratio of monosaccharides also differs between the two strains, indicating that EPS from the respective strains may be useful for different purposes.

3.4. Functional group analysis

In order to investigate the functional groups of the purified EPSs of *R.marinus* DSM 4252^T and *R.marinus* MAT 493 FT-IR spectroscopy was used (Fig. 5), and band assignments were made according to literature data.

The IR spectra of the purified EPSs of *R.marinus* DSM 4252^T from all media showed the same functional groups (Fig. 5A) and exhibited a broad peak at around 3335 cm^{-1} (range 3600–3200 cm^{-1}) for O—H stretching vibration of the polysaccharide (Kavita, Singh, Mishra, & Jha, 2014) and two weak C—H stretching bands at 2924 and 2855 cm^{-1}. The peak at 2359 cm^{-1} was attributed to NH stretching absorption band and the peak at 1652 cm^{-1} corresponded to a C=O stretching vibration of the N-acetyl group or protonated carboxylic acid (Ahluwalia & Goyal, 2005; Lillo, Cabello, Cespedes, Caro, & Perez, 2014). Also, at 1540 cm^{-1} a peak was observed which was assigned to the N—H deformation vibration of an amine group (Lillo et al., 2014). The peak at 1521 was assigned to the secondary amid group (Ahluwalia & Goyal, 2005). Another peak at 1418 cm^{-1} could be attributed to the symmetric stretching of the COO^- group (Zhao, Yang, Yang, Jiang, & Zhang, 2007). The peak at 1217 cm^{-1} corresponded to an O-S-O group that is an evidence of sulfate esters (Na et al., 2010) and the peak at 1103 cm^{-1} might be assigned to O-acetyl ester linked uronic acid (Kavita et al., 2014). The strong absorption at 1039 cm^{-1} in the range of 1200–1000 cm^{-1} which is anomeric region, was attributed to C—O—C and C—O groups in polysaccharides and suggested that the monosaccharide in the EPS has pyranose ring (Vijayabaskar, Babinastarlin, Shankar, Sivakumar, & Anandapandian, 2011). The weak absorption at 910 and 890 cm^{-1} was assigned to the coexistance of α and ß glycosidic bond (Lim et al., 2005). The peak at 818 cm^{-1} can determined the exact position of 6- sulfate of D-galactose unit (C6-O-S) (Maciel et al., 2008; Prado-Fernández, Rodríguez-Vázquez, Tojo, & Andrade, 2003). The weak absorption at 845 cm^{-1} demonstrated the presence of 4-sulfate of D-galactose (C4-O-S) (Prado-Fernández et al., 2003). The peak at 770 cm^{-1} might be attributed to the (S-F) stretching absorption band (Pretsch, Fernández, Alvarez, 2000) and the absorption peak at 600 cm^{-1} was attributed to stretching of alkyl- halides (Kavita et al., 2014).

According to FT-IR band assignments of the purified EPSs of *R.marinus* DSM 4252^T from marine broth with and without added sugars, the EPS contains sulfated polysaccharides of complex structure containing uronic acids. Sulfated exopolysaccharide derivatives are known to have advantageous properties, in partic-

Table 3
The monosaccharide composition of EPS produced by the *R.marinus* strains DSM 4252^T and MAT 493.

Type of sugar	*R.marinus* DSM 4252^T			*R. marinus* MAT 493			
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose	Mannose
Marine broth	1	3.4	5.76	4.99	1	1.47	1.24
Marine broth + glucose	1	2.45	4.93	1	1.59	1.51	1.27
Marine broth + sucrose	1	1.57	3.35	1	4.71	3.6	3.34
Marine broth + maltose	1	2.13	4.11	1	3.75	3.02	1.87
Marine broth + lactose	1	1.57	3.72	–[a]	–[a]	–[a]	–[a]

[a] Quantification of monosaccharide components from MAT 493 grown in marine broth + lactose, not possible due to overlapping peaks.

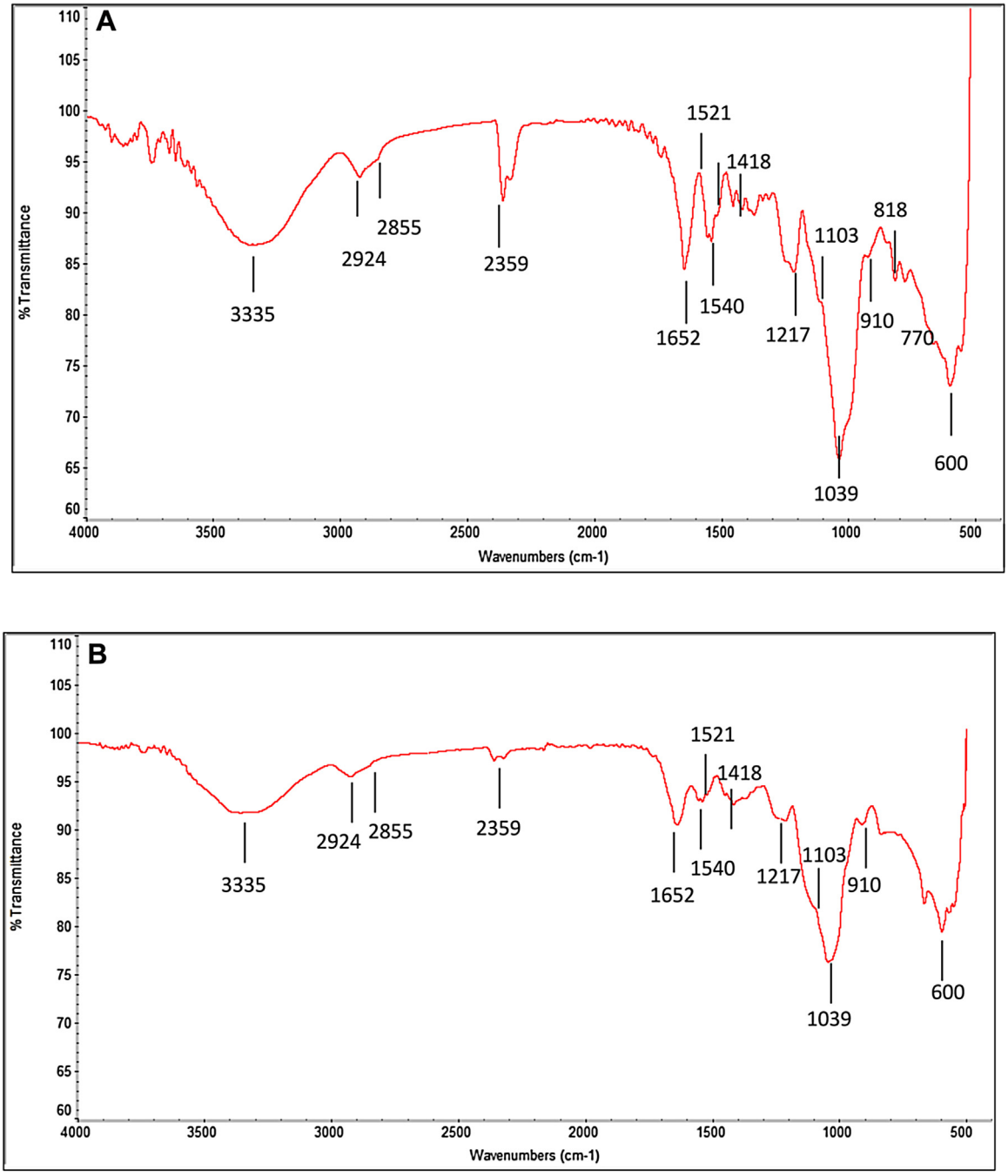

Fig. 5. FT-IR spectrum of the purified exopolysaccharide from (A) *R.marinus* DSM 4252^T and (B) *R.marinus* MAT 493.

ular as therapeutic substances. Best known are heparin (extracted from porcine intestinal mucosa as anticoagulant and antithrombotic agent in the prevention and treatment of venous thrombosis) and fucoidan from Brown seaweed, which has been reported having a range of bioactive properties. Sulfated EPS from bacterial origin are less well known, but mauran, a highly polyanionic sulfated

EPS produced by the halophilic bacterium *Halomonas maura*, has been reported to have antioxidant, antihemolytic and antithrombogenic activities (Raveendran et al., 2013). Novel polysaccharides from bacterial origin offer an alternative to the well-known animal varieties and will also expand the potential range of activities and potency of EPS derived health promoting agents.

The FT-IR spectra of the purified EPSs of *R.marinus* 493 (Fig. 5B) was in principle similar to FT-IR spectra of *R.marinus* DSM 4252^T. However, the peak at 910 cm^{-1} had strong absorption which corresponded to a ß-glycosidic bond and the peak at 818 cm^{-1} was absent.

4. Conclusion

In conclusion, both *R.marinus* DSM 4252^T and *R.marinus* MAT 493 produced exopolysaccharides. Different nutritional conditions influenced the production of the EPSs. The highest EPS production efficiency was however for both strains found in marine broth supplemented by lactose followed by a maltose supplemented marine broth. Monosaccharide analysis showed that the produced EPSs are heteropolysaccharides mainly consisting of xylose and arabinose. The FT-IR spectrum of the EPSs showed the presence of sulfate and carboxyl groups which demonstrated that they contain uronic acids. It also revealed the presence of amino sugars together with acetyl group. The unusual functional groups and monosaccharide composition makes the EPS of *R. marinus* interesting for further studies, motivating more detailed analysis of its chemical structure and such studies are in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.08.062.

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