Natural and engineered hydroxyectoine production based on *Pseudomonas stutzeri* *ectABCD/ask* gene cluster †

Running title:

*P. stutzeri* hydroxyectoine gene cluster

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† Supplemental material for this article may be found at http://aem.asm.org/.
Abstract

In this study we report the presence of a functional hydroxyectoine biosynthesis gene cluster \textit{ectABCD/ask} in \textit{Pseudomonas stutzeri} type strain DSM5190\textsuperscript{T} and evaluate the suitability of \textit{P. stutzeri} DSM5190\textsuperscript{T} for hydroxyectoine production. Furthermore we present heterologous \textit{de novo} production of the compatible solute hydroxyectoine in \textit{Escherichia coli}. In this host the \textit{P. stutzeri} gene cluster remained under the control of its salt-induced native promoters. We also noted the absence of trehalose, when hydroxyectoine genes were expressed, as well as a remarkable inhibitory effect of externally applied betaine on hydroxyectoine synthesis. The specific heterologous production rate in \textit{E. coli}, under the conditions employed, exceeded that of the natural producer \textit{Pseudomonas stutzeri} and, for the first time, enabled effective hydroxyectoine production at low salinity (2\%) with the added advantage of simple product processing due to the absence of other co-solutes.

Introduction

As reviewed extensively elsewhere halotolerant and halophilic microorganisms can adapt to extreme salinity by accumulation or biosynthesis of a range of osmolytes (8, 14, 21, 32, 36). Extreme halophiles like the archaeon \textit{Halobacterium salinarum} usually recruit inorganic ions while others, in particular highly adaptive organisms, make use of a more flexible strategy and employ \textit{compatible solutes} (also called \textit{extremolytes}) to obtain osmotic equilibrium. Unlike inorganic ions and even at molar concentrations these organic low molecular weight osmolytes do not interfere negatively with normal cell metabolism – even at molar concentrations. In addition to their function as osmoprotectants for whole living cells compatible solutes also protect biological macromolecules against physical stress \textit{in vitro}. Therefore, they have found their way into a range of biochemical applications (25, 33) and
even skin care products (5).
The compatible solute ectoine (12) is often detected as the main solute in chemoheterotrophic eubacterial halophiles of broad salt tolerance. Its biosynthesis (supplement, figure S01) proceeds as an off-branch from the aspartate amino acid family. Enzymes for the three final biosynthetic steps are usually encoded in an ectABC-type cluster (29) although there are notable exceptions which have an incomplete cluster or even only a solitary ectC (22). The ectoine derivative hydroxyectoine is usually formed by a subsequent biosynthetic step (6), provided the required enzyme (ectoine hydroxylase, often EctD) is encoded in the genome. According to current knowledge hydroxylation of ectoine seems to be linked to conditions where survival strategies become important, as for example imminent desiccation at extreme salinity and elevated temperature in the Halomonadaceae (15). Therefore, the industrial production strain for ectoine, Halomonas elongata, can be manipulated to increase its hydroxyectoine content (to a maximum of approx. 50%), but as yet not to such an extent that hydroxyectoine becomes the sole compatible solute. Fermentation under extreme conditions and subsequent chromatographic separation procedures increase production costs. As hydroxyectoine is in high demand due to its superior protective effect on whole cells (30) and biological macromolecules (4, 17, 27), the search is on for natural and heterologous production strains.

Since the work of Smith & Smith (41) we know that the primary compatible solute of Pseudomonas species and Rhizobia is N-γ-acetylglutaminyl glutamine 1-amide (NAGGN). So far this compound has been found only in combination with other co-solutes, e.g. trehalose, glucosyl glycerol or mannitol (9, 18, 34). Although Pseudomonas stutzeri is a well investigated organism (23) hydroxyectoine production was reported only recently as a surprise discovery in the type strain DSM5190\textsuperscript{T} by our group (20). This is the first time ever that NAGGN was encountered in combination with hydroxyectoine as a co-solute in a
Pseudomonas species. Subsequently an ectABC-type gene cluster was detected in the genome sequence of P. stutzeri A1505 (44). Downstream of ectABC two open reading frames were located and annotated as putative proline hydroxylase (PST_0178) and aspartokinase (PST_0177). Both the similarity of PST_0178 to other ectoine hydroxylases and the proximity of PST_0177 to the ectABC gene cluster indicated a complete hydroxyectoine biosynthesis gene cluster including an aspartokinase. Until now such an arrangement was only known from Marinomonas sp. MWYL1 [CP000749] and a few α-proteobacteria, e.g. Hyphomonas neptunium [CP000158]. As the availability of aspartic acid semialdehyde was reported to be critical for heterologous ectoine production (2), a complete biosynthetic cluster including aspartokinase appears to be an ideal prerequisite for genetic engineering. However, experimental evidence of the function of this constellation in the γ-proteobacterium Pseudomonas stutzeri and its potential use for heterologous expression was still lacking. Thus, the aim of this study was not only to demonstrate that the putative ectABCD/ask gene cluster has a metabolic function, but also to evaluate hydroxyectoine production for industrial use in both the natural producer P. stutzeri and the heterologous host E. coli.

Materials and Methods

Strains and culture conditions. Pseudomonas stutzeri DSM 5190T (37) and E. coli DH5α (16) were obtained from the DSMZ (Braunschweig, Germany). We used mainly minimal medium MM63 (24) for growth and production experiments. Luria Bertani (LB) medium (1) was employed for cloning purposes and to study uptake of solutes from complex medium. Antibiotics were added and NaCl content was modified where necessary. P. stutzeri and E. coli were grown at 37 °C, liquid cultures with shaking at 180 rpm. Growth data were collected from cultures in shaking flasks with appendages for OD measurements.

Molecular biology methods. We amplified the complete P. stutzeri hydroxyectoine gene
cluster including the aspartokinase by polymerase chain reaction and cloned it into pUC18 (31) making use of primers clus_for (5'-ATC AGA TCG CGG AGC TCG GG-3') and clus_rev (5'-CCG GAC TCG ATC ACA TAT GTC TTA-3') located upstream and downstream the ectABCD/ask cluster (numbered PST_00181 to PST_00177 respectively) and modified for NdeI and SacI restriction sites (underlined). We included a sequence 400 base pairs upstream of ectA to ensure that expression of the whole gene cluster remained under control of the native promoters. In addition, to exclude expression under control of the pUC18 lac-promoter, the gene cluster was oriented in the opposite direction with respect to this promoter. The construct was named pSB01. In a second construct, named pSB02, the aspartokinase was excised from pSB01 with ApaI and BstXI. Both plasmids were transformed into E. coli DH5α. We named the resulting organisms E. coli DH5α pSB01 and E. coli DH5α pSB02 respectively (see supplement, figure S02 for plasmid maps).

**Analysis of solute content.** If not noted otherwise, cell material was harvested from an exponentially growing shaking culture and freeze dried. Extraction followed a modified protocol by Bligh & Dyer (3) as described previously (13). Solute content was analyzed by isocratic HPLC on a NUCLEOSIL® 100-3 NH2 RP column (Macherey-Nagel, Düren, Germany) and 80/20% (v/v) acetonitrile/water at a flow rate of 1mL min⁻¹ as mobile phase. Additionally, ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 DPX spectrometer. Spectra were calibrated to the internal standard trimethylsilylpropionic acid (TMSP).

**Sequencing.** Sequencing reactions were carried out by Sequiserve (Vaterstetten, Germany).

**Results**

**Sequence of P. stutzeri 5190° ectABCD/ask.** Complete sequence data of P. stutzeri DSM
5190\textsuperscript{T} ectABCD/ask are available via the EMBL gene bank [FN868642]. Comparison with the A1501 genome [CP000304], shows high homology as expected: ectA, ectB, ectC, ectD and ask displayed 5, 27, 1, 6 and 14 differences in the DNA sequence, respectively. Most differences in the nucleotide sequence are silent at the amino acid level. There is only one exchange of glycine against serine and one of glutamine against lysine in EctB, as well as one of glutamate against aspartate in EctC.

**Solute spectrum of* P. stutzeri* DSM5190\textsuperscript{T}**. In response to increasing salinity the organism synthesized hydroxyectoine, NAGGN and trehalose *de novo* as main osmolytes. At all salinities tested, hydroxyectoine was the main compatible solute (> 50 %). In addition, very small amounts of ectoine were detectable at 2 % NaCl and higher (figure 1). When grown on complex medium (LB) the organism also accumulated large amounts of betaine (comparable to those of hydroxyectoine) and histidine (4% w/w of total solutes), either directly from the medium or from suitable precursors (See supplementary figure S03 for a $^{13}$C-NMR spectrum of extract from a culture grown in LB). In the presence of sufficient amounts of betaine, biosynthesis of other solutes was reduced by approximately 10 % (not shown).

In mineral-salt medium MM63, the relative proportion of solutes remained independent of the NaCl concentration (figure 1) and the actual amounts were largely growth-phase independent (supplementary figure S04). Within experimental error, total solute content increased in near-linear fashion from 1 % to 5 % NaCl (760 µmol (g DBM x h)$^{-1}$ at 5 % NaCl). Above 7.5 % NaCl the organism did not grow well and measurements did not yield reproducible results. In medium MM63, *P. stutzeri* DSM5190\textsuperscript{T} displayed a maximum growth rate of 0.2 (h$^{-1}$) at 2 % NaCl.

**Heterologous production of hydroxyectoine in* E. coli* DH5\alpha**. Expression of the complete ectABCD/ask gene cluster under the control of the native *P. stutzeri* promoters (pSB01)
successfully led to salt-induced production of hydroxyectoine (figure 2). Contrary to the situation in the donor, however, a remarkable growth-phase dependence was observed: hydroxyectoine production was clearly delayed and increased at the expense of ectoine when entering stationary phase. In late stationary phase, the final contribution of ectoine to the solute pool was below 5% of total (at 3% NaCl) or undetectable. The total hydroxyectoine content displayed a more than 20-fold increase from 25 µmol to over 500 µmol/g dry biomass when salinity was raised from 1% to 2% NaCl. Beyond that salinity no further increase was observed (Figure 2). Only negligible traces of trehalose were detected (not shown). Against expectations a comparison of pSB01 and pSB02 (gene cluster without aspartokinase) revealed that hydroxyectoine production in \textit{E. coli} was not dependent on co-expression of aspartokinase (not shown). Neither plasmid conferred enhanced osmotolerance to \textit{E. coli} DH5α.

**Growth in supplemented medium.** \textit{E. coli} cells harbouring pSB01 (Figure 3) or pSB02 (not shown) accumulated betaine in very large amounts when supplemented with this solute. In this situation, total biosynthesis of ectoines was reduced to approximately one tenth compared to cells grown in minimal medium.

**Specific hydroxyectoine production rate.**

As the product hydroxyectoine is directly related to the biomass grown at a certain salinity, the specific production rate (µmol/g DBM x h) is a simple function of hydroxyectoine content and growth rate. As shown in Table 1, the natural producer \textit{P. stutzeri} DSM5190$^\top$ and donor of the \textit{ectABCD/ask} gene cluster has its highest specific hydroxyectoine production rate at 5% NaCl (76.8 µmol/g DBM x h). The heterologous production system in \textit{E. coli} DH5α pSB01 reaches a 2.2-times higher specific production rate (175 µmol/g DBM x h) at a much lower salinity of only 2% NaCl. The higher productivity of the genetically-engineered strain
is caused by two factors: a) the apparent repression of other endogenous compatible solutes such as trehalose enabling a higher hydroxyectoine content and b) its faster growth rate in medium MM63 (0.35 h⁻¹ as compared to 0.2 h⁻¹ at 2 % NaCl).

**Discussion**

*P. stutzeri* **5190**<sup>T</sup> compatible solutes. *P. stutzeri* **5190**<sup>T</sup> is the first *Pseudomonas* sp. where hydroxyectoine production was reported (20) and the complete *ectABCD/ask* gene cluster identified. Compared to other halotolerant microorganisms, the salinity-dependent total solute content is in the expected range, but production of hydroxyectoine along with trehalose and NAGGN as co-solutes is unusual. Trehalose is commonly employed as an osmolyte only at lower salinities (8, 14, 36), a fact that can be contributed to its relatively high energy cost, approximately double that of ectoine (32). In addition, hydroxylation of ectoine has so far always been linked to extreme conditions triggering survival strategies (15). In particular, the seemingly unnecessary hydroxylation step under standard growth conditions appears to lack economic reason. With respect to these considerations and in unison with the pronounced natural competence of several *P. stutzeri* subspecies (23), we were intrigued by the idea that genes for hydroxyectoine biosynthesis might have been acquired by lateral gene transfer. Unfortunately, we did not find significant variations in codon usage or in GC-content compared to the rest of the *P. stutzeri* genome to support this theory, at least not in the A1501 genome. Neither could we find unusual trehalose biosynthesis pathways which might help to explain the use of trehalose as a third solute together with NAGGN and hydroxyectoine. Therefore, the presence of the ectoine/hydroxyectoine biosynthetic pathway may be specific for the genomovar *P. stutzeri*, which is characterized by its true marine lifestyle. This group of organisms is very versatile, uses cheap substrates (as for example ethylene glycol) and is easy to cultivate, even under high cell-density conditions. *P. stutzeri* strains therefore qualify as
potential industrial production strains. As use of complex media leads to accumulation of external compatible solutes (in particular glycine betaine, which represses hydroxyectoine biosynthesis), production processes are confined to mineral-salt media devoid of complex components such as yeast extract. Unfortunately, the growth rate of \textit{P. stutzeri} DSM5190\textsuperscript{T} in medium MM63 at 2\% NaCl is considerably lower than that of \textit{E. coli} (Table 1). Although media improvements are certainly an option, the intrinsic disadvantage of \textit{P. stutzeri} for hydroxyectoine production remains, namely that hydroxyectoine is always produced in combination with other solutes. This not only reduces content and hydroxyectoine productivity but also imposes the need for subsequent down-stream separation procedures to obtain a pure product.

While uptake of betaine in complex medium was to be expected because it is energetically favourable to \textit{de novo} biosynthesis of solutes, accumulation of histidine came as a surprise. Its function remains unclear, especially as this compound has so far not been reported to play a role in osmoadaptation. Thus, it is very well possible that scavanging histidine in connection with osmotic adaptation is only a side-effect of other transport processes. Genes encoding for histidine uptake systems like \textit{hisJQMP} (7) or the histidine utilization pathway (\textit{hut}) (45) underly complex but not yet fully understood regulation. According to current knowledge, there is no osmolarity dependent regulation. In the \textit{P. stutzeri} A1501 genome [CP000304], no transport system is annotated and similarities to verified His- and Hut-proteins are insufficient to propose homologues.

**Heterologous expression in \textit{E. coli} DH5\textalpha.** In order to avoid the problem of by-product separation, we aimed at establishing a heterologous production process to obtain almost pure hydroxyectoine. So far, \textit{de novo} heterologous production has only been reported for ectoine (e.g. 2, 29, 35, 40) but not for hydroxyectoine. In all these studies a coherent \textit{ectABC} gene cluster was used. Bestvater \textit{et al.} identified the availability of the immediate precursor
aspartylphosphate, and thus the reaction catalyzed by aspartokinase, as a metabolic bottleneck in ectoine biosynthesis by way of expression of the ectABC gene cluster of *Marinococcus halophilus*. Only in the presence of a deregulated aspartokinase (plasmid pAKECT1) did they achieve a maximum specific production rate of 140 µmol (g DBM and h)^{-1} at 2 % NaCl (2). This heterologous productivity was as high as that of the industrial production strain *H. elongata* at 3 % NaCl (10). In view of these reports, the availability of a complete ectABCD/ask gene cluster for hydroxyectoine biosynthesis in *P. stutzeri* 5190^T seemed very encouraging, especially as the presence of the aspartokinase gene promised optimal results without the need for further pathway engineering.

Expression of the complete *P. stutzeri* ectABC/ask gene cluster in *E. coli* DH5α proved successful, albeit with delayed conversion of ectoine into hydroxyectoine. As demonstrated in fig. 2, a minimum of 2 % NaCl was required for efficient hydroxyectoine synthesis. Total hydroxyectoine content in stationary phase was the same at 2 % and 3 % NaCl, approx. 500 µmol (g DBM)^{-1} and more than twice as high as in the donor strain at 2 % NaCl. With a maximum productivity of 175 µmol (g DBM and h)^{-1} at 2 % NaCl, the heterologous expression record for ectoine by Bestvater *et al.* (2) has now been exceeded without the need for engineering aspartokinase.

Although the drastic increase from 1 % NaCl to 2 % NaCl indicates a somewhat deregulated function of the *P. stutzeri* ectABCD/ask gene cluster in *E. coli*, it is worthy of note that under given conditions *de novo* biosynthesis of trehalose is abolished to almost negligible levels and that the presence of external betaine shuts down hydroxyectoine biosynthesis (Figure 3). One can therefore conclude that at least part of the regulation associated with the native gene cluster still functions in the host. However, contrary to our expectations and contrary to results by Bestvater (2), production of hydroxyectoine was not changed significantly in construct pSB02, which lacks the ask gene. The three *E. coli* aspartokinases ThrA, MetL and LysC can provide the neccessary precursor for hydroxyectoine biosynthesis. Nevertheless, the
concentrations seem to be insufficient due to feedback regulation of the enzyme, at least with the vector construct used by Bestvater. Thus, the association of ask with the ectABCD gene cluster as well as possible implications of this proximity (28) and likely differences of the enzymes involved might be an interesting object for further research but is beyond the scope of this project.

**Comparison of hydroxyectoine production systems.** Hydroxyectoine is at present produced on an industrial scale with *H. elongata* using the "bacterial milking" technique developed by Sauer and Galinski (38) or an improvement of this technique based on the continuous fermentation of this organism (26), followed by chromatographic separation of the two ectoines. In order to increase the hydroxyectoine content, *H. elongata* has to be grown at increased salinity and temperature (unpublished data). Recent work on related organisms such as *Halomonas boliviensis* (42) and *Chromohalobacter salexigens* (43) seems to confirm the situation within the *Halomonadaceae* that hydroxyectoine is always produced in combination with ectoine (e.g. 55% of total ectoines in *H. boliviensis* at 18.5% NaCl) and that its relative proportion increases with temperature.

The other native strains which have been employed so far for hydroxyectoine production are from the Gram-positive genus *Marinococcus*. Early work with *Marinococcus* strain M52 has revealed a growth-phase dependent hydroxyectoine synthesis enabling a high proportion of hydroxyectoine in stationary phase cells. Contrary to *Halomonas* cells, *Marinococcus* sp. cannot be "milked" by simply diluting the medium (11). However, as shown by Schiraldi and co-workers (39), osmotic downshock in combination with heat will trigger permeabilization of cells. Unfortunately, *Marinococcus* sp. produce growth-inhibiting components like acetate and are therefore not suitable for batch- and fed-batch fermentation strategies. The maximum growth rate depicted in Table 2 is only achieved at the beginning of a batch culture and is not representative for the entire growth phase. This draw-back can in principle be resolved by using a dialysis reactor system (19) or a microfiltration technique (39) at the expense of
markedly reduced growth rates. These technically demanding fermentation strategies have so far not been exploited for industrial application.

In this study we have presented two alternative production systems: wild-type *P. stutzeri* DSM5190<sup>T</sup> and *E. coli* expressing the *ectABCD/ask* gene cluster from *P. stutzeri* under the control of the donors promoter region. Both have the advantage that the precursor ectoine is almost completely transformed into hydroxyectoine at low salinity and normal growth temperature. Whereas the donor produces hydroxyectoine in combination with trehalose and NAGGN, the heterologous production system has hydroxyectoine as its sole compatible solute. A comparison of the specific production rates of the donor and the host (Table 1) demonstrates the superiority of the heterologous production system, in particular at low salinity (2 % NaCl). With a maximum production rate of 175 µmol (gDBM and h)<sup>-1</sup> and the additional advantage of relatively low salinity (2 % NaCl), heterologous hydroxyectoine production in *E. coli* is superior to any other strategy published so far (Table 2).

**Conclusions**

In conclusion, *P. stutzeri* DSM5190<sup>T</sup> lends itself as a new potential candidate for the production of the compatible osmolyte hydroxyectoine. Since the strain shows a high physiological variability (23), a range of approaches to further improve cultivation for industrial scale applications seems feasible. With respect to growth rate (0.16h<sup>-1</sup>) and yield (480 µmol hydroxyectoine per g dry biomass), 5 % NaCl appear to be optimum process conditions. As *P. stutzeri* 5190<sup>T</sup> produces NAGGN and trehalose as side-products, the use of deletion strains for trehalose and NAGGN in combination with optimized mineral-salt media may provide a promising perspective.

The heterologous *E. coli* production system based on the *ectABCD/ask* gene cluster from *P. 
stutzeri DSM5190\textsuperscript{T} suffers the stigma of genetically-modified organisms (GMO) and the costs of maintenance of the plasmid, for example by the addition of antibiotics. On the other hand, the heterologous system provides a number of advantages, mainly higher specific production rate than the donor and any other available system so far (175 \(\mu\text{mol (gDBM h)}^{-1}\)), high hydroxyectoine content at low salinity (500 \(\mu\text{mol (gDBM)}^{-1}\) at 2 \% NaCl) and almost complete absence of other contaminating compatible solutes (> 95 \% purity).

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Main solutes synthesized de novo in MM63 by *P. stutzeri DSM5190<sup>T</sup>* are trehalose (white), NAGGN (light grey) and hydroxyectoine (dark grey). Ectoine (black) is present only in minor amounts. Relative to dry biomass the amount of all solutes increases with salinity as does – consequently – the total amount (given in µmol / g dry biomass). Growth rates are indicated by circles and given in h<sup>-1</sup>. The organism’s growth optimum in minimal medium is around 2 % salinity but growth rates do not vary much between 1% and 5 %. Due to poor growth of the strain, data could not be reproduced sufficiently at salinities above 7.5 %.

**Figure 1 - *P. stutzeri DSM5190<sup>T</sup>* salinity dependent growth rate and solute biosynthesis.**
When expressing the *ectABCD/ask* gene cluster in *E. coli* DH5α the strain produces ectoine and hydroxyectoine (as indicated on the x-axis) depending on medium salinity (given in % NaCl w/w) and growth phase (bars in different shades of grey). Ectoine levels decrease while hydroxyectoine levels increase over time.
Figure 3 – Heterologous solute production is reduced by betaine uptake.

\( E. \ coli \) DH5\( \alpha \) pSB01 production of hydroxyectoine and ectoine (as indicated on the x-axis) in minimal medium at 3% salinity. When supplemented with 2 mM betaine, betaine is taken up and production of ectoines is reduced to approximately one tenth of the original value.
Table 1 - Specific hydroxyectoine production rates of donor and acceptor strain.

<table>
<thead>
<tr>
<th>Strain vector</th>
<th>Salinity (%)</th>
<th>Growth rate (h⁻¹)</th>
<th>Yield (µmol/gDBM)</th>
<th>Specific production rate (µmol/gDBM h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. stutzeri DSM5190ᵀ</td>
<td>1.0</td>
<td>0.15</td>
<td>140 HOE, 55 T, 45 N</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.20</td>
<td>220 HOE, 90 T, 90 N</td>
<td>44.0</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>0.18</td>
<td>250 HOE, 110 T, 110 N</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.16</td>
<td>480 HOE, 120 T, 160 N</td>
<td>76.8</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>0.12</td>
<td>580 HOE, 150 T, 215 N</td>
<td>69.6</td>
</tr>
<tr>
<td>E. coli DH5a pSB01</td>
<td>1.0</td>
<td>0.32</td>
<td>25 HOE</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.35</td>
<td>500 HOE</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>0.2</td>
<td>500 HOE</td>
<td>100</td>
</tr>
</tbody>
</table>

Solute content is shown for hydroxyectoine (HOE), Trehalose (T) and NAGGN (N). Ectoine is neglected here due to low amounts (< 5 % of total solutes). See supplemental figure S05 for a comparative graphic representation of the data for 1% to 3% NaCl.
Table 2 - Comparison of hydroxyectoine production systems

<table>
<thead>
<tr>
<th>Strain vector</th>
<th>Salinity (% NaCl)</th>
<th>Growth rate (h⁻¹)</th>
<th>Content (µmol/gDBM)</th>
<th>Specific production rate (µmol/gDBM h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. stutzeri</em> DSM5190&lt;sup&gt;T&lt;/sup&gt;</td>
<td>5.0</td>
<td>0.16</td>
<td>480</td>
<td>76.8</td>
<td>this study</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α pSB01</td>
<td>2.0</td>
<td>0.35</td>
<td>500</td>
<td>175</td>
<td>this study&lt;sup&gt;1)&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Marinococcus</em> strain M52</td>
<td>10.0 max. 0.25</td>
<td>860</td>
<td>215</td>
<td>(11) &lt;sup&gt;2)&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Marinococcus</em> strain M52</td>
<td>10.0 0.20 0.03</td>
<td>670 603</td>
<td>134 18</td>
<td>(39) &lt;sup&gt;3)&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>H. boliviensis</em></td>
<td>18.5 multi-step process</td>
<td>950</td>
<td>169</td>
<td>(42) &lt;sup&gt;4)&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Maximum specific hydroxyectoine production rates were calculated on the basis of growth rates and biomass content.

<sup>1)</sup> Trehalose production is suppressed almost completely.

<sup>2)</sup> Organism does not grow exponentially, maximum growth rate only observed at the beginning of batch-phase; hydroxyectoine is retained within biomass upon osmotic downshock.

<sup>3)</sup> Cells were first grown in batch culture (µ = 0.2) and subsequently subjected to continuous dialysis in order to remove growth-inhibiting compounds (µ = 0.03).

<sup>4)</sup> Cells were first grown at optimal salinity for biomass production (step 1) and then transferred to a medium of higher salinity for solute production (step 2). Here the highest specific production rate during the hydroxyectoine production phase is compared, not accounting for the time needed for step 1 (24 h) and an adaptation phase (3 h) at the beginning of the production phase.