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Original article

Bacterium *Hafnia alvei* secretes L-methioninase enzyme: Optimization of the enzyme secretion conditions

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ABSTRACT

I isolated bacteria from blue cheese in order to find bacterial strains secreting L-methioninase enzyme, and optimized the conditions for the most efficient enzyme secretion. The efficient isolate, identified according to the 16S rRNA gene sequence analysis, was *Hafnia alvei* belonging to Enterobacteriaceae. I confirmed that the *H. alvei* strain harbored the methionase gene, *mdeA* (1194 bp). The environmental (pH, temperature) and nutritional (carbon and nitrogen sources and Mg concentration) factors influencing the L-methioninase production of *H. alvei* were optimized. The highest yield of L-methioninase enzyme was reached after 48 h of incubation when the acidity of the growing medium was adjusted to pH 7.5 and the temperature was 35 °C. The following concentrations of the supplements increased the L-methioninase yield in the medium: galactose (2.0 g L⁻¹), MgSO₄ (0.25 g L⁻¹), L-methionine as an inducer (2.0 g L⁻¹), and L-asparagine as an additional N source (1.5 g L⁻¹). I introduce a bacterial strain of *H. alvei* that is previously unreported to secrete L-methioninase enzyme and show that a carbon source is a mandatory supplement whereas L-methionine is not a mandatory supplement for L-methioninase enzyme production of *H. alvei*.

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1. Introduction

Bacteria secrete enzymes that are commonly used in many different medical applications. One of the interesting enzymes is methionine-gamma-lyase (MGL), often called L-methioninase. Recently, MGL has been extensively studied for its several potential medical applications, for instance, in cancer therapy and as an antimicrobial drug (El-Sayed, 2010; Maggi and Scotti, 2019; Suganya et al., 2017)

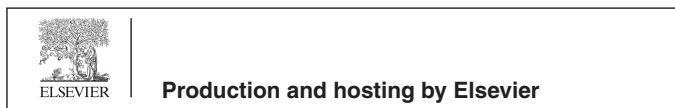
Several organisms are known to secrete MGL. Among bacteria, *Pseudomonas putida* is one of the most commonly studied species (Selim et al., 2015). Other species reported as intracellular MGL producers are, for instance, *Brevibacterium linens* (Dias and Weimer, 1998; Suwabe et al., 2011), *Pseudomonas ovalis* (Tanaka et al., 1976), *Aeromonas* sp. (Tanaka et al., 1985), *Citrobacter freundii* (Manukhov et al., 2005), *Lactococcus lactis* (Martínez-Cuesta et al., 2006), *Idiomarina* sp. (Song et al., 2015), *Streptomyces* sp. (Huang

et al., 2014) and recently, *Enterobacter Cloacae* (Prihanto et al., 2018). Investigating new microorganisms with high MGL production efficiency as well as the optimum conditions to improve the yield of enzyme are still needed to be able to minimize the production costs.

Nutrients in the growth medium are known to affect MGL production greatly. Supplements, such as carbon and nitrogen sources and trace elements have been observed to increase the MGL production of microbes (El-Sayed, 2009; Swathi, 2015). Metal salts, such as Mn, Mg, Cu and Co have been observed to increase the production, although not remarkably (El-Sayed, 2009). The most important supplement has been L-methionine, which promoted, for instance, the MGL production of a fungus *Aspergillus flavipes* (El-Sayed, 2009). Thus, L-methionine has been suggested to be an inducer and a mandatory supplement for MGL production (Khalaf and El-Sayed, 2009). However, other studies suggest that the microbes, namely a bacterium *Pseudomonas putida* (Tan et al., 1997) and a fungus *Geotrichum candidum* (Bonnarme et al., 2001) were able to produce MGL independently of L-methionine.

Taking these previous observations into consideration, I designed our optimization procedure to find the best growing conditions for the most efficient bacterium, which I aimed to find from blue cheese. I used several subsequent incubations to find the

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mandatory supplements and the optimal conditions to produce MGL enzyme the most efficiently.

2. Materials and methods

2.1. Samples and medium

Samples (25) were collected from blue cheese, placed in sterile bags, transported to the laboratory in an ice box and stored at 4 °C. The bacteria were cultivated in modified mineral malt medium M9 (pH 7.0 ± 0.2). M9 contained Na₂HPO₄·2H₂O (6.0 g L⁻¹), KH₂PO₄ (3.0 g L⁻¹), NaCl (0.5 g L⁻¹), L-methionine (1.0 g L⁻¹), MgSO₄·7H₂O (0.25 g L⁻¹), CaCl₂ (0.014 g L⁻¹), glucose (2.0 g L⁻¹) and agar 15 g L⁻¹.

2.2. Preliminary screening of the bacterial isolates

A total of 25 potential bacterial isolates was screened preliminary for their MGL secretion using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) as a dye on modified M9 medium as described by Sharma et al. (2014). The isolate with the highest potential to secrete MGL was selected for further experiments.

2.3. Molecular characterization of the bacterial strain

Genomic DNA was extracted from the bacteria grown in Luria-Bertani medium to an exponential phase. Bacterial culture was centrifuged at 12,000 rpm for 15 min and aliquots of 10 ml Supernatant were harvested and washed once with sterile distilled water. DNA was extracted using GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions.

Two primer sets were used. For the species identification, 16S rDNA universal oligonucleotide primers were used as described by Buonauro et al. (2001). To find the methioninase gene, *mdeA* gene in the bacteria, a forward (5'GCAACGAAGTATGGAAGTGC3') and a reverse (5'TAATAAATCGCCTTATTAAGG3') primer were designed and synthesized to recover an amplicon of 1204 bp including the full-length gene (1194 bp) along with 10 nucleotides preceding the 5' end of the gene. The designing of the primers was based on the sequence of the *mdeA* gene in the *Hafnia alvei* complete genome sequence (CP009706.1) with the protein accession no. AIU72023.1 (Tan et al., 2014), which is available in the National Center for Biotechnology Information (NCBI).

The DNA sequences of the 16S rRNA gene and the *mdeA* gene were amplified in separate PCRs in a thermal cycler (Bibby Scientific, UK) in a reaction mixture (25 µL) containing 2 µL DNA, 1 µL of each primer (10 pmoles), 12.5 µL master mix (2x) and 9.5 µL dH₂O. The following thermal cycle was used: initial denaturation for 5 min at 94 °C, 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C (16S rRNA gene) or at 60 °C (*mdeA* gene), extension for 2 min at 72 °C, and a final extension for 10 min at 72 °C.

Sanger sequencing was carried out at Beijing Genomic Institute (BGI), Hong Kong, China, for the 16S rRNA gene. The sequence was compared to the NCBI database using the BLAST software. The obtained sequences were aligned in Ugene (Okonechnikov et al., 2012) using the T-Coffee algorithm (<https://www.ebi.ac.uk/>, EMBL-EBI, Cambridgeshire, UK) and a phylogenetic tree was generated using iTOL, an interactive tree tool (<http://itol.embl.de/index.shtml>) (Letunic and Bork, 2011).

2.4. Preparation of inoculum

A loop full of a 24 h-culture of the selected bacterial isolate was transferred to the modified M9 medium (30 ml) in a test tube and incubated at 35 ± 2 °C at a constant temperature for 24 h.

2.5. Optimization of the MGL enzyme production

Enzyme production was optimized by varying one factor at a time in triplicate Erlenmeyer flasks (250 ml) with 100 ml of the sterile fermentation M9 medium. The medium was inoculated with 10 ml of the bacterial seed medium.

In the optimization procedure, different factors were tested subsequently, and the best alternative of the first test was chosen for the next step. The optimization procedure included the following steps. (1.) As a preliminary step, four temperatures (25, 30, 35, 40 ± 2 °C) were used for three incubation periods (24, 48, 78 h). (2.) Nine different pH values (pH 4–8) were then tested at the best temperature and time period observed. For the final experiments, the pH value of the medium was adjusted to 7.5 ± 0.2 and the flasks were incubated at 35 ± 2 °C. Some of the subsequent treatments were carried out for all three incubation periods (24, 48, 72 h) if stated below. The optimization continued with testing (3a.) different carbon sources (glucose, mannitol, sucrose, xylose, maltose, lactose, galactose, fructose, glycerol; no addition as the control) that were first added at a concentration of 2.0 g L⁻¹, and the (3b.) best carbon source was then tested at five concentrations (1.0–5.0 g L⁻¹). Then, (4.) six different nitrogen sources including three different amino acids (L-methionine, L-asparagine, L-glutamine) and three organic N sources (yeast extract, peptone or tryptone) (no addition as the control) were added at the concentration of 1.25 g L⁻¹. The final optimized incubation was a mixture of the best N source at five concentrations (0.75–1.75 g L⁻¹) and L-methionine (2.0 g L⁻¹) as the inducer for the three incubation periods. Finally, (5.) an additional incubation was carried out to understand if the amino acid L-methionine is able to delay the growth retardation of the bacteria; magnesium sulfate (MgSO₄·7H₂O) was added as a sole supplement at four concentrations (0.15–0.55 g L⁻¹), each concentration for the three incubation periods.

The MGL production of the bacteria was assessed using a spectrophotometric assay based on the amount of ammonia liberated using Nessler reagent as described by Imada et al. (1973) with some modifications described by El-Sayed, 2009. The enzymatic activity of the bacteria was described as units (U) of L-methioninase defined as the amount of enzyme that liberates one µmole of ammonia per min. Enzyme yield was expressed as U mL⁻¹ medium. The absorbance at 480 nm was measured using a spectrophotometer with enzyme and substrate blanks as controls.

2.6. Statistical analysis

The effects of different factors on MGL production were studied using one-way ANOVA followed by Duncan's New Multiple Range test.

Table 1

BLAST analysis of the partial 16S rDNA of the L-methioninase secreting *Hafnia alvei* isolate (acc. no. MK072731) versus the closest taxa in the gene bank.

Species	Accession	Identity	Coverage
<i>Hafnia alvei</i> strain JCM 1666	NR112985	100%	100%
<i>Hafnia alvei</i> , strain 196.12.1	MG859578	97.72%	100%
<i>Hafnia alvei</i> , strain F9	KT767908	97.71%	100%
<i>Hafnia alvei</i> , strain F28	KT767875	97.71%	100%
<i>Hafnia alvei</i> , strain F13	KT767861	97.71%	100%
<i>Hafnia alvei</i> , strain F10	KT767858	97.71%	100%
<i>Hafnia paralvei</i> , strain ATCC 29927	NR116898	99.00%	98.77%
<i>Rouxiella chamberiensis</i> , strain 130333	NR135871	98.00%	97.66%
<i>Hafnia psychrotolerans</i> , strain DJC1-1	NR134741	96.00%	97.05%

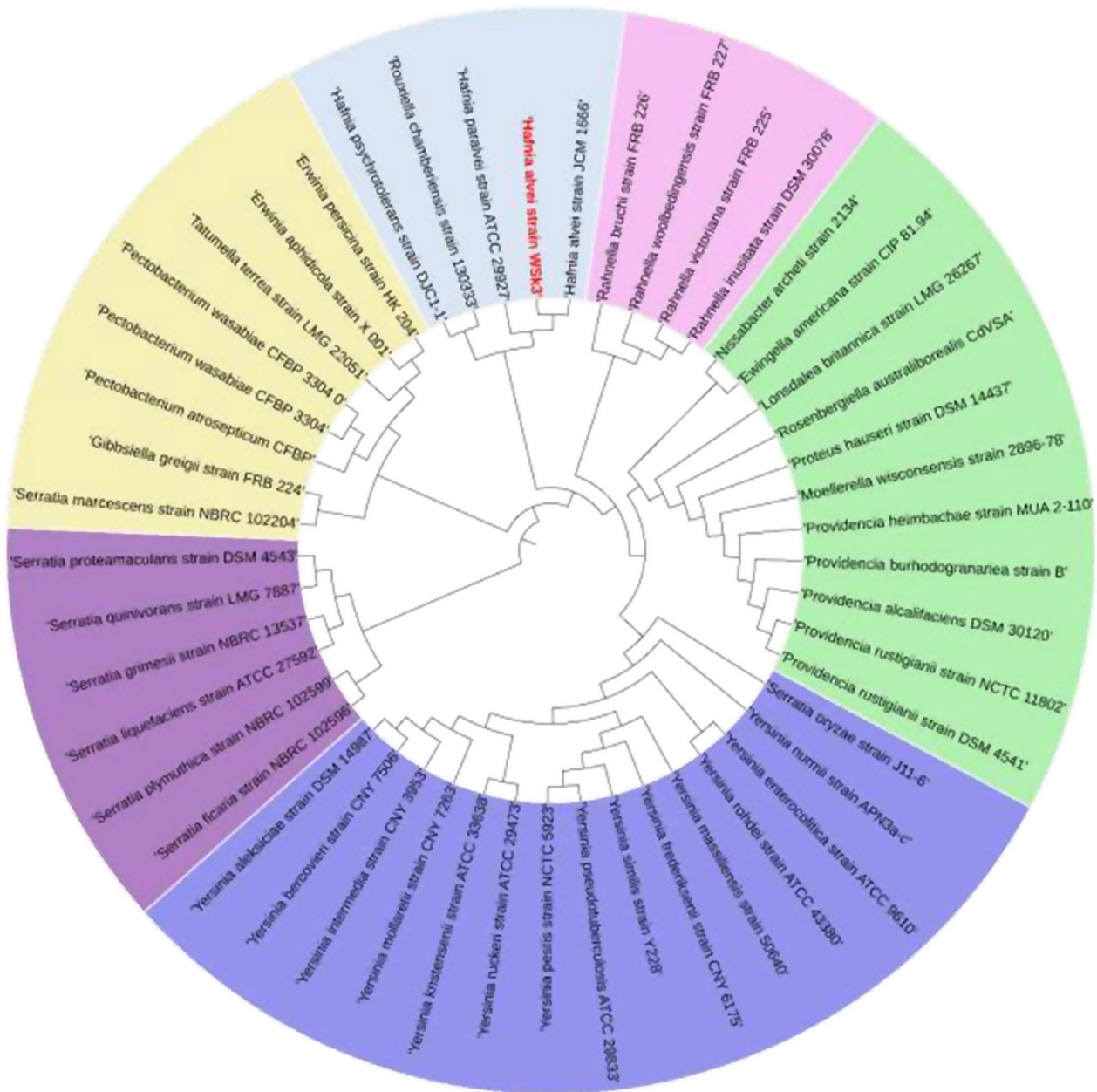


Fig. 1. Phylogenetic tree showing the position of the L-methionase secreting *H. alvei* strain WSK3 (with acc. no. MK072731) and the closest related strains based on partial 16S rRNA gene sequences.

3. Results

3.1. Identification of bacterial strains

Twenty-five bacterial isolates, assessed as different species after morphological inspection, were obtained from the samples. The partial 16 S rDNA sequencing revealed that the isolates included ten species of both *Pseudomonas* and *Streptomyces* and five species of *Hafnia*. After the preliminary screening, we chose the best isolate to secrete MGL. The isolate was identified to *Hafnia alvei* with 97.7–100% identity and 100% coverage (Table 1). The strain was named WSK3 and given the accession no. MK072731. The phylogenetic tree indicated the occurrence of six clusters (Fig. 1). *Hafnia alvei* strain WSK3 belonged to a cluster together with four strains of *H. alvei* and one of *Rouxiiella chamberiensis*. The closest two clusters included *Rahnella* sp. in one cluster and the species of *Serratia*,

Erwinia, *Tatumella*, *Pectobacterium* and *Gibbsiella* in the other cluster.

The *H. alvei* strain WSK3 harbored the L-methioninase (*mdeA*) gene (Fig. 2). PCR yielded an amplicon of 1204 bp including the gene with the expected full-length of 1194 bp and 10 nucleotides preceding the 5' end of the gene. Nucleotide number of the gene, disregarding the stop codon, indicates that the number of deduced amino acids is 397. The length of this polypeptide chain is identical to that of *Hafnia alvei* with accession no. AIU72023.1 (Tan et al., 2014). The *mdeA* gene has given the accession no. MN566443.

3.2. Optimization procedure

The preliminary incubation revealed that the highest MGL production by *H. alvei* was obtained at 35 °C after a 24 h-incubation. The MGL enzyme production dropped with the elevated

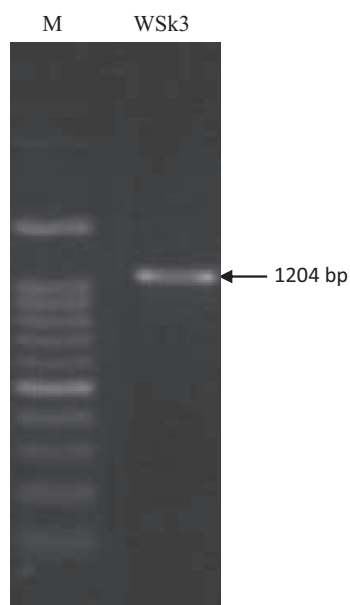


Fig. 2. Amplicon (1204 bp) generated from PCR including the full-length *l*-methioninase gene *mdeA* (1194 bp) of *H. alvei* strain WSk3 along with 10 nucleotides preceded the gene at the 5' end. M: 100-bp DNA ladder (DM-M-002-500, Auckland, New Zealand).

temperature and incubation time. The optimal pH for the MGL production was pH 7.5.

Galactose as the sole carbon source resulted in significantly the highest MGL production (49 U m L⁻¹) (Table 2). Galactose was followed by mannitol, xylose, lactose, and fructose (30 U m L⁻¹). The respective control value was 8 m L⁻¹. The concentration of 2.0 g L⁻¹ galactose was significantly the most efficient giving the activity of 50 U m L⁻¹ (Table 2).

From the six different nitrogen sources, *l*-asparagine was significantly the best substitute for *l*-methionine (Table 3). The enzyme activity was 71 U m L⁻¹. When different concentrations of *l*-asparagine were tested, enzyme activity was significantly the highest (97 U m L⁻¹) with the *l*-asparagine concentration of 1.5 g L⁻¹ after the incubation period of 48 h (Fig. 3). The prolonged growth of the bacteria decreased enzyme activity.

Magnesium sulfate at the 0.25 g L⁻¹ concentration resulted significantly the highest enzyme activity (60 U m L⁻¹) after 24 h (Fig. 4). The prolonged growth of the bacteria decreased enzyme activity.

The highest *l*-methioninase activity in the medium was reached in the following conditions: at 35 °C after 24 h incubation in pH 7.5, and galactose (2.0 g L⁻¹), *l*-methionine as an inducer (2.0 g L⁻¹), and *l*-asparagine as an additional N source (1.5 g L⁻¹) as the supplements.

4. Discussion

To the best of our knowledge, this is the first study describing the ability of the bacterium *Hafnia alvei* to secrete MGL enzyme.

The MGL production by *H. alvei* was at the same level than previously reported for other microorganisms, such as *Streptomyces variabilis*, *Achromobacter starkeyi*, *Serratia marcescens*, *Candida tropicalis* and *Yarrowia lipolytica* (Bondar et al., 2005; El Awady et al., 2017; Ruiz-Herrera and Starkey, 1970).

The optimum temperature for MGL production by *H. alvei* was 35 °C, similar to several fungi, such as *Aspergillus ustus* (Abu-Tahon and Isaac, 2016) and *A. flavipes* (El-Sayed and Shindia, 2011) and bacteria *Pseudomonas putida* (Takakura et al., 2004), *Aeromonas* sp. (Nakayama et al., 1984), *Brevibacterium Linens* (Pinnamaneni et al., 2012) and *Citrobacter intermedius* (Faleev et al., 1996).

The maximum MGL production by *H. alvei* increased when the bacteria were grown on alkaline medium. The enzyme production by *H. alvei* was highest at pH 7.5 declining in both more alkaline and acidic conditions. The same pH optimum has been reported for other microbes (Arfi et al., 2006; Dias and Weimer, 1998; El-Sayed, 2010). As a summary of the basic conditions, they seem to be much the same for different fungal and bacterial species.

The most important supplement for *H. alvei* to produce MGL appeared to be an extra carbon source, namely galactose, at the concentration of 2 g L⁻¹. Galactose and lactose were observed to be best carbon sources for a bacterium *Lactobacillus plantarum* to enhance MGL production (Zhang et al., 2019). In our study, in the absence of any carbon source (control), the MGL production was low; the production was about one sixth of the production when galactose was present. This indicates that a carbon source is mandatory as a co-dissimilator for the MGL production of bacteria *H. alvei*, as observed previously for fungal species *Trichoderma harzianum* (Salim et al., 2019) and *A. flavipes* (Khalaf and El-Sayed, 2009).

In order to find other mandatory supplements, we supplied the media with different nitrogen sources and finally added *l*-asparagine with the amino acid *l*-methionine. In order to understand if *l*-methionine is able to delay the growth retardation of the bacteria, an additional incubation was carried out with Mg. The production of MGL enzyme decreased when the bacteria were incubated longer than 24 h, Mg as the sole supplement. At latest, the production decreased after 48 h when *l*-asparagine and *l*-methionine were supplemented. One reason for growth retardation may be the inactivation of the enzyme due to some sort of proteolytic activity. Another reason may be that the bacteria reached their plateau, however, was different in Mg and *l*-asparagine treatments; the bacteria reached the plateau later in the latter treatment. This indicates that *H. alvei* was able to overcome the problems of proteolytic activity and growth retardation in case the essential compounds, i.e. the amino acids, were present.

l-methionine (0.8%) has been observed to be the best nitrogen supplement for the maximum production of MGL by the fungal species *A. flavipes* and *A. ustus* (Abu-Tahon and Isaac, 2016; Khalaf and El-Sayed, 2009). Although bacteria do not grow on *l*-methionine, perhaps due to their inability to metabolize the deaminated (α -keto methionine) and demethylated (α -keto butyric acid and methanol) residues of *l*-methionine, *l*-methionine is an inducer of MGL production. In our incubation, the bacteria were

Table 2
Effects of different carbon sources and different concentrations of galactose on *l*-methioninase enzyme activity (U mL⁻¹) (mean, n = 3, SD = 2–4) of *Hafnia alvei*.

Carbon source (2.0 g L ⁻¹)										
Control	Glucose	Mannitol	Sucrose	Xylose	Maltose	Lactose	Galactose	Fructose	Glycerol	
8 ^c	13 ^c	30 ^b	10 ^c	30 ^b	13 ^c	30 ^b	49 ^a	30 ^b	13 ^c	
Galactose concentration (g L ⁻¹)										
0	1	2	3	4	5					
4 ^c	45 ^b	50 ^a	47 ^b	47 ^b	44 ^b					

Means followed by the same letter are not significantly different at $\alpha = 0.05$.

Table 3
Effects of different nitrogen sources on L-methioninase enzyme activity (U mL^{-1}) (mean, $n = 3$, $\text{SD} = 2\text{--}4$) of *Hafnia alvei*.

Nitrogen sources (1.25 g L^{-1})						
Control	L-methionine	L-asparagine	L-glutamine	Yeast extract	Peptone	Tryptone
30 ^{cd}	59 ^b	71 ^a	31 ^c	14 ^e	18 ^{de}	26 ^{cd}

Means followed by the same letter are not significantly different at $\alpha = 0.05$.

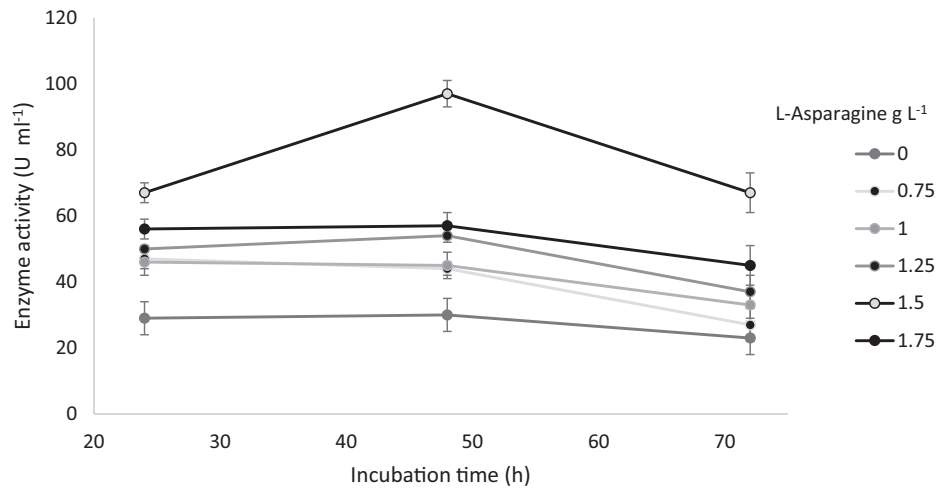


Fig. 3. L-methioninase enzyme activity of the test bacterium *Hafnia alvei* at increasing concentrations of L-asparagine as a supplement (each mixed with 2.0 g L^{-1} L-methionine) after different incubation times *in vitro* (24, 48 and 72 h).

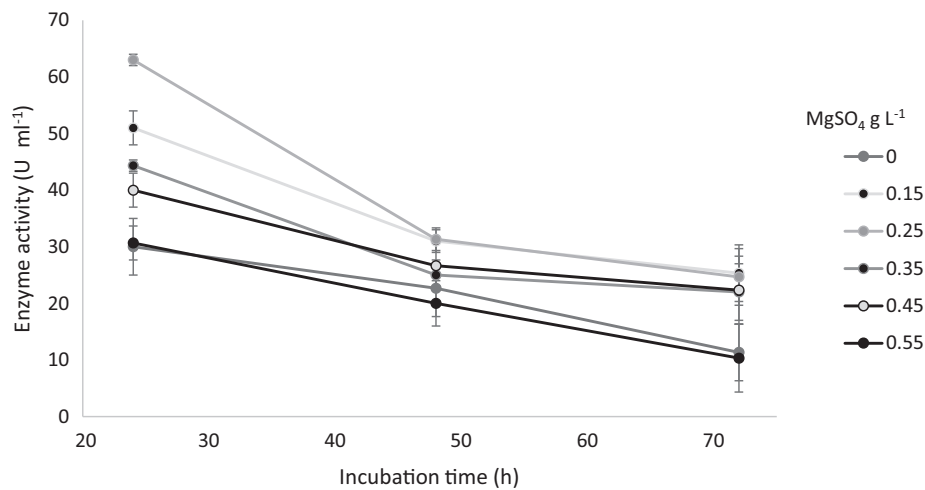


Fig. 4. L-methioninase enzyme activity of the test bacterium *Hafnia alvei* at increasing concentrations of magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) as a sole supplement after different incubation times *in vitro* (24, 48 and 72 h).

possibly able to utilize L-methionine in the presence galactose as a growth-supporting organic compound as suggested for glucose (Lockwood and Coombs, 1991).

Different nitrogen sources, such as peptone and yeast extract in the medium increased the production of MGL, as previously reported for *Geotrichum candidum* (Arfi et al., 2006) and *Candida tropicalis* (Selim et al., 2015). In our study, supplementing the medium with different nitrogen sources showed that the nitrogen compounds in general stimulated the MGL production, even if its inducer L-methionine was not present. Moreover, the mixture of L-methionine and L-asparagine resulted in the highest enzyme production by *Hafnia alvei*. Therefore, in our case for *H. alvei*, the production of MGL was independent on L-methionine. The same

independence was observed previously for fungi *Pseudomonas putida* (Tan et al., 1997) and *Geotrichum candidum* (Bonnarme et al., 2001). However, other studies have reported that the MGL production of *A. flavipes* and *Yarrowia lipolytica* were dependent on the presence of L-methionine in their growth media (Bonnarme et al., 2001; Khalaf and El-Sayed, 2009). As a summary of our nitrogen incubations, *H. alvei* was independent on L-methionine in the growth medium although it produced more enzymes in the presence of L-methionine.

As a summary about the comparison of our study to previously published studies, I suggest that different fungal and bacterial species have the much the same optimal growing conditions for their maximum MGL production. However, regarding mandatory

supplements in the growth medium, some differences between the species seem to exist.

In conclusion, our results suggest the optimum conditions to produce L-methioninase taking advantage of a bacterium *H. alvei*. *Hafnia alvei* did not need L-methionine as a mandatory supplement for its growth, an observation that adds knowledge about mandatory supplements of different species. Further optimizing of the conditions will enable the development of commercial production of L-methioninase. The new species offers more possibilities to produce L-methioninase for cancer therapy and as a drug for several microbial and cardiac diseases.

Declaration of Competing Interest

The author declare that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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