J Food Sci Technol
https://doi.org/10.1007/s13197-021-05090-9

ORIGINAL ARTICLE

Detection and relative quantification of amine oxidase gene (*yobN*) in *Bacillus subtilis*: application of real-time quantitative PCR

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Revised: 28 January 2021 / Accepted: 1 April 2021

Abstract Degradation of undesirable biogenic amines (BAs) in foodstuffs by microorganisms is considered one of the most effective ways of eliminating their toxicity. In this study, we design two sets of primers for the detection and quantification of the amine oxidase gene (*yobN*) and endogenous (housekeeping) gene (*gyrB*) in *Bacillus subtilis*. Moreover, these sets can be used for relative quantification of *yobN* by real-time PCR (qPCR). We also tested the degradation of BAs by three bacterial strains (*B. subtilis* strains: IB1a, CCM 2216, CCM 2267) in a mineral medium over a two-day period. Their degradation abilities were verified by high performance liquid chromatography with UV detection (HPLC/UV). According to the results, two strains significantly ($P < 0.05$) reduced histamine, tyramine, putrescine, and cadaverine. Moreover, our results indicate that the degradation ability of *B. subtilis* strains could be limited by sporulation because the gene encoding amine oxidase *(yobN)* is no longer expressed in the spores.

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Keywords Biogenic amines degradation · Histamine · QPCR · Primers

Introduction

Biogenic amines (BAs) are low-molecular-weight nitrogen compounds that are formed by some bacterial species in food and beverages during fermentation. They are indispensable components of living cells, but they may be toxic to human health in higher concentrations (Silla Santos 1996). The most dangerous of the BAs is histamine, which is responsible for the majority of food poisonings. Parente et al., 2001 pointed out that levels of histamine greater than 100 mg/kg can be health threatening, thus its quantity in foodstuffs must be monitored. European legislation (Commission Regulation (EC) No 2073/2005 2005) lays down food safety criteria for histamine in fishery products of up to 100 mg/kg and for fishery products, which have undergone enzyme maturation treatment in brine, of up to 200 mg/kg. However, high levels of BAs may occur in all fermented foodstuffs and beverages, where the threat of their increased accumulation is to be mainly found in cheeses, sausages and wine. Concentrations of BAs exceeding 1 g/kg have been reported in cheese, including histamine and tyramine as the most commonly present BAs (Fernández et al. 2007; Alvarez and Moreno-Arribas 2014). Some European countries recommend or suggest histamine limits in wine, which has a negative economic impact on the producers (Capozzi et al. 2012).

Removing histamine and other BAs formed is very complicated because of their persistence. They resist high temperatures, even autoclaving (Zaman et al. 2010). Therefore, attention has focused on preventing their formation, such as, using fresh raw materials, taking hygienic

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precautions as well as technological procedures. Nevertheless, it is not always possible to prevent BAs from forming, because their presence is the result of the technological procedure (Alvarez and Moreno-Arribas, 2014). Thus, attention has focused on other possibilities. One of them is irradiation, which can effectively degrade histamine molecules, but there is a potential risk of generating free radicals (Cardozo et al. 2014). Another possibility could be the addition of essential oils. Latorre-Moratalla et al., 2010 found that *Satureja thymbra* essential oil may inhibit the growth of decarboxylase-positive spoilage bacteria. Cai et al. 2015 successfully used spearmint oil as an antimicrobial agent for fish fillets, which had a positive influence on amine reduction.

Probably, the most effective solution is to use microorganisms, which are able to degrade amines formed as a part of starter and/or adjunct cultures. This ability is based on the fact that some microorganisms can produce amine oxidases (AOs), which are a large group of enzymes catalysing the degradation of BAs to substances that can be utilised by microorganisms as a source of energy and growth. These enzymes and deamination pathways have been described in several studies (Yagodina et al. 2002; Sekiguchi et al. 2004; Wang et al. 2013).

A powerful tool for searching for strains with degradation abilities could be real-time PCR (qPCR). This advanced technique offers the advantages of speed, sensitivity, simplicity and the specific detection and quantification of target genes in one step (Landete et al. 2007). Quite large number sets of primers have been designed to search for strains that produce BAs (Landete et al. 2011; Alvarez and Moreno-Arribas 2014; Guo et al. 2015; O'Sullivan et al. 2015), whereas when it comes to recognizing degrading strain, the situation is different. Eom et al., 2015 have published primers for the detection of the amine oxidase gene *yobN* in *Bacillus subtilis* strains, which have showed a high potential capacity to degrade BAs (Zaman et al. 2010; Kim et al. 2012). However, the annealing temperature of primers is only 48 °C, which is at a level where nonspecific ordinary primering can occur (Hecker and Roux 1996). Hence, suitable specific primers for the correct detection of promising *B. subtilis* strains for degrading BAs are still missing.

The purpose of this study is to design new primers for the specific detection of the amine oxidase gene (*yobN*) in *Bacillus subtilis* strains, which are able to degrade the most common BAs present in fermented foodstuffs.

This article is based on the previous study Butor et al. (2017). In this study, five strains able to degrade BAs were isolated from 408 food samples. These strains belonged to the species: *Bacillus subtilis*, *Bacillus pumilus*, *Enterobacter cloacae*, *Rhizobium radiobacter* and *Acinetobacter pitii*. By far the best results were seen in *B. subtilis* strain IB1a, so the attention was focused on this species. However, during storage, this strain lost the ability to degrade BAs, so other strains of *B. subtilis* (CCM 2216 and CCM 2267) were tested.

Material and methods

Strains and cultivation conditions

The *Bacillus subtilis* strains used in this study (IB1a, CCM 2216, CCM 2267) were obtained from the bacteria collection at the Department of Environmental Protection Engineering at Tomas Bata University in Zlín (strain IB1a), the Czech Republic or from the Czech Collection of Microorganisms (CCM 2216 and CCM 2267).

Five millilitres of 24-hour culture (ca. 10^8 CFU) was inoculated into media (50 mL) to obtain the initial concentration of bacteria (7.1 ± 0.3 log CFU/mL). Cultivation was performed at 30 ± 1 °C with shaking (200 RPM), in mineral medium 1 (MM1) (KH_2PO_4, $Na_2HPO_4{\cdot}2H_2O$, $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, NaCl, trace elements: $MnSO_4{\cdot}5H_2O$, H_3BO_3, $ZnSO_4{\cdot}7H_2O$, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, $Co(NO_3)_2{\cdot}6H_2O$, $CuSO_4{\cdot}5H_2O$; pH = 7.1 ± 0.1) containing 0.05% (w/v) glucose, and modified nutrient broth (0.15% (w/v) NaCl, 0.1 5% (w/v) beef extract, 0.25% (w/v) peptone; pH = 7.1 ± 0.1). Both media were supplemented with 0.02% (w/v) biogenic amine (histamine, tyramine, putrescine, and cadaverine; each in this concentration), and hydrochloric acid (0.1 M or 1 M HCl) was added to adjust a pH of the medium to 7.1 ± 0.1. For the control sample for qPCR, medium without any biogenic amines was used.

At specified times (0, 12, 24 and 48 h), the growth of the cells was monitored by plate method. Cultured samples were serially diluted with sterile phosphate buffer (1:9), and 100 µL of each sample was loaded on the plate with MRS agar. The cells present on the plate count agar following growth at 30 °C for 24 h were counted and the number of cells was expressed as CFU/mL. Samples for spore determination were heated at 80 °C for 10 min and then were serially diluted and inoculated on plates the same way as bacterial count. At the same time, were collected 5 mL of each cultured media for the relative expression level and biogenic amine degrading capability analysis.

Primer design

Specific gene primers were designed from conserved sequences of the amine oxidase gene *yobN*. In this study, endogenous gene primers were designed for the DNA gyrase β subunit (*gyrB*) (Caamaño-Antelo et al., 2015). The sequences of the amine oxidase gene *yobN* and

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endogenous gene (*gyrB*) for 44 different *Bacillus subtilis* strains were obtained from the National Centre for Biotechnology Information (National Center for Biotechnology Information 2019). New sets of primers for target genes were designed based on Primer Design genefisher2 (Giegerich et al. 1996). Furthermore, the properties of sets of primers were verified using the NCBI Primer-Blast tool. Primers in this study were synthetized by Merck (Darmstadt, Germany).

DNA extraction, Polymerase chain reactions (PCR)

To evaluate the primers' specificity, DNA was extracted from bacterial cells. Genomic DNA was prepared from 5 mL of bacterial strains that were grown in a modified nutrient broth. Pellets of these strains were obtained by centrifuging at 3000 × *g* for 5 min. DNA was extracted using High Pure PCR Template Preparation Kit (ROCHE, Germany) according to the manufacturer´s instructions. The purity and concentration of the DNA was measured using a Multimode Microplate Reader Infinite 200 PRO (Tecan, Switzerland).

PCR was performed by using a commercial mix, G2 Hot Start Green Master Mix (ROCHE, Germany). The reaction volume, 25 µL, included 12.5 µL of the commercial mix (ROCHE, Germany), 800 nmol/L of forward primer, 800 nmol/L of reverse primer and 10–100 ng of template. In addition, a negative control sample without template was prepared.

The PCR products were separated in 1% (w/v) agarose gel in TAE buffer with ethidium bromide by agarose electrophoresis run for 25 min at 90 V on a 1% gel. The GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, USA) was used as molecular weight marker.

The partial nucleotide sequence of the amplified genes *yobN* and *gyrB* were verified by sequencing with our set of primers (Table 1). PCR products were purified using NucleoMag® Tissue (Macherey–Nagel, Germany). The resulting sequences were compared against NCBI database using Basic Local Alignment Search Tool program (NCBI, 2009).

Table 1 Primer sequences for amine oxidase gene (*yobN*) responsible for BAs degradation and house-keeping gyrase gene (*gyrB*) used in qPCR

Primers	Sequence 5´-3´	Description
*yobN*6-L	GCTATACATGGGCCGATGAGG	qPCR
*yobN*6-R	CCGCAGAATACGGGTACTGG	qPCR
*gyrB*1-L	AGCGGAGATGACGTAAGGGA	qPCR
*gyrB*1-R	TCTTGCTCTTGCCGCCATTA	qPCR

Reverse transcriptase and qPCR

RNA isolation was done by High Pure RNA Isolation Kit (ROCHE, Germany). First strand cDNA was synthetized from 1 µL RNA using a Transcriptor First Strand cDNA Synthesis Kit (ROCHE, Germany).

qPCR was performed by using thermocycler CFX 96 Real-Time (Bio-Rad, Hercules, CA, USA) with the commercial kit Fast start universal SYBR Green Master (ROCHE, Germany). The total reaction volume, 25 µL, included 12.5 µL of ROCHE mix, 300 nmol/L of forward primer, 300 nmol/L of reverse primer and 1–2 µg DNA. The qPCR conditions were: initial denaturing at 95 °C for 3 min, followed by 45 cycles each comprising 95 °C for 30 s denaturing, 60 °C for 30 s annealing, and 72 °C for 1 min of extension, final extension was performed at 72 °C for 5 min. Data was normalized to *gyrB* expression.

The baseline and cycle threshold were automatically calculated using C1000 Touch Thermal Cycler equipped with a CFX 96 Touch™ System Software, version 2.1 (Bio-Rad, CA, USA). The melt curve analysis was performed on the same device (CFX 96 Real-Time) after the completion of qPCR.

Determination of biogenic amine content

The degrading capacity of the strains tested was experienced in mineral medium 1 and modified nutrient broth. Mixtures were derivatised using dansylchloride (Sigma-Aldrich, USA) according to (Dadáková et al. 2009).

BAs (histamine, tyramine, putrescine and cadaverine) were detected using high-performance liquid chromatography, Dionex HPLC UltiMate 3000 (Thermo Fischer Scientific, Waltham, Massachusetts, USA), following preceding derivatization using dansylchloride (Dadáková et al. 2009). The chromatographic column used for separation was an Agilent Zorbax RRHD Eclipse Plus C18 with the dimensions of 50 × 3.0 mm, 1.8 µm (Agilent, Paolo Alto, USA), spectrophotometric detection was carried out at a wavelength of 254 nm and a column temperature of 30 °C. The flow rate was 0.453 mL/min. The detection and separation of biogenic amines was performed according to (Smělá et al. 2004; Dadáková et al. 2009). Data were acquired and evaluated using Chromeleon™ 6.8 software (Thermo Fisher Scientific, USA).

Statistical evaluation

Non-parametrical analyses of variance from the Kruskal–Wallis and Wilcoxon tests (Unistat® 6.5 software; Unistat, London, UK) were used to evaluate the results obtained (the significance level was 0.05). Non-parametrical tests were used due to absence of normal distribution in some

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cases (Shapiro–Wilk test were applied). To estimate of the dependence of threshold cycle on DNA concentration regression line (linear least squares method) was used (Unistat® 6.5; software Unistat, London, UK). Tests of regression parameters were also performed. Prerequisite of tests used were also estimated using the same programme (the significance level was also 0.05).

Results and discussion

The ability to degrade BAs depends not only on the species but also on the strains. Thus, testing suitable strains by conventional techniques is unreliable or labour and time consuming. Molecular methods, especially real-time PCR with specific gene detection, is an interesting alternative (Marcobal et al. 2006). To examine the expression of the amine oxidase gene we designed and tested gene-specific primers. We then verified the ability of strains to degrade BAs using HPLC/UV. We also monitored the growth of cells during the degradation process.

Specific primer design

In this study, we designed three sets of primers for the amine oxidase gene (*yobN*) in *B. subtilis,* which is responsible for the degradation of BAs. Due to normalization of the target gene with an endogenous standard, we designed and tested primers for the gyrase gene (*gyrB*). According to the PCR tests, the set of primers were chosen, which do not form dimers or nonspecific products. The new sets of primers anneal to the amine oxidase gene of *Bacillus subtilis* strains and *B. subtilis* subsp. *subtilis*. No amplification occurred for the strains *B. subtilis* subsp. *altitudinis*. The final length of the PCR product for the detection of the amine oxidase gene (*yobN*) is 162 bp, and for the detection of the endogenous gene (*gyrB)* primers with the length 216 bp were selected (Table 1). The sequencing followed by analysis in BLAST (NCBI, 2009) confirmed that PCR products corresponded to the *yobN* and *gyrB* partial nucleotide sequences.

qPCR efficiency values with our sets of primers were in optimal range between 90 and 110% (Broeders et al. 2014), which corresponds to the slope of the long-linear phase of the amplification reaction between −3.58 and −3.10 (Fig. 1; P‹0.05). Post-amplification melting-curve analysis (data not shown), confirmed that the chosen sets of gene-specific primers do not form dimers or non-specific products (P ‹ 0.05). To calculate the relative expression, the Pfaffl method was used.

Expression of Gene Encoding Amine Oxidase

qPCR is nowadays a common method for measuring gene expression. This quantitative analysis requires no postprocessing, results are obtained quickly and, therefore, it could be used for the routine detection of bacterial strains that have potential to degrade histamine and other BAs (Wong and Medrano 2005). Some bacteria, such as *B. subtilis, B. polymyxa, Staphylococcus carnosus,* have been shown to reduce histamine and tyramine through the production of amine oxidase enzymes (Zaman et al. 2014; Eom et al. 2015; Kung et al. 2016).

We checked for the presence of the amine oxidase gene *yobN* and endogenous gene *gyrB* in bacterial strains by PCR (Fig. 2). To study the expression of the target gene in the selected bacterial strains, we performed qPCR analysis. However, qPCR data confirmed that only strains CCM 2216, CCM 2267 were able to express amine oxidase at higher levels (Fig. 3; P ‹ 0.05). Strain CCM 2267 exhibited a slightly higher relative expression level 3.07 ± 0.13 compared to 2.66 ± 0.16 attained by the strain CCM 2216. These values were obtained after 12 h of cultivation, when the cells were in the exponential phase of the growth. This was followed by a significant decline in relative expression level of the strain CCM 2267 to 1.35 ± 0.21 after 24 h and after 48 h, the relative expression level of this strain was equalized with the control cultured in MM1 without BA. For the strain CCM 2216, the decrease was somewhat slight and values of relative expression 1.35 ± 0.13 were recorded at the end of the cultivation (P ‹ 0.05). On the other hand, qPCR results showed no expression in the IB1a strain. Their ability to degrade BAs in culture was confirmed by HPLC/UV analysis. Figure 3 confirms that the decrease in total BAs content is higher (P‹ 0.05) in strains with higher relative expression of the target gene *yobN*.

Obtained PCR products of the *yobN* and *gyrB* had melting temperatures 76 ± 0.5 °C and 78 ± 0.5 °C respectively.

Detection of biogenic amine content

High performance liquid chromatography is the most commonly used technique because of its great versatility, efficiency, sensitivity and reproducibility, and is therefore the official technique for analysing histamine in foods (Commission Regulation (EC) No 2073/2005 2005; Marcobal et al. 2006). In our study, we determined the accurate degradation capacity of the selected strains with the amine oxidase gene using the HPLC/UV method (Figs. 3 and 4).

B. subtilis CCM 2216 significantly ($P < 0.05$) reduced histamine (18 ± 3.20%), cadaverine (18 ± 0.50%) and putrescine (14 ± 3.77%) within 48 h when compared to the initial concentration. *B. subtilis* CCM 2267 showed

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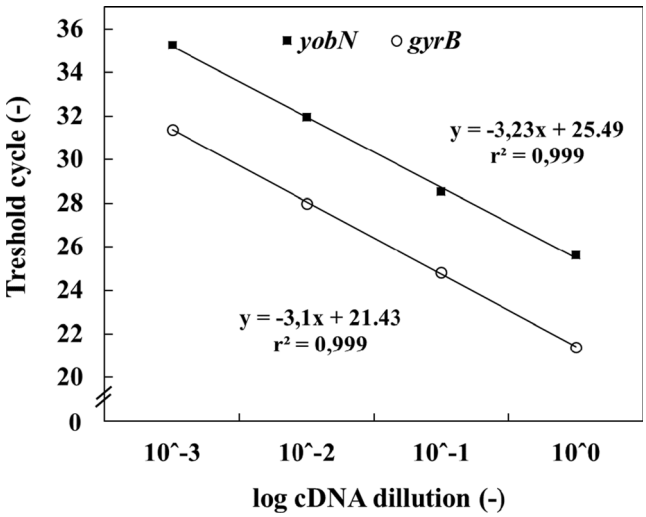

Fig. 1 qPCR standard curves of amine oxidase gene (*yobN*) and endogenous (housekeeping) gene (*gyrB*). The templates were cDNA purified from bacterial cells grow in MM1 after 48 h cultivation

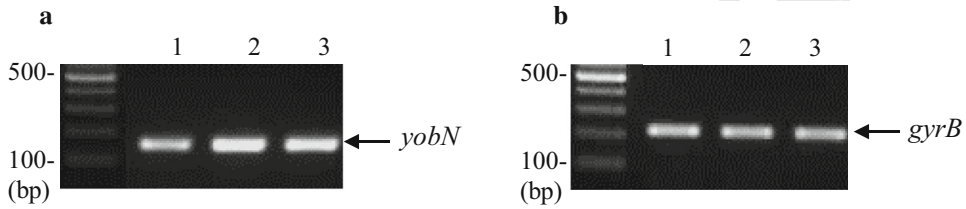

Fig. 2 PCR testing of new primers. **a** DNA fragments of amine oxidase gene (*yobN*) were amplified by primers *yobN*6-L and *yobN*6-R from *Bacillus subtilis* strains: IB1a (1), CCM2267 (2), and CCM2216 (3). **b** DNA fragments of endogenous gene (*gyrB*) were amplified by primers *gyrB*1-L and *gyrB*1-R from *Bacillus subtilis* strains: IB1a (1), CCM2267 (2), and CCM2216 (3)

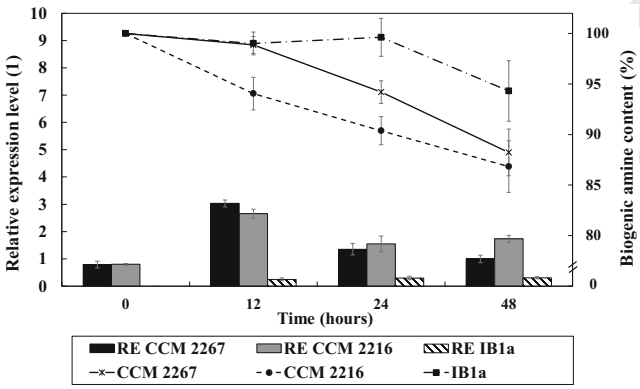

Fig. 3 Comparison of relative expression levels of the amine oxidase gene *yobN* in *Bacillus subtilis* strains performed by qPCR method with the biogenic amines content in media determined by HPLC/UV during 48 h of cultivation

significant ($P < 0.05$) tyramine degradation 24 ± 3.51%, histamine degradation 14 ± 4.39% within 48 h. Decrease of cadaverine and putrescine was low (6–3%; $P \geq 0.05$).

B. subtilis IB1a did not show any expression according to the qPCR data. However, the strain showed slight, but significant, ($P < 0.05$) degradation of histamine (6.75 ± 0.33%), tyramine (3.5 ± 0.75%), cadaverine (6.25 ± 0.35%), and putrescine (6.25 ± 1.64%). This degradation could be explained by transglutaminase activity during sporulation, which has been demonstrated by Kobayashi et al. 1996. Transglutaminase catalyses the acyl transfer reaction between the donor, the -carboxymide group of the protein bound glutamine residue, and the acceptor, the amino group of the primary amine, diamine or polyamine in peptides or proteins. Transglutaminase induces the formation of ε-(γ-glutamyl)lysine cross-link in proteins via acyl transfer between the ε-amino groups of the lysine residue and the ε-amide group of the glutamine residue. Transglutaminase modifies protein molecules by cross-linking, binding low-molecular-weight compounds, and deamination reactions. Amino acids are cross-linked to the proteins in the presence of transglutaminase and improve the nutritional value (Chanarat et al. 2012; Yerlikaya et al. 2015). The influence of transglutaminase on the reduction of BAs was observed in some studies (Yerlikaya et al. 2015; Lu et al. 2017).

Growth of cells

During degradations tests, the growth of cells was also monitored to examine differences in the quantity and the bacterial growth curves for the different strains. The

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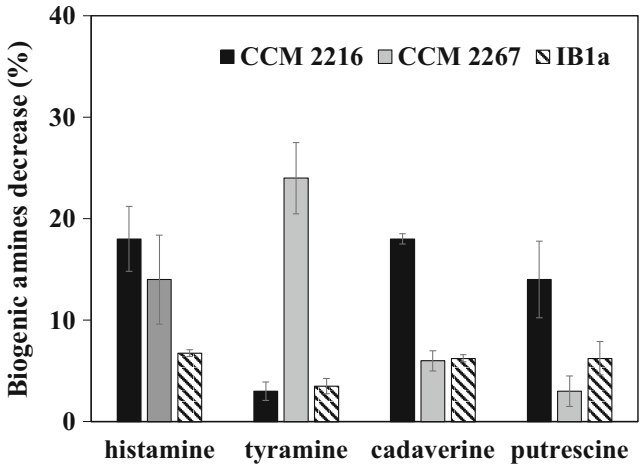

Fig. 4 Decrease of histamine, tyramine, cadaverine and putrescine measured by HPLC/UV after 48 h cultivation with *Bacillus subtilis* strains (CCM 2216, CCM2267 and IB1a). Reaction was carried out in MM1 inoculated by at 30 °C, pH 7.5 ± 0.1 under shaking condition (200 rpm) for 48 h

findings of growth bacteria in media supplemented by BAs during 48 h of cultivation are reported in Fig. 5.

The highest CFU/mL level ($1.9 \cdot 10^8$ CFU/mL) was reached by *B. subtilis* CCM 2216 after 24 h cultivation, which was then followed by a steep fall (Fig. 5; $P < 0.05$). The population of strain CCM 2267 started at $2.3 \cdot 10^7$ CFU/mL, the highest level was reached after 12 h' of incubation ($1.5 \cdot 10^8$ CFU/mL) and then continued decline at $7.1 \cdot 10^7$ CFU/mL after 48 h. These processes were confirmed by RNA amounts obtained from cells for qPCR. When initial values started at 36 ng/µL, they rose after 12 h' of cultivation to 2000 ng/µL and then dropped to 800 ng/µL after 48 h. A similar course of events was observed for *B. subtilis* IB1a, which reached a maximum ($1.1 \cdot 10^8$ CFU/mL) after 24 h' cultivation. Based on these findings, sporulation was subsequently tested, which could influence degradation of BAs because the metabolism of spores is suppressed.

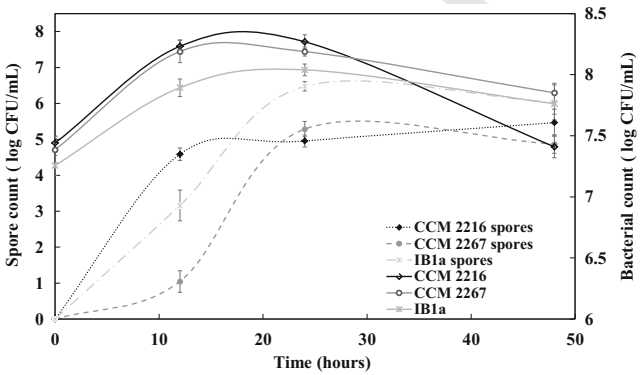

Fig. 5 Comparison of *Bacillus subtilis* strains growth and the number of spores in MM1 supplemented by histamine, tyramine, cadaverine and putrescine at 30 °C, pH 7.5 ± 0.1 under shaking condition (200 rpm) during 48 h of cultivation. The bacterial growth was determined by the colony counting method

Spore formation

B. subtilis belong to the three classes of Gram-positive bacteria (Bacilli, Clostridia, and Negativicutes), that respond to nutrient limitation by forming an endospore. The endospore is a metabolically dormant and environmentally resistant cell, able of surviving a wide range of environmental stresses, such as heat, desiccation, and ultraviolet radiation (Galperin et al. 2012; Huang and Hull 2017).

Spores were noticed after 12 h of cultivation in all three strains and their quantity grew during the entire time of cultivation for strain CCM 2216 (Fig. 5). *B. subtilis* CCM 2267 and IB1a reached a maximum after 24 h' of cultivation and there then followed a slight decrease after 48 h. The greatest quantity amount of spores was observed for the strain IB1a ($2.97\ 10^6$ CFU/mL) after 24 h of cultivation.

The growth and degradation were also tested in modified nutrient broth, because this medium contains peptone, which may support the growth of the cells more than is the case for MM1. The results showed ten times higher CFU/mL values with the same growth evolution (data not shown). However, greater quantities of bacteria cells did not influence the expression of amine oxidase, degradation of BAs or sporulation. This may indicate, that bacteria prefer using peptone and bovine extract as a source of nitrogen than biogenic amines.

No differences in the number of bacteria or spores were observed during cultivation in medium with/without BAs. Thus, the chosen concentration of BAs (0.2 g/L) probably does not limit or support cell growth.

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Conclusions

The aim of this study was the quick identification of histamine and other strains of *Bacillus subtilis* that degrade BAs using PCR methods. In this paper, two sets of primers were designed to detect the amine oxidase gene *yobN* and endogenous gene *gyrB*. The higher annealing temperature (60 °C) of these primers favours specificity by diminishing nonspecific priming. We have demonstrated that the primers allow the detection and quantification of target genes by qPCR. Using this method enables faster and easier searching for the strains capable of reducing histamine and tyramine, the two abundant toxic BAs in foodstuffs and beverages.

In conclusion, we confirmed that the *Bacillus subtilis* strains CCM 2216 and CCM 2267 are able to reduce histamine, tyramine, cadaverine, and putrescine. However, it should be mentioned that we noticed significant formation of spores during the cultivation of all tested *B. subtilis* strains. Therefore, it is necessary to consider the cultivation conditions for any potential industrial application.

Acknowledgement The financial support from the Grant Agency of the Czech Republic (GAČR No. 17-09594S) and the Internal Grant Agency of the Tomas Bata University in Zlín (No. IGA/FT/2020/009) is greatly acknowledged.

Author contributions Supervision: Buňková L.; methodology: Buňková L. and Jančová P.; investigation: Pištěková H., Šopík T., Maršálková K. and Berčíková L.; writing-original draft: Pištěková H.; formal analysis: Buňka F.; writing review and editing: Pištěková H., Jančová P., Buňková L., Buňka F.; project administration: Buňková L.; funding acquisition: Buňková L. and Buňka F.

Funding This work was supported by the Grant Agency of the Czech Republic (GAČR No. 17-09594S) and the Internal Grant Agency of the Tomas Bata University in Zlín (project IGA/FT/2020/009).

Declarations

Conflict of interest The authors declare no conflict of interest.

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