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BIOGENIC AMINES DEGRADATION BY MICROORGANISMS ISOLATED FROM CHEESE

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ABSTRACT

The aim of this study was the isolation and characterization of microorganisms able to degrade biogenic amines and their identification. Individual microorganisms were obtained by isolation from commercially available foodstuffs and food produced in the technological laboratories of Faculty of Technology, Tomas Bata University in Zlín and subsequently identified by MALDI-TOF MS. The results of MALDI-TOF MS identification were verified by 16S rRNA sequenation. In this work was studied the ability of 5 bacterial strains positive to biogenic amines degradation isolated from dairy products to decrease biogenic amines content in vitro and quantified reduction in the concentration of biogenic amines tryptamine, β-phenylethylamine, putrescine, cadaverine, histamine and tyramine. The level of degradation (decrease of biogenic amines) was determined on the base of the ability to grow in media with biogenic amines as the sole source carbon and nitrogen. The isolated strains with the ability of degradation of one or more biogenic amines were cultured in medium supplemented with relevant biogenic amines, the media derivatized with dansyl chloride and these amines separated by HPLC at a wavelength of 254 nm. From five tested strains identified as Bacillus subtilis, Bacillus pumilus, Enterobacter cloacae, Rhizobium radiobacter and Acinetobacter pitii, isolated from gouda type cheese, the greatest ability of degradation was observed in Bacillus subtilis, which was capable to degrade almost all amount of histamine, cadaverine and putrescine. Other four strains showed a lower rate of degradation than Bacillus subtilis, but the ability to degrade biogenic amines with these microorganisms was still significant.

Keywords: biogenic amines; *Bacillus*; degradation; cheese

INTRODUCTION

Biogenic amines (BA) are volatile basic nitrogen compounds of low molecular weight that are characterized by biological activity. They occur naturally in living organisms as metabolic intermediates and products of proteins and amino acids. These compounds are synthetized and degraded during normal metabolism of animals, plants and microorganisms. Biogenic amines are formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones **(Askar and Treptow, 1986; Maijala et al., 1993)**. These compounds can be divided according to their chemical structure of aromatic (β-phenylethylamine and tyramine), aliphatic (putrescine, cadaverine, spermidine and spermine) and heterocyclic (histamine and tryptamine). According to the number of amino groups in the molecule can be biogenic amines classified as monoamines - histamine, tyramine and tryptamine; diamines - putrescine and cadaverine, and polyamines – spermine, spermidine and agmatine **(Bardócz et al., 1993)**. They play an important role in many human physiological functions, such as cerebral activity, gastric acid production, and immune responses **(Shalaby, 1996)** or cell growth and differentiation **(Ladero et al., 2010)**. During microbiological activity BA may accumulate in food in higher concentrations. Excessive oral intake of BAs can cause health problems such as nausea, headache, rash, and changes in blood pressure **(Ladero et al., 2010; Lovenberg, 1973)**. Possible health complications relate primarily susceptible individuals whose detoxification systems work insufficiently from genetic causes or as a result of pharmacological treatment **(Bodmer et al., 1999)**, e.g., as antidepressants such as monooxidase inhibitors (MAOIs) **(Hernandez-Jover et al., 1997; Yongmei, et al., 2009)**. Due to the potential adverse effects of BAs on human health, it is necessary to prevent their accumulation in food **(EFSA, 2011)**. For foods or their parts that have passed through fermentation process, or were exposed to microbial contamination during production or storage can be expected a higher probability of BA occurrence **(Mariné-Font et al., 1995)**. Foods that often contain elevated levels of BA are fish, fish products and

derivatives and fermented products **(ten Brink et al., 1990; Halász et al., 1994)**. Cheeses usually contain significant amounts of biogenic amines and in some cases have been reported high levels of BA **(Joosten and Stadhouders, 1987; Buňková et al., 2013)**. Cheese is the one of most common fermented food associated with the BA poisoning **(Joosten, 1988, Tawfik et al., 1992)**. The term „cheese reaction" is used for tyramine intoxication. Small amounts of BA were detected in milk powder **(Voigt et al., 1974)**, yoghurts and in large quantities in many varieties of cheese **(Stratton et al., 1991; Buňková et al., 2013)**. The most important biogenic amines occurring in cheese are histamine, tyramine, tryptamine, putrescine, cadaverine and β-phenylethylamine **(Tawfik et al., 1992)**. During the cheese ripening casein is slowly degraded by proteolytic enzymes, it is leading to increased free amino acids content **(Foster et al., 1958; Joosten and Olieman, 1986)**. These amino acids can then be used for further reactions catalysed by specific bacterial decarboxylases and lead to the formation of CO_2 and amines. Therefore, the content of biogenic amines (particularly histamine, putrescine and cadaverine) gradually increases, and varies with increasing cheese ripening time **(Degheidi et al., 1992)**. Concentration of tyramine and histamine higher than 1 g/1 kg was recorded in cheese. It was demonstrated that tyramine and histamine are also the most frequent BA in foodstuffs. Their concentrations in cheese were recorded as the highest **(Stratton et al., 1991; Fernandez et al., 2007)**. Due to adverse effects on health, BA accumulation in foods should be prevented **(EFSA, 2011)**. Content of biogenic amines in foods fluctuates and also the composition of microflora is changing. One of the most important role play the actual quality and conditions of raw materials, for fermented products, then added a suitable choice of starter cultures. Other, no less important factors affected the BA formation or reduction is e.g. pH, NaCl concentration, the temperature or metabolic and biochemical activity of present microflora **(Gücükoğlu and Küplülü, 2010; Buňková et al., 2009)**. To reduce BAs accumulation in food many various strategies were suggested, such as inhibition of bacteria that can produce BAs, reducing the number of BAs producers using pasteurization of milk for cheese production, reducing proteolytic activity (therefore reduce the availability of amino acid precursors of biogenic amines). Another way to reduce the amount of BAs in food is their direct removal from foodstuff. The only known method to remove the BAs that were already created in foods is their removal by means of enzymes or microorganisms which are able to degrade BA. Possibility of BA removal from food in this way is based on the fact that aminooxidases, which are responsible for the BA detoxification, received in the diet have been found in some microorganisms **(Alvarez and Moreno-Arribas, 2014)**. The use of such strains seem appropriate strategy to reduce the value of BA in foods where it is very difficult to avoid: accumulation BAs due to the presence of BA-producing bacteria, including lactic acid bacteria (LAB), which are often part of the normal microflora of the food, thereby BA are present in the final stages of the manufacturing process **(Fadda et al., 2001)**. It is known that some representatives of the genera *Brevibacterium*, *Bacillus*, *Lactobacillus*, *Pediococcus*, or *Micrococcus* are capable to degrade the BA **(Herrero-Fresno et al., 2012, Postollec et al., 2011)**. It is important to note known that microbial decarboxylase activity is very variable, while in most cases, species specific. The detection of these bacteria is thus important to estimate the content of BA in foods and the associated risks and the effort to prevent their accumulation in fermented foods **(Martinez et al., 2011; Postollec et al., 2011)**.

MATERIAL AND METHODOLOGY

Isolation of microorganisms

Microorganisms were isolated from commercially available food. Food-samples were sterilely removed and 10 x diluted with saline and resuspended in Stomacher. Ten microliters of bacterial suspension was inoculated into tubes with 5000 µl of mineral medium MM1 according **Vítková (2016)** with biogenic amines (tryptamine, phenylethylamine, putrescine, cadaverine, histamine and tyramine). These samples were cultivated for one week at 30 °C. Partial results were read every 24 hours. The basic ability of degradation was identified on the basis of ability to grow in mineral medium supplemented with BAs, but the inability to grow in mineral medium without the addition of BAs (control). Of the 408 food samples, 5 different strains able to degrade biogenic amines were isolated. All of them were obtained from gouda type cheese. These 5 species of microorganisms were transferred from tubes to plates with medium Nutrient agar (HiMedia; India) and cultured for 48 hours at 30 °C.

MALDI-TOF MS analysis

Samples for analysis were prepared by isolating a pure culture that has been grown for 24 hours at 30 ° C, then was resuspended and transferred to 150 µL of sterile distilled water in a microtube. 450 µL of 96% ethanol was added. Samples were frozen at -70 °C and identified by MALDI-TOF MS system according to **Gregova et al. (2012)**.

Table 1 The HPLC gradient elution program.

Time (min)	10% acetonitrile (%)	10% acetonitrile (%)
0.1	41	59
1.9	37	63
3.5	18	82
4.0	0	100
9.5	0	100
11.5	41	59
15.5	41	59

Table 2 PCR conditions.

Step	Temperature (°C)	Time	Number of cycles
Denaturation	94	10 min	1
Amplification	94	30 s	35
	57	30 s	
	72	60 s	
Final extension	70	10 min	1

Note: *Sequencing was carried out in SEQme s.r.o. (SEQme, Dlouhá 176, Dobříš, Czech Reublic). Sequenation results were processed and evaluated by BLAST algorithm **(Simon et al., 2006)**.

HPLC analysis

Concentration of given biogenic amines in individual experiments was determined by HPLC. Broth after culturing of the tested isolates was centrifuged at 4600 rpm for 10 minutes and the supernatant was diluted 1:1 (v/v) with perchloric acid (c = 1.2 mol.L^{-1}). The acidified mixture was subjected to derivatisation according to **Dadáková et al. (2009)**. 1,7-heptanediamine was used as an internal standard. Derivatised samples were filtered through a syringe filter with a porosity of 0.22 µm, and applied to a column (Agilent Eclipse Plus C18 RRHD, 50 x 3.0 mm, particle size 1.8 mm) chromatography system (Column Thermostat Agilent 1260 Infinity; autosampler LabAlliance, USA; binary pump LabAlliance, USA; UV/VIS DAD detector Agilent Technologies). Separation of dansylderivate of biogenic amines was proceede with a gradient elution and detection was carried out by UV spectrophotometry (λ = 254 nm). Conditions for derivatisation, separation and detection of monitored biogenic amines according to **Smělá et al. (2004)** and **Dadáková et al. (2009)** are summarized in Table 1.

Measurement of the optical density of the cells

Measuring of optical density of the cells was detected the growth of bacterial cells in an appropriate cultivation medium MM1 for a defined time interval. Measurements were carried out using a spectrophotometer TECAN. Culture was in microtitre plates monitored at 600 nm against negative control (pure medium without bacterial biomass).

Statistical analysis

The non-parametrical analyses of variance from the Kruskal-Wallis and Wilcoxon tests (Unistat® 6.5 software; Unistat, London, UK) were used in order to evaluate the obtained results (the significance level was 0.05).

PCR and strain identification by sequencing

Polymerase chain reaction (PCR) and 16S rRNA sequencing was used to verify identification of bacterial strains. DNA isolation was performed according to study of Simmon et al., 2006 and Christensen and Bisgaard, 2010. For amplification forward primer 341F (5'-CCTA CGGGAGGCAGCAG-3') and reverse primer 907R (5'-CCGTCAATT CCTTTGAGTTT-3') were used (Zhao, et al., 2008). PCR was performed using commercial kit G2 Hot Start Green Master Mix (ROCHE, Germany). The reaction volume included 10 µL of commercial master mix, 800 nmol.L-1 of forward primer, 800 nmol.L-1 of reverse primer and 1 µL of DNA. Negative control was performed – the same volumes of primers and master mix,

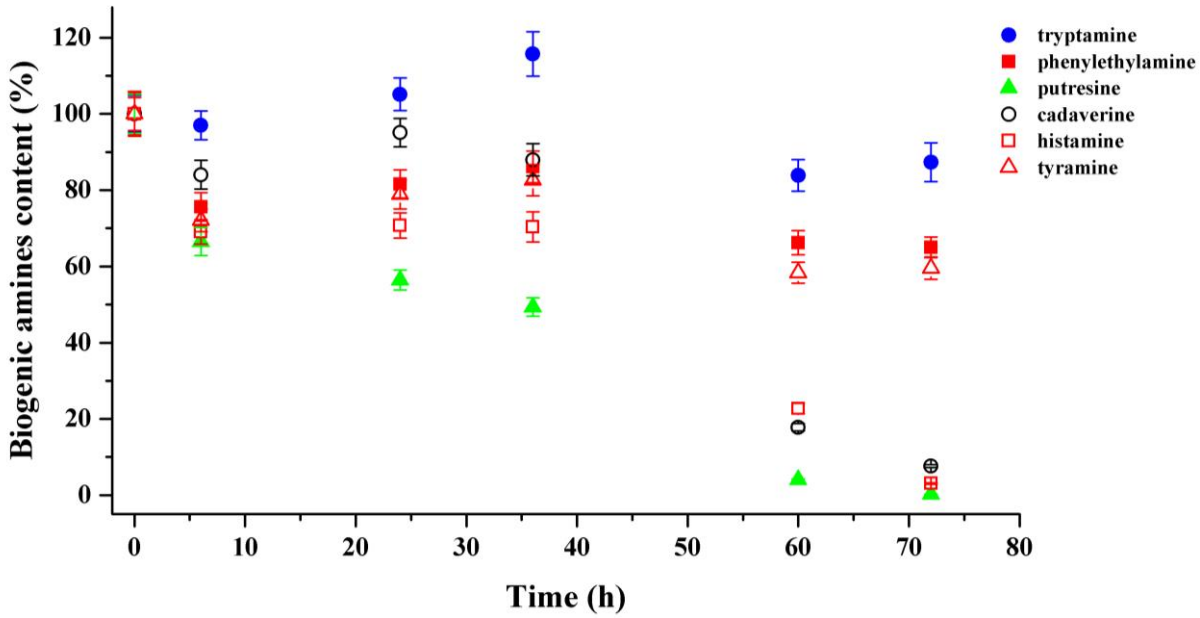

Figure 1 Changes in BAs concentration during the 72 hour cultivation of *Bacillus subtilis* 1a (the values were expressed as mean with standard deviatioin depicted as the bars).

Table 3 Changes in concentration of biogenic amines due to *Bacillus subtilis* 1a after 6 and 72 hours of cultivation.

BA*	after 6 hours of cultivation **	after 72 hours of cultivation ***
tryptamine	97.00	87.32 ††
phenylethylamine	75.63 †	65.03 ††
putrescine	66.52 †	0.23 ††
cadaverine	84.06 †	7.60 ††
histamine	69.05 †	3.05 ††
tyramine	72.11 †	59.55 ††

Note:

* percent concentration of biogenic amines in the sample after cultivation (in comparison with the start concentration).

** the means followed by "†" in superscript differ from the start concentration (p <0.05).

*** the means followed by "††"in superscript differ from the concentration measured after 6 hours of cultivation (p <0.05).

without a DNA as template. PCR conditions are shown in Table 2.

RESULTS

From 408 food samples were isolated 5 strains able to degrade biogenic amines. They were identified as *Bacillus subtilis* 1a, *Bacillus pumilus* 13b, *Enterobacter cloacae* 16b, *Rhizobium radiobacter*16a and *Acinetobacter pitii* 5a.

During the cultivation of all tested degraders amounts of all monitored biogenic amines in broth slightly declined after a 6-hour cultivation (Table 3, Figure 1). After a 24-hour cultivation concentration of 6 biogenic amines moderately increased ($p \geq 0.05$) while the concentration of putrescine was still declining (p <0.05). In the time of 36 hours started sharply to fall concentration of all biogenic amines. Within the times between 60 and 72 hours there was again a slight increase in concentration. *Bacillus subtilis* 1a showed the best ability to reduce biogenic amines in the medium. The greatest reduction of the BAs concentration was observed with putrescine, cadaverine and histamine (p <0.05) (Figure 1). Total volumes of putrescine and histamine after the 72-hour cultivation were nearly zero (p <0.05). As can be seen in Figure 1, the graph demonstrates that the amounts of BAs started sharply to increase after the 6-hour cultivation. *B. subtilis* 1a had ideal conditions for growth in that particular time. Thus, it grew and consumed free substrate from the medium and as a result it produced BA's as secondary metabolites. This metabolic pathway is probably more energetically preferable for this microorganism, therefore it consum of free substrate instead of using BAs. The results of the tests for biogenic amines degradation by *B. subtilis* 1a are summarized in Table 3. As it shown in Figure 1, concentration of BAs in samples gradually declined. After a depletion of free nutrients from substrate is organism forced to use carbon and nitrogen bound in BAs. This phenomenon was described in other studies **(Latorre-Moratalla, et al., 2010; Alvarez and Moreno-Arribas, 2014; Zaman, et al., 2011)**. Significant decrease in the concentration of biogenic amines in the sample was observed for other four isolates (p <0.05) – *Enterobacter cloacae* 16b, *Bacillus pumilus* 13b, *Rhizobium radiobacter* 16a and *Acinetobacter pitii* 5a.

None of these four strains showed such a high degradation potential as *Bacillus subtilis* 1a. By *Enterobacter cloacae* 16b was degraded 15.5% of tryptamine, 25.8% of putrescine, 45% of phenylethylamine, 24.1% of cadaverine, 19.5% of histamine and 38.5% of tyramine. Ability of *Bacillus pumilus* 13b to degrade biogenic amines was lower compared with *Enterobacter cloaceae* 16b but still significant (p <0.05). The levels of biogenic amines have been reduced by about one quarter. *B. pumilus* is most applied in the reduction of cadaverine, wherein the concentration of this BA was decreased by 29% (p <0.05). *Rhizobium radiobacter* 16a showed almost an equal ability to degrade each of the six investigated biogenic amines such as *B. pumilus* 13b (p <0.05).

Significant differences in the reduction of the concentration were observed only with phenylethylamine, when its amount after 72-hour cultivation lowered to a half (p <0.05). *Acinetobacter pitii* 5a was able to reduce the quantity of biogenic amines also about one quarter. An exception was histamine whose concentrations were decreased only by 3% ($p \geq 0.05$). Comparison of biogenic amines degradation efficiency can be seen in Table 4.

Table 4 Comparison of changes in concentrations by individual species after 72 hours of cultivation.

BA*	***Bacillus subtilis* 1a ****	***Bacillus pumilus* 13b**	***Enterobacter cloacae* 16b**	***Rhizobium radiobacter* 16a**	***Acinetobacter pitii* 5a**
tryptamine	12.68 †c	8.55 †b	15.46 †c	9.65 †b	3.25 †a
phenylehtylamine	34.97 †b	27.7 †a	43.96 †c	43.31 †c	23.77 †a
putrescine	99.77 c	27.57 †b	25.80 †b	25.14 †b	21.85 †a
cadaverine	92.40 b	28.68 †a	24.05 †a	28.30 †a	25.04 †a
histamine	96.95 c	20.55 †b	19.57 †a,b	20.43 †b	16.36 †a
tyramine	40.45 †c	36.23 †a	38.49 †a,b	39.75 †a,b	37.42 †a

* percentage reduction in the concentration of biogenic amines (in comparison with the start concentration).

** the means followed by "†" in superscript differ from the start concentration (p <0.05); the means within a line (the difference between the species) followed by superscript letters differ (p <0.05).

DISCUSSION

In this study the biogenic amines degradation ability of microorganisms isolated from ripened cheese was studied. The results obtained from 5 samples isolated from commercially available cheese show that in these 5 selected isolates studied the ability to degrade biogenic amines in time in *in vitro* conditions. *Bacillus subtilis* 1a showed the highest degradation activity of biogenic amines compared to other tested degraders. It was observed 100% degradation of putrescine, 97% degradation of histamine, and 92% degradation of cadaverine, other biogenic amines were degraded to 50%. Other 4 degraders showed less degradation activity. All isolates have reduced amounts of biogenic amines in broth by less than 50%. *Acinetobacter pitii* 5a and *Bacillus pumilus* 13b have the highest degradation activity for tyramine, *Rhizobium radiobacter* 16a and *Entrerobacter cloacae* 16b most degraded phenylethylamine. **Lee et al. (2015)** observed also in *Bacillus subtilis* degradation of histamine in the broth of 74% within 24 hours, 100% degradation of histamine showed different isolated species *B. polymyxa*. Another study described in *B. subtilis* degradation of histamine by 27 – 60%, putrescine 7 to 30% and cadaverine 22 to 29% at 24 hours in *in vitro* conditions **(Zaman et al., 2010)**. The study of **Eom et al. 2015** suggesting that histamine degradation was apparently mediated by suppression of histamine H3 receptor expression. According to the results of it was suggested that the *B. subtilis* might affect the synthesis and degradation of BAs, such as histamine and tyramine, by decreasing the expression of histidine and tyrosine decarboxylase-related genes (hdc and tydc). *Bacillus subtilis* 1a is able to significantly degrade BAs in mineral medium. This strain degraded the entire volume of histamine, putrescine and cadaverine. This fact points to the possibility of application of this strain as a part of the starter cultures for cheese, fermented meat products and fermented beverages where histamine, putrescine and cadaverine occur in high concentrations **(Latorre-Moratalla, et al., 2010; Silla Santos, 1996)**. Cheese, especially that made from raw milk, in which a specific non-starter microflora is essential for the organoleptic characteristics of the final product, is a particular technological challenge because it is a complex ecosystem involving many various microorganisms with different metabolic instruments, including amino acid decarboxylase enzymes that are responsible for high amounts of biogenic amines **(Fernández et al., 2007)**. Consistent with previously reported results, the addition of this strain as positive starter cultures could help to reduce the most dangerous BAs, such as histamine that is considered as the most toxic biogenic amine. Must not forget the fact that in many cases, the accumulation of BAs has been attributed mainly to the activity of the non-starter microflora **(Valsamaki et al., 2000)**. Furthermore, cadaverine and putrescine can react with nitrite to form heterocyclic carcinogenic nitrosamines, nitrosopyrrolidine or nitrosopiperidine **(Silla Santos, 1996)**. It is important to say that during the first 12 hours of cultivation volume of histamine increased which was highlighted in earlier studies **(Zaman, et al., 2011)**. This fact can be a problem when adding *B. subtilis* to starter cultures for food with a short fermentation time. The fact that *B. subtilis* addition to started microflora may change the composition of microflora due to competitive action on other strains should not be forgotten. This effect could devalue the final product during the ripening process **(Linares et al., 2011)**. The use of BA-degrading bacteria to reduce the BA content of foods would be to eliminate them from the food matrix. This might to be the strategy of choice with those fermented foods in which it is difficult to avoid the presence of BA-producing LAB because they are part of the usual microbiota, and consequently BA are present at the final stages of the manufacturing process **(Alvarez and Moreno-Aribas, 2014)**.

CONCLUSION

In conclusion it can be emphasized that all studied strains, primarily *B. subtilis* 1a, exhibited the ability to degrade biogenic amines *in vitro* and they could be used as microbiological indicators to prevent BAs accumulation in food. However, they require additional studies to verify the ability of biogenic amines degradation in the fermentation conditions and normal manufacturing process.

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