Effects of NaCl concentration and initial pH value on biogenic amine formation dynamics by *Enterobacter* spp. bacteria in model conditions

GABRIEL GREIF - MÁRIA GREIFOVÁ - JOLANA KAROVIČOVÁ

Summary

Effects of NaCl concentration and initial pH value on biogenic amine formation by the bacteria *Enterobacter aerogenes* and *Enterobacter cloacae* in GTY broth (glucose, tryptone, yeast autolysate) were studied. Sterile GTY broth of various NaCl concentrations and initial pH values was inoculated with 5 x 10^3 CFU.cm^{-3} *E. aerogenes* and/or *E. cloacae* and cultured at 37 °C under stationary conditions. Biogenic amines were determined by the HPLC-UV method as Dansyl derivatives. Amines specific formation rates (ν_P) were calculated from parameters of the function $c_P = f(\tau)$. Under the model conditions used, *E. aerogenes* produced cadaverine and histamine, with the highest cadaverine and histamine concentrations measured in broth containing 3 % NaCl (1 807 μg.cm^{-3} and 341 μg.cm^{-3}, respectively). *E. cloacae* only produced putrescine from ornithine, and the highest putrescine concentrations (320.6 μg.cm^{-3}) were measured at the NaCl concentration of 0.5 % and pH 6 (548.2 μg.cm^{-3}).

Keywords

Enterobacter; putrescine; cadaverine; histamine

Biogenic amines in foods originate from two sources. They represent the natural component of cell structures of plants, animals and microorganisms, or may arise during the process of production and storage as the result of metabolic action of microorganisms. Biogenic amines thus become indicators of microbial contamination, and their presence and concentrations may be used as a food quality parameter [1, 2].

Bacterial decarboxylases are mostly specifically oriented towards a certain amino acid, above all the L-forms. Bacterial lines have been reported in the literature that decarboxylate amino acids [3-12].

Bacteria of the *Enterobacteriaceae* species (above all *Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumoniae, Proteus vulgaris, Serracia marcescens*), being the contaminating microflora, are responsible for the formation of cadaverine, putrescine and histamine in foods of plant and animal origin. These bacteria are part of the intestinal microflora of humans and domestic animals. They also occur in the natural environment. Being thermolabile, their presence in diary products as well as other heat-treated foods suggest either unsatisfactory pasteurisation regime or poor hygienic and sanitation standard of the respective production plant [13].

Formation of amines in fermented foods and beverages depends on the presence of free amino acids (the quantities of free amino acids depend on the degree of proteolysis), as well as on the presence of microorganisms able to produce amines, and on technological parameters (pH, NaCl concentration, temperature, water activity) [14-23].

Enterobacter aerogenes, *Enterobacter cloacae* are among the important producers of diamines (cadaverine, putrescine) and histamine. High concentrations of biogenic amines in food may result in failure of the detoxifying system of adult individuals to eliminate these substances from the body. This may be explained by the presence of inhibitors. Putrescine and cadaverine, amines with weaker pharmacological effects compared with histamine or tyramine, block the activity of monoamine oxidase (MAO) and diamine oxidase (DAO), which explains the fact that higher concentrations of histamine and tyramine were measured in the blood when ingested in the pres-

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ence of cadaverine and putrescine as compared with the intake of water solutions of these amines alone [24-27]. The activity of amine oxidases has also been reported to be weaker in patients with allergies who take amine oxidase-based drugs as well as in patients with digestive system conditions such as gastritis and gastric ulcers compared with healthy individuals. Other factors reducing the performance of the detoxifying system include alcohol, coffee, tea, cigarettes and some medicines (ambroxol hydrochloride, dihydralazine, isoniazide, clavulanic acid, promethazine, verapamil - α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]methylamino]propyl]-3,4-dimethoxy-α-(1-methylethyl) benzeneacetonitrile) [28-30].

Since biogenic amines represent significant indicators of bacterial contamination of foods and beverages, and as their higher concentrations may affect the safety of food, this work aimed at studying the effects of model conditions (NaCl concentration, initial pH value) on the formation of cadaverine, putrescine, and histamine by bacteria of the *Enterobacteriaceae* species (*Enterobacter aerogenes, Enterobacter clo*acae) in GTY broth (glucose, tryptone, yeast autolysate).

MATERIALS AND METHODS

Microorganisms used

Enterobacter aerogenes CCM 2531, *Enterobacter cloacae* CCM 1903 (Czech Collection of Microorganisms, Brno, Czech Republic).

Inoculum preparation and substrate culture

Suspensions of bacterial cells of the strain studied was prepared of 24-hr culture grown on oblique GTY (glucose, tryptone, yeast autolysate), in diluting solution (0.85 % NaCl and 0.1 % peptone) at the concentration of 3 x 10^8 CFU.cm^{-3}, which corresponds to McFarland´s opacity scale Grade I [31].

By decadic dilutions, inoculum was prepared to be subsequently applied (in the volume of 1 cm^3) into 9 cm^3 sterile GTY broth in test tubes. To dilute the inoculum, 9 parallel test tubes were used, thereof 3 for the concentration 10^4 CFU.cm^{-3}. One cm^3 of this concentration was used to inoculate 20 parallel test tubes containing GTY broth (9 cm^3 each), giving initial suspension density in the order of 10^3 CFU.cm^{-3}.

Culture conditions

E. aerogenes and *E. cloacae* were cultured in GTY broth under stationary conditions in a thermostat at 37 °C; the culturing conditions are shown in Tab. 1. Samples for analytical determination of biogenic amines were taken at pre-determined time intervals, with every sample being taken from a previously not sampled test tube.

Chemical analyses

- pH determination - Conductometer type OK-104 (Radelkis, Budapest, Hungary),
- determination of NaCl concentration [32].

Biogenic amines analyses

Biogenic amines (putrescine, cadaverine, histamine) were determined as Dansyl derivatives using the modified HPLC-UV method [33].

Growing media containing the tested microorganisms were mixed with 5% solution of trichloracetic acid in 1:3 (vol : vol) ratio. After thorough mixing and subsequent centrifugation at 7 000 rpm, the supernatant was filtered through a microfilter (0.2 μm) and used for derivatisation.

To derivatise amines in the growing media containing the microorganisms studied, 600 μl filtrate, 300 μl deionised water, and 100 μl saturated solution of sodium carbonate were used. The amines are non-dissociated in basic environment. The mixture was heated to 40 °C for 20 min. Then, 1 cm^3 acetonitrile was added. After thorough shaking, 100 μl Dansyl chloride in acetone (50 mg.cm^{-3}) were added, the mixture was repeatedly thoroughly shaken and heated to 40 °C for 1 hour. After this time, 100 μl sodium glutamate solution (50 mg.cm^{-3}) were added to the mixture to remove excess Dansyl chloride. The repeatedly

Tab. 1. Experimental conditions used to evaluate effects of pH and NaCl concentration on biogenic amines formation by *Enterobacter aerogenes* and *Enterobacter cloacae.*

Enterobacter aerogenes									Constant values
pH	5.8	6.3	6.6	7.1	7.8	–	–	–	NaCl 0.2 %
NaCl [%]	–	0.2	1.2	1.9	3.0	4.5	6.8	–	pH 7.0 ± 0.2
Enterobacter cloacae									
pH	5.8	6.0	6.2	7.0	7.6	8.1	8.9	–	NaCl 0.2%
NaCl [%]	0	0.5	1.0	2.0	3.0	4.0	5.0	7.0	pH 7.0 ± 0.2

Culturing temperature 37 °C, culture medium: GTY broth + 0.1 % lysine; 0.1 % histidine; 0.1 % arginine; 0.1 % ornithine; 0.1 % tyrosine.

shaken mixture was heated to 40 °C for 1 hour, again. After stopping the derivatisation reaction, the content of the test tube was cooled down to 20 °C, 1 cm^3 ethyl acetate was added, and the mixture was again thoroughly shaken on a shaker. In this way, Dansyl derivatives of the amines were extracted to organic phase. The organic phase was then separated from the water phase, and aliquots of the organic phase (the upper layer) were pipetted into dry (aluminium foil-covered, to keep it in dark) ground-glass stoppered test tubes and expanded to 1 cm^3 with methanol. Thus prepared sample was then applied to chromatographic column.

Analytical conditions

Liquid chromatograph, manufactured by Laboratorní přístroje (Prague, Czech Republic) consisting of:

- high-pressure pump HPP 5001 (Laboratorní přístroje),
- doser LCI 30 with 20 μl dosing loop (Laboratorní přístroje),
- UV–VIS detector LCD 2040 (254 nm) (Laboratorní přístroje),
- recorder TZ 4620 (Laboratorní přístroje),
- column Nucleosil 120, C18, (250 x 4 mm), 5 μm (Watrex, Bratislava, Slovakia),
- mobile phase methanol : acetonitrile : water (2 : 1 : 1 v/v/v), mobile phase flow rate 1 $cm^3.min^{-1}$.

Qualitative analysis of biogenic amines

Internal standard (2,7-diamine heptane) method was used for qualitative analysis. The method consists of the addition of standard solution of the respective amine; the elution time of the analyte studied is determined and compared with the elution time of the standard.

Quantitative analysis of biogenic amines

For quantitative analysis, the method of analytical line was used, with increasing concentrations of Dansyl derivative of the respective amine injected onto the liquid chromatographic column.

Calculation of amines formation characteristics

Amines formation characteristics were calculated from the parameters of the calculated curve defined as the function of product concentration (c_P) on time (τ):

$$c_P = Ce^{-e^{B(\tau - M_P)}} \quad (1)$$

$$\nu_P = \frac{BC}{e} \quad (2)$$

where c_P is product concentration at time τ, C is maximum product concentration in stationary phase (the upper asymptote), B is the slope of the straight line at point M, M_P is the time of the maximum amine specific formation rate, ν_P is the amine specific formation rate. Point M is defined by the relation

$$\frac{d^2 c_P}{d\tau^2} = 0 \quad (3)$$

Average concentration values for the amines studied and their standard deviations (for the different sampling intervals) were calculated from two parallel determinations. The course and the formation curve parameters according to equation (1) were calculated using the software TableCurve 2D for Windows (Jandel Scientific, California, USA) and Origin 3.5 (Microcal Software, Northampton, USA), with the curve selection being evaluated based on the parameters (correlation coefficient r^2, significance test of the curve - t-test, and Fischer test).

RESULTS AND DISCUSSION

Effects of NaCl concentration on biogenic amines formation

The study of biogenic amines formation by the bacteria *E. aerogenes* in GTY broth in the presence of various NaCl concentrations (see Tab. 1) was based on published data [33, 34] suggesting that the above strain is able to produce enzymes L-lysine decarboxylase (EC 4.1.1.18) and L-histidine decarboxylase (EC 4.1.1.22), which in turn form cadaverine and histamine.

Effects of the substrate on the activity of the decarboxylation enzymes of *E. aerogenes* were studied by measuring amine concentrations in the media at pre-set time intervals (Fig. 1). The confidence intervals shown in the Figures were calculated from two parallel determinations of the amines.

The results (Fig. 1) show that cadaverine and histamine formed at all the NaCl concentrations in GTY broth used. Maximum cadaverine concentrations were measured in the broth containing 3 % NaCl at 13 hrs (1 807 $\mu g.cm^{-3}$) (Fig. 1A), whereas histamine concentrations were the highest at the same NaCl concentration at 26 and 32 hrs of culturing (341 $\mu g.cm^{-3}$) (Fig. 1B). The results further showed that cadaverine was produced at the *E. aerogenes* cell density of 10^6–10^7 CFU.cm^{-3} and histamine at 10^8 CFU.cm^{-3}. Gradual increasing NaCl concentration within the interval (0.6–3.0 %) impacted favourably on the activity of the decarboxylation enzymes of *E. aerogenes*. However, NaCl concentrations exceeding 3.0 % had inhibi-

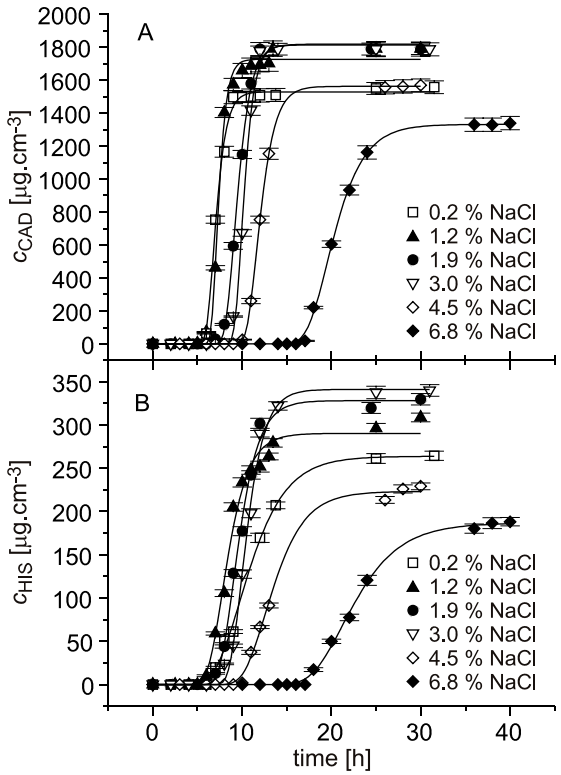

Fig. 1. Effects of NaCl concentration on cadaverine (A) and histamine (B) formation by the bacteria *Enterobacter aerogenes* CCM 2531 in GTY broth at 37 °C.

tory action on bacterial growth, which became probably reflected in the concentration of the decarboxylation enzymes at the initial suspension density (5.10^3 CFU.cm^{-3}). Also, the NaCl concentration in the medium studied at 37 °C, inoculated with *E. aerogenes* affected the specific formation rates (ν_P) of the amines studied. The highest specific formation rates of cadaverine ν_{CAD} and histamine ν_{HIS} were observed at the NaCl concentration of 1.2 % and 3.0 % respectively (Fig. 2). Equations (1) and (2) were used to calculate specific formation rates of the biogenic amines studied. The effect of NaCl concentration on specific formation rates of biogenic amines by *E. aerogenes* at the initial cell suspension density (in the order of 10^3 CFU.cm^{-3}) is illustrated in Fig. 2, and may be described by the following quadratic equations:

$$\nu_{CAD} = 814.17 + 49.79\, c_{NaCl} - 21.12\, c^2_{NaCl}$$
$$\nu_{HIS} = 32.01 + 32.19\, c_{NaCl} - 5.28\, c^2_{NaCl}$$

which may be used to theoretically determine NaCl concentrations at which $\nu_{CAD} \approx 0$ and $\nu_{HIS} \approx 0$. This, however, may not hold for higher initial cell suspension densities of the microorganism used (10^6 and/or 10^7 CFU.cm^{-3}), which are densities of *E. aerogenes* cell suspension able to produce measurable quantities of cadaverine [35].

M_{CAD}, i. e. the time at which a maximum of cadaverine specific formation rate (ν_{CAD}) is achieved, extended exponentially with the increasing NaCl concentrations for *E. aerogenes* cultured in GTY broth at 37 °C; in respect of M_{HIS} (the time at which a maximum of ν_{HIS} is achieved) this however was only true for NaCl concentrations exceeding 1.2 %. M_{HIS} was greater (by 1 hr) at the NaCl concentration in GTY broth of 0.6 % than at the NaCl concentrations of 1.2 % and 1.9 %; this is the reason for using quadratic function to describe this relation (Fig. 2).

WENDAKOON and SAKAGUCHI [36] studied the effects of NaCl addition to the medium (mackerel broth) on the formation of biogenic amines by the *E. aerogenes* strain ATCC 43175, and reported 6-fold increase in histamine and almost 4-fold increase in cadaverine formation in the presence of 1 % NaCl compared to the controls (without NaCl addition). Addition of 2 % NaCl only caused a slight increase in amine formation, and additions of 3 % or more lacked any stimulatory effect on amine formation. The aforementioned results suggest that NaCl concentrations of 0.5–2.5 % as used in the canning and meat industry may, in particular in respect of heat non-treated products, result in replication of the microorganism mentioned and production of high cadaverine and histamine

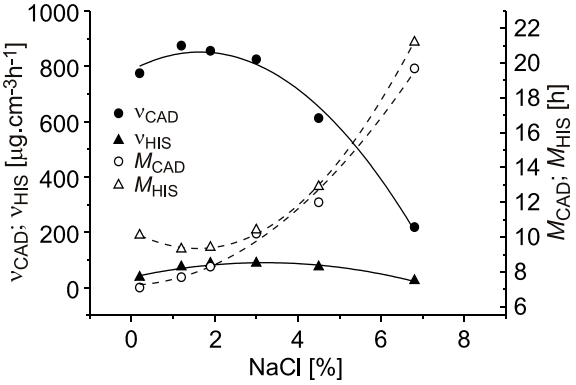

Fig. 2. Effects of NaCl concentration on specific formation rates of biogenic amines ν_P by the bacteria *Enterobacter aerogenes* CCM 2531 and time M, at which ν_P reaches maximum values in GTY broth at 37 °C.

ν_{CAD} – specific formation rate of cadaverine, ν_{HIS} – specific formation rate of histamine, M_{CAD} – time at which ν_{CAD} reaches maximum values, M_{HIS} – time at which ν_{HIS} reaches maximum values.

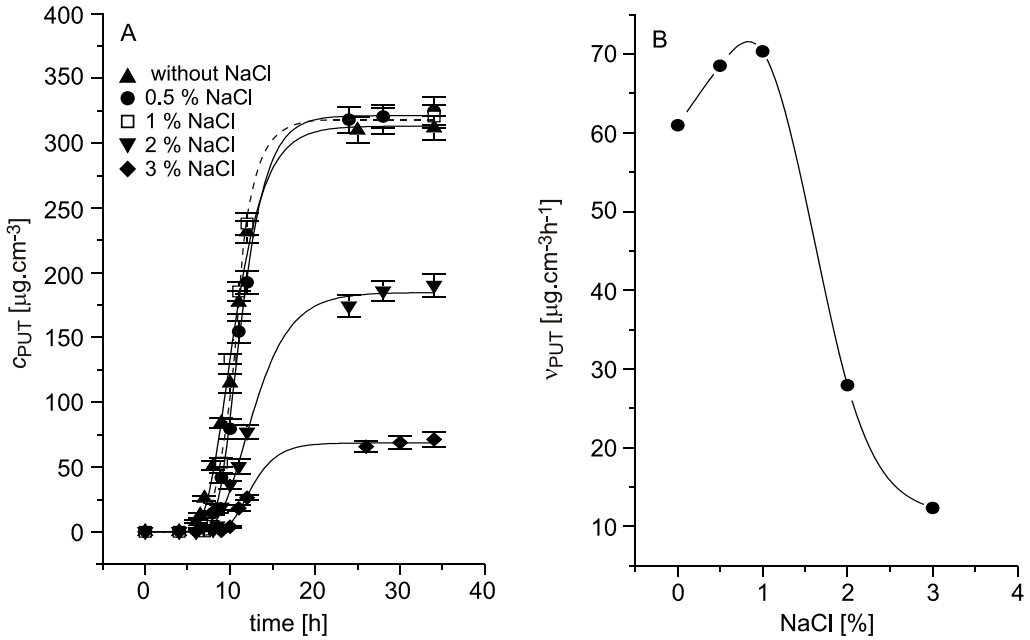

Fig. 3. Effects of NaCl concentration on putrescine formation (A) and specific putrescine formation rate v_{PUT} (B) by *Enterobacter cloacae* CCM 1903 in GTY broth at 37 °C.

concentrations, in particular if the technological procedures and good manufacturing practice principles are not adhered to.

There are reports in the literature of the ability of *Klebsiella pneumoniae* strains to be able to produce also cadaverine and histamine at suboptimal temperatures, in particular in fish [37, 38].

Although *E. cloacae* strain was reported to produce putrescine from arginine [39], the *E. cloacae* strain CCM 1903 used by us produced in GTY broth and at the initial NaCl concentrations (Tab. 1) upon stationary culture at 37 °C putrescine from ornithine as the direct precursor rather than from arginine. No putrescine could be detected in GTY broth supplemented with 0.1% arginine with the inoculated *E. cloacae* strain CCM 1903. Addition into GTY broth of ornithine as the direct precursor resulted in *E. cloacae* CCM 1903 producing putrescine. Due to this, 0.1% ornithine was added to the culture medium. The results are shown in Fig. 3A.

The highest putrescine concentrations (309.6 µg.cm^{-3}; 320.6 µg.cm^{-3} and 317.4 µg.cm^{-3}) produced by *E. cloacae* were measured in GTY broth containing 0 %; 0.5 % and 1 % NaCl, respectively, at 28 hrs of culturing under the above conditions (Fig. 3A). For the *E. cloacae* - produced enzyme L-ornithine decarboxylase (EC 4.1.1.17) activity and putrescine formation, the optimum conditions included NaCl concentrations of 0.5–1 % NaCl in GTY broth at 37 °C. Putrescine specific formation rates (v_{PUT}) were also the highest in these conditions (Fig. 3B). NaCl concentrations exceeding 2 % affected significantly both, putrescine specific formation rates and final putrescine concentrations of 11 µg.cm^{-3} at 72 hrs in the presence of 4% NaCl. NaCl concentrations in the medium of 5 % and 7 % completely inhibited L-ornithine decarboxylase (no putrescine could be detected).

Effect of initial pH value on biogenic amines formation

E. aerogenes in GTY broth at 37 °C produced cadaverine at lower initial values of culture medium pH. The highest cadaverine concentrations were found in GTY broth at initial pH value of 5.8 at 30 hrs of culturing (2 863 µg.cm^{-3}) (Fig. 4A). At higher initial pH values, highest cadaverine concentrations were measured as early as at 11 hrs (2 480 µg.cm^{-3}; initial pH 7.1), 12 hrs (2 503 µg.cm^{-3}; initial pH 6.6) and 11 hrs (2 490 µg.cm^{-3}; initial pH 6.3). At these initial pH values however, cadaverine concentrations decreased during the subsequent hours (Fig. 4A), which may be connected with mutual interactions between cadaverine formed and other components of the given media. *E. aerogenes* is able to produce 3-hydroxy-2-butanone and 2,3-butanediol by utilising glucose via pyruvate. In addition, lactic acid,

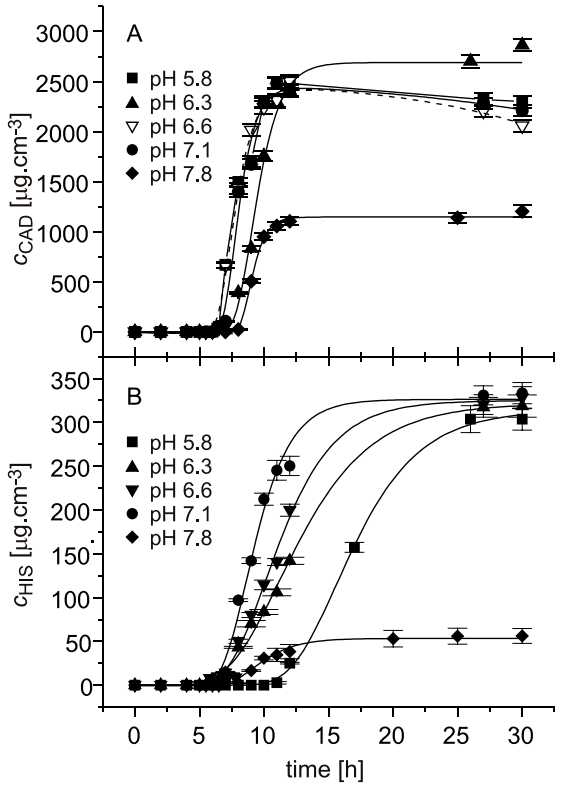

Fig. 4. Effects of pH on cadaverine (A) and histamine (B) formation by *Enterobacter aerogenes* CCM 2531 in GTY broth at 37 °C.

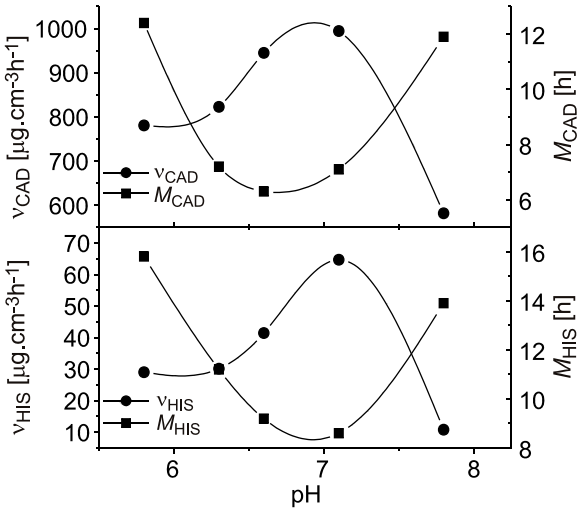

Fig. 5. Effects of pH on specific formation rates of biogenic amines ν_P by *Enterobacter aerogenes* CCM 2531 and time *M*, at which ν_P reaches maximum values in GTY broth at 37 °C.

ν_{KAD} – specific formation rate of cadaverine, ν_{HIS} – specific formation rate of histamine, M_{KAD} – time at which ν_{KAD} reaches maximum values, M_{HIS} – time at which ν_{HIS} reaches maximum values.

acetic acid and formic acid are formed, the latter getting subsequently decomposed to hydrogen and carbon dioxide. Such an environment however may also cause cells to die or even become lysed, with diamine oxidases released into the environment, which also may contribute to cadaverine disappearance.

The results of the measurements illustrated in Fig. 4B suggest that histamine concentrations formed by *E. aerogenes* in GTY broth at 37 °C were significantly smaller than cadaverine concentrations. The highest histamine concentration in the medium studied was measured at 25 hr (333.4 µg.cm^{-3}) at the initial substrate pH value of 7.3. At initial pH values of the GTY broth of 5.8–6.6, histamine concentrations of 309.5–328.3 µg.cm^{-3} were measured at 25 hrs and 30 hrs of culturing (Fig. 4B). Increasing the substrate (GTY broth) initial pH value to 7.8 was followed by not only inhibition of *E. aerogenes* growth but also by reduced cadaverine and histamine formation (Fig. 4A, B). Maximum histamine concentration measured in this case was 54.7 µg.cm^{-3}.

TAYLOR, LEATHEWOOD and LIEBER [40] claimed that the microorganism responsible for histamine formation in lactic bacteria-fermented cabbage is *E. aerogenes*. This statement may be agreed with but partly since substantial amounts of histamine are formed in fermented cabbage at the end of fermentation, when substrate pH ranges between 3.7–3.5 [21, 22, 41].

Cadaverine specific formation rate several times exceeded histamine specific formation rate (Fig. 5), which above all was associated with the maximum amine concentrations at the stationary phase (the upper asymptote) (Fig. 4A, B). Moreover, Fig. 5 suggests that the optimum pH value for the specific formation rates of the biogenic amines studied at the temperature optimal for *E. aerogenes* growth is 7.0–7.2. These pH values closely correlate with growth and specific growth rates of the microorganism studied.

In GTY broth of the initial substrate pH (Tab. 1) and at the temperature of 37 °C, *E. cloacae* produced putrescine (Fig. 6A). The highest putrescine concentrations (546.4 µg.cm^{-3}; 548.2 µg.cm^{-3} and 539.4 µg.cm^{-3}) were measured for initial substrate pH values of 5.8; 6.0; and 6.2 (Fig. 6A). At higher initial substrate pH values (7.1 to 8.9), *E. cloacae* produced markedly smaller amounts of putrescine, and this may have been closely connected with intracellular biochemical processes as well as with ornithine transport inside the cell. Specific formation rate of putrescine by *E. cloacae* in GTY broth at 37 °C was the highest at pH 6.2 (130.26 µg.cm^{-3}h^{-1}) at time M_{PUT} = 10.9 hrs

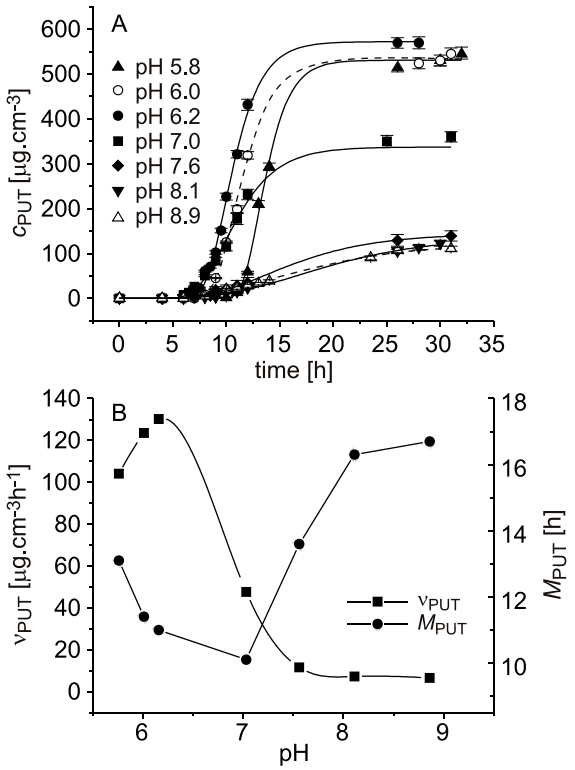

Fig. 6. Effects of pH on putrescine formation (A), specific rates of putrescine formation v_{PUT} (left axis) by *Enterobacter cloacae* CCM 1903, and time M_{PUT}, at which v_{PUT} reaches maximum values (right axis) (B) in GTY broth at 37 °C.

(Fig. 6B). At higher initial substrate pH values, the specific formation rate of putrescine dropped significantly, which above all is connected with the maximum final putrescine concentrations achieved during the culturing process under the given conditions. Also, the time M_{PUT} was observed to become extended, however at substrate pH values exceeding 7.1 only (Fig. 6B).

Based on the results obtained, it may be stated that markedly higher putrescine concentrations were formed within pH ranges more acid that optimal for *E. cloacae,* and this allows the assumption that this microorganism reacts to substrate pH by enhanced production of substances of basic pH, including biogenic amines.

CONCLUSION

The above discussed results as well as other results and experience from our laboratories regarding analysis of biogenic amines in foods allow the assumption that analytical determination of microbial products (biogenic amines) is required apart from microbiological tests of input raw materials. Such determinations provide for a feedback on multiplication of bacteria, in particular of those of the family *Enterobacteriaceae,* and thus on the quality of the input raw materials. This has also been pointed out by authors of the references [2, 42]. Thus, in applying HACCP (Hazard Analysis Critical Control Points) principles in production plants focusing on mainly the production of rapidly deteriorating foods of animal as well as plant origin, it is recommended to monitor diamine levels (cadaverine, putrescine) at selected Critical Control Points. Significant quantities of histamine are formed upon processing of fish which contain higher concentrations of free histidine compared with meat of warm-blooded animals or milk; a contribution to this is also made by a different microflora, e.g. *Morganella morgani*, *Klebsiella pneumoniae, Hafnia alvei, Vibrio.*

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