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ScienceDirect
Food Chemistry 101 (2007) 1230–1238
Food Chemistry
www.elsevier.com/locate/foodchem

Formation of pyrazines and 2-acetyl-1-pyrroline by *Bacillus cereus*

An Adams, Norbert De Kimpe *

Department of Organic Chemistry, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

Received 9 November 2005; received in revised form 24 January 2006; accepted 10 March 2006

Abstract

The production of alkylpyrazines and 2-acetyl-1-pyrroline by different *Bacillus cereus* strains, which has been previously reported, was studied in detail. *B. cereus* ATCC 27522 produced the highest amounts of flavour compounds when grown as surface cultures on plate count agar. Pyrazine, methylpyrazine, 2,5-dimethylpyrazine, trimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were produced in low amounts. Since they were also detected in control flasks, an enzymatic formation was concluded to be unlikely. Only the production of 3-ethyl-2,5-dimethylpyrazine was in all cases significantly different from the control. Detailed precursor studies revealed that the production of 2-acetyl-1-pyrroline by *B. cereus* ATCC 27522 proceeds via acetylation of 1-pyrroline, a metabolic degradation product of proline and ornithine. Comparison of results obtained from dynamic headspace and simultaneous steam distillation – solvent extraction showed that the use of a non-thermal extraction method is essential to obtain reliable results on the biological formation of these Maillard flavour compounds.

Keywords: *Bacillus cereus*; Bioflavour; Alkylpyrazines; 2-acetyl-1-pyrroline; Maillard flavour

1. Introduction

Microorganisms are essential for the development of the desired flavour of various fermented food products. In addition, microbial de novo production or bioconversion of natural precursors leads to flavouring substances that can be labelled as 'natural', and represents as such an interesting area in the field of food science.

Pyrazines comprise a group of heterocyclic nitrogen-containing compounds which contribute significantly to the unique roasted flavour of many heated food products (Maga & Sizer, 1973; Maga, 1992). Alkylpyrazines are important products of the Maillard reaction, formed usually at temperatures above 100 °C (Koehler & Odell, 1970). Trialkylated pyrazines such as 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine were shown to be impact flavour compounds of coffee (Blank, Sen, & Grosch, 1992), roasted sesame seeds (Schieberle, 1993), and roasted beef (Cerny & Grosch, 1993). A whole range of flavour-significant alkylpyrazines was detected in maple syrup (Akochi, Alli, Kermasha, Yaylayan, & Dumont, 1994), roasted peanuts (Leunissen, Davidson, & Kakuda, 1996), and in dough and breads (Martínez-Anaya, 1996). Various reports were made in the literature on the microbial origin of pyrazines in fermented food products, i.e. in fermented soybeans, cocoa, and cheese (Rizzi, 1988). Tetramethylpyrazine, for instance, was found to be responsible for the characteristic odour of natto, a Japanese fermented soybean product, which is usually inoculated with *Bacillus subtilis*, also known as *Bacillus natto* (Kosuge, Adachi, & Kamiya, 1962). Production of alkylpyrazines as a result of *B. subtilis* fermentation of soybeans has been extensively described (Ito, Sugawara, Miyanohara, Sakurai, & Odagiri, 1989; Larroche, Besson, & Gros, 1999; Owens, Allagheny, Kipping, & Ames, 1997). Also during the fermentation of cocoa beans, the production of alkylpyrazines by *B. subtilis* and *Bacillus megaterium* was shown to play a role in flavour formation (Selamat, Harun, & Ghazali, 1994). In an investigation of the contribution of

* Corresponding author. Tel.: +32 9 264 59 51; fax: +32 9 264 62 43.
E-mail address: norbert.dekimpe@UGent.be (N. De Kimpe).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.03.027

microbial metabolites to cocoa flavour Romanczyk, McClelland, Post, and Aitken (1995) observed the production of 2-acetyl-1-pyrroline, together with some alkylpyrazines, by *Bacillus cereus* strains isolated from cocoa fermentation boxes. 2-Acetyl-1-pyrroline is a very important Maillard flavour compound, with potent cracker-like flavour characteristics, and contributes significantly to the flavour of a large number of heated food products, in particular of rice (Demyttenaere, Abbaspour Tehrani, & De Kimpe, 2002). The production of 2-acetyl-1-pyrroline by *B. cereus* was the first report on the microbial origin of this interesting flavour compound. Later, the production of 2-acetyl-1-pyrroline by *Penicillium nalgiovense* on Mediterranean dried sausages was described (Stahnke, 2000) and 2-acetyl-1-pyrroline was reported as one of the 'mousy' heterocycles in wine, produced by heterofermentative wine lactic acid bacteria and the spoilage yeasts *Brettanomyces* and *Dekkera* (Grbin & Henschke, 2000; Costello & Henschke, 2002).

According to the experiments described by Romanczyk et al. (1995), only specific *Bacillus* strains were able to produce 2-acetyl-1-pyrroline in well-defined circumstances. A series of ^{13}C- and ^{15}N-labelling experiments showed that *B. cereus* utilized glucose as carbon source, and glutamic acid or proline as nitrogen source for the formation of 2-acetyl-1-pyrroline. Sampling was performed by simultaneous steam distillation – solvent extraction of two-day old surface cultures.

The main purpose of the presented work was to investigate the biological origin of pyrazines and 2-acetyl-1-pyrroline in *B. cereus* cultures and to exclude any heat-induced formation of these Maillard flavour compounds. Thermal formation of these flavour compounds is possible, as well during sterilisation of the culture media as during the steam distillation process. In the present investigation, alternative detection methods were evaluated, and the influence of different precursors was studied.

2. Materials and methods

2.1. B. cereus strains

B. cereus strains DSM 487 and DSM 2896 were bought from the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), *B. cereus* strains ATCC 14737 and ATCC 27522 from ATCC (American Type Culture Collection, Rockville, MD) in the form of freeze-dried cultures.

2.2. Collection of volatiles from bacterial surface cultures

B. cereus surface cultures were cultivated in 500-ml erlenmeyers filled with 50 ml of standard plate count agar (PCA, Oxoid Ltd., Drongen, Belgium). Inoculation was performed with 0.5 ml of one-day old liquid cultures (nutrient broth, Difco Laboratories, Detroit, Mich.). After one day of incubation at 30 °C, the surface cultures were sampled by dynamic headspace purge and trap at room temperature (Demyttenaere, Herrera, & De Kimpe, 2000). Purified air was swept over the surface cultures at a flow rate of 30 ml/min. Volatiles were adsorbed on Tenax TA traps (60/80 mesh), packed into pyrex glass tubes between two glasswool plugs. Prior to headspace sampling, the Tenax tubes were conditioned at 250 °C for two hours under continuous nitrogen flow. Samples were taken at regular time intervals by desorption of the Tenax tubes by eluting with three volumes of 2.5 ml of diethyl ether. Effluents were collected in 10-ml conical glass tubes and concentrated until 500 µl using a gentle nitrogen flow. For quantitative experiments, collidine (2,4,6-trimethylpyridine, +99%, Acros Organics, Geel, Belgium) was added to the extract. Where possible, cultures were cultivated triplicate.

2.3. Addition of supplements

L-Proline, L-ornithine monohydrochloride, L-lysine, L-lysine monohydrochloride, L-glutamic acid, 4-aminobutanal diethyl acetal, 1,3-dihydroxyacetone dimer, starch (soluble), and D-glucose (Acros Organics, Geel, Belgium) were added before or after medium sterilization as indicated. When supplements were added to the medium after sterilization, the compounds were sterilized as concentrated solutions using filter sterilization.

2.4. Likens–Nickerson extraction

For the simultaneous steam distillation – solvent extraction, specific Likens–Nickerson glassware was used. The culture medium (PCA) from two petri dishes (40 ml) or from one erlenmeyer (50 ml) was placed in a 500-ml round-bottom flask, and 300 ml of distilled water was added. After continuous extraction for 1.5 h, the solvent (20 ml diethyl ether) with analytes was dried with $MgSO_4$, filtered and concentrated using a gentle nitrogen flow.

2.5. GC–MS analysis

For the analysis of the extracts, a Hewlett–Packard 6890 GC Plus coupled with a HP 5973 MSD (Mass Selective Detector-Quadrupole type), equipped with a CIS-4 PTV (Programmed Temperature Vaporization) Injector (Gerstel, Mulheim-an-der-Ruhr, Germany), and a EC5-MS capillary column (30 m × 0.25 mm i.d.; coating thickness 0.25 µm) was used. Working conditions were: injector 250 °C; transfer line to MSD 260 °C; oven temperature start 35 °C, hold 5 min, programmed from 35 to 60 °C at 2 °C min^{-1} and from 60 to 250 °C at 20 °C min^{-1}, hold 5 min; carrier gas (He) 1 ml min^{-1}; splitless; ionization EI 70 eV; acquisition parameters in full scan mode: scanned m/z 40–200 (0–20 min), 40–400 (>20 min). For SIM (Selected Ion Monitoring) mode the acquisition parameters are displayed in Table 1. Analytes were identified by comparison of their mass spectra with mass spectral libraries (Nist98 and Wiley 6th), and by calculation of retention

Table 1
Acquisition parameters for GC–MS analysis of headspace extracts in SIM mode

Compound	Time frame (min)	Selected ions (*m*/*z*)
Pyrazine	1.0–7.0	80, 53, 52
Methylpyrazine	7.0–11.0	94, 67, 53
2,5-Dimethylpyrazine	11.0–14.2	108, 81, 42
2-Acetyl-1-pyrroline	14.2–17.0	83, 111, 69
Collidine (internal standard)	17.0–19.0	121, 106, 79
Trimethylpyrazine	19.0–19.5	122, 81, 42
3-Ethyl-2,5-dimethylpyrazine	19.5–32.0	136, 135, 108

indices and comparison with literature data (Adams, 1995). Standards (pyrazine, 2,5-dimethylpyrazine, 2-ethyl-3,5(6)-dimethylpyrazine from Acros, 2-methylpyrazine from Aldrich, Bornem, Belgium and 2,6-dimethylpyrazine and 2,3,5-trimethylpyrazine from Janssen Chimica, Beerse, Belgium) were used for the calculation of response factors needed for quantification.

2.6. Synthesis of 1-pyrroline

The synthesis of 1-pyrroline was performed according to a method developed for the synthesis of 2,3,4,5-tetrahydropyridine (Nguyen Van & De Kimpe, 2000). In a dried 250-ml flask, 0.25 mol (33.3 g) of *N*-chlorosuccinimide was dissolved in 220 ml of dry diethyl ether. Equimolar amounts of pyrrolidine (17.8 g) dissolved in 10 ml of diethyl ether were carefully added, while stirring in an ice bath. The combined reagents were allowed to react for 4 h at room temperature. After filtration and evaporation to one third of the volume, two equivalents of potassium hydroxide in methanol (30 g KOH in 120 ml methanol) were added and the mixture was refluxed for 3 h. The reaction mixture was poured in 100 ml of water, extracted with diethyl ether (two times 50 ml), washed with water (100 ml) and dried ($MgSO_4$). The obtained yield of 1-pyrroline was rather low (20%) since the evaporation of methanol involved substantial losses of volatile 1-pyrroline.

3. Results and discussion

3.1. Comparison of different B. cereus strains

In first instance, the production of 2-acetyl-1-pyrroline **1** was studied by Likens–Nickerson extraction of surface cultures of *B. cereus* ATCC 27522 and ATCC 14737 cultivated in Petri dishes, in order to reproduce the results obtained by Romanczyk et al. (1995), using the same experimental procedure. The results are displayed in Table 2. In the extracts of blank plate count agar, no 2-acetyl-1-pyrroline was detected. The production of 2-acetyl-1-pyrroline increased from day 1 until day 3 after inoculation and decreased afterwards. *B. cereus* ATCC 27522 displayed the highest production of the rice flavour compound. These results are in agreement with the findings of Romanczyk et al. (1995) although significantly higher amounts of 2-acetyl-1-pyrroline were reported by those authors (i.e. maximum levels of 87 μg 2-AP/kg by *B. cereus* ATCC 27522 as compared to 26 μg/kg in Table 2). *B. cereus* DSM 487 and DSM 2896 did not produce any flavour compounds in detectable amounts.

Table 2
Production of 2-acetyl-1-pyrroline (μg/kg PCA) by two *B. cereus* strains grown on plate count agar Petri dishes (at 32 °C), as measured by Likens–Nickerson extraction

Days after inoculation	*B. cereus* ATCC 27522	*B. cereus* ATCC 14737
0	n.d.[a]	n.d.
1	8.5	n.d.
2	23.5	n.d.
3	26.2	6.0
4	6.3	2.6

[a] Not detected.

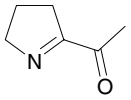
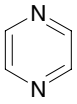
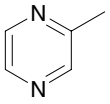
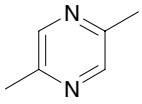
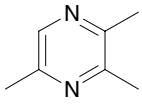
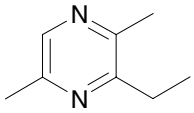
3.2. Influence of various precursors on the production of alkylpyrazines

Proline, ornithine hydrochloride, lysine, glutamic acid, glucose, starch, and 1,3-dihydroxyacetone were evaluated as precursors for flavour production by *B. cereus* in several experiments (data not shown). The most important volatile metabolites detected were pyrazine **2**, methylpyrazine **3**, 2,5-dimethylpyrazine **4**, trimethylpyrazine **5** and 3-ethyl-2,5-dimethylpyrazine **6**, of which the flavour properties are displayed in Table 3. It must be noted that under the given analytical conditions (EC-5 capillary column) 2,5-dimethylpyrazine and 2,6-dimethylpyrazine cannot be separated from each other. Both compounds co-elute since the difference in linear retention index amounts only one unit. Based on small differences in the mass spectrum of both compounds, the flavour compound produced by the bacteria was tentatively identified as 2,5-dimethylpyrazine, but the presence of small amounts of 2,6-dimethylpyrazine cannot be excluded.

When lysine was added to the culture medium (1% and 2%), the highest amounts of pyrazines were found. Likens–Nickerson extraction of 2-day old Petri dish cultures of *B. cereus* ATCC 27522 grown on plate count agar enriched with 1% lysine yielded 6.1 mg 2,5-dimethylpyrazine per kg. The amounts of pyrazines produced by the bacterial cultures were, however, not significantly higher than the amounts recovered from blank culture media enriched with lysine. Since lysine is a basic amino acid that is very reactive in the Maillard reaction, it can be stated that the formation of pyrazines in these cultures is probably chemical in origin. Standard tests showed that during autoclaving (1.2 bar, 20 min) of culture media browning and pyrazine formation occurs upon alkalisation to pH 9 or higher.

Among the other precursors tested, ornithine hydrochloride was a good precursor for the production of alkylpyrazines, and the presence of starch increased especially the production of 2,5-dimethylpyrazine. No consistent differences with the control were found for the other precursors tested.

Table 3
Pyrazines identified in the headspace of *Bacillus cereus* surface cultures with their flavour properties and linear retention indices (LRI)

Compound	Structure	Odour description	Odour threshold (ng/l, air)[a]	LRI (EC-5)
2-Acetyl-1-pyrroline	 **1**	Popcorn, scented rice	0.02[b]	920
Pyrazine	 **2**	Nutty	>2000[c]	724
Methylpyrazine	 **3**	Nutty, green	>2000	818
2,5-Dimethylpyrazine	 **4**	Nutty	1720	910
Trimethylpyrazine	 **5**	Roasted	50	1000
3-Ethyl-2,5-dimethylpyrazine	 **6**	Earthy, roasted	0.011	1089

[a] Wagner et al. (1999).
[b] Schieberle (1991).
[c] Odor threshold (pyrazine) > odor threshold (methylpyrazine) (Buttery et al., 1999).

3.3. Influence of ornithine as a precursor

Romanczyk et al. (1995) investigated the production of 2-acetyl-1-pyrroline but did not provide any details on the production of alkylpyrazines by *B. cereus*.

In this study, the production of pyrazines by *B. cereus* ATCC 14737 and *B. cereus* ATCC 27522 on plate count agar supplemented with ornithine hydrochloride was investigated, in comparison with control flasks (non-inoculated plate count agar supplemented with ornithine hydrochloride). Sampling was performed by dynamic headspace sampling of 50-ml surface cultures. The production of pyrazines on ornithine-supplemented medium increased with time. The results of the 5th and the 6th headspace sample (starting 10 days after inoculation and each of 6-days duration), which yielded the highest amounts of pyrazines, are displayed in Table 4. From these results can be concluded that the tested *B. cereus* strains significantly enhanced the formation of pyrazines, especially of 2,5-dimethylpyrazine, on ornithine-containing medium. *B. cereus* ATCC 27522 was a more efficient strain for pyrazine production than *B. cereus* ATCC 14737, and there was a significant difference with the control. However, this difference became notable only after a considerable amount

Table 4
Amounts of pyrazines (μg/kg) detected in the headspace of blank and *B. cereus* inoculated plate count agar, supplemented with 2% ornithine hydrochloride (after medium sterilization)

Sample	Culture/control	MP[a]	DMP	TriMP	EDMP
Hsp 5	Blank	0.23 ± 0.18	0.94 ± 0.27	n.d.[b]	n.d.
	B. cereus ATCC 14737	0.94 ± 0.50	15.04 ± 8.47	n.d.	n.d.
	B. cereus ATCC 27522	8.74 ± 1.57	56.09 ± 3.16	0.54 ± 0.49	1.66 ± 0.48
Hsp 6	Blank	0.07 ± 0.14	0.55 ± 0.50	n.d.	n.d.
	B. cereus ATCC 14737	1.03 ± 1.58	13.94 ± 6.20	1.20 ± 1.26	0.01 ± 0.02
	B. cereus ATCC 27522	3.21 ± 1.43	26.17 ± 4.89	1.61 ± 0.58	1.44 ± 0.62

Average results of the 5th (6-day dynamic headspace started on the 10th day after inoculation) and 6th headspace sample (6-day dynamic headspace started on the 16th day after inoculation) with standard deviation ($n = 3$).
[a] MP, methylpyrazine; DMP, 2,5-dimethylpyrazine; TriMP, trimethylpyrazine; EDMP, 3-ethyl-2,5-dimethylpyrazine.
[b] n.d., not detected.

of time (10 days). The standard deviations obtained from the repetitions of the experiments are sometimes high, indicating that the bacterial flavour formation is sometimes irreproducible. 2-Acetyl-1-pyrroline **1** was not detected in the headspace samples of this experiment.

It is generally believed that most pyrazines associated with microbial fermentations are not directly formed by enzyme-catalyzed reactions (Rizzi, 1988). The metabolic activities of the microorganisms rather generate various precursors, such as α-acetolactate, acetoin, free amino acids, ammonia, etc., which are converted to pyrazines by non-enzymatic chemical reactions. Since the same pyrazines were also detected in the headspace of blank culture media, it is believed that, also in the present investigation, the activities of *B. cereus* increase the formation of efficient precursors from ornithine hydrochloride for the chemical formation of pyrazines.

The increasing pyrazine production as a function of time may be correlated with the release of the free δ-amino group of ornithine from the hydrochloride salt. As in the experiments described by Romanczyk et al. (1995), ornithine was applied to the cultures as the monohydrochloride, since it is commercially available as such. However, the results of ornithine hydrochloride enrichments can therefore not simply be compared with the results of other precursors, for instance of lysine, the higher homologue of ornithine, since the δ-amino group of ornithine is not available for chemical reactions in ornithine hydrochloride. Therefore, the production of flavour compounds by *B. cereus* ATCC 27522 on media supplemented with ornithine hydrochloride was compared with the production on media supplemented with lysine hydrochloride in another experiment. This time, the precursors were added prior to medium sterilization. The results of this experiment are shown in Table 5. The amounts of pyrazines produced from cultures supplemented with lysine hydrochloride and ornithine hydrochloride were, in general, not significantly different from each other, and were not significantly different from the control flasks (sterile PCA with supplements). As expected, the high amounts of pyrazines resulting from the addition of lysine described before are not found in this experiment since the reactive ε-amino group of lysine is not readily available in the hydrochloride salt. Only the formation of 3-ethyl-2,5-dimethylpyrazine, an interesting flavour compound with a low odour threshold, was in all cases significantly different from the control

Table 5
Amounts of 2-acetyl-1-pyrroline and pyrazines (μg/kg) detected in the headspace of blank and *B. cereus* ATCC 27522 inoculated PCA, supplemented with 2% ornithine hydrochloride (Orn.HCl) or 2% lysine hydrochloride (Lys.HCl) (added prior to medium sterilization)

Sample	Culture/control	Supplement	Pyr[a]	MP	DMP	2-AP	TriMP	EDMP
Hsp 1	Blank	Lys.HCl	68.30 ± 8.53	9.04 ± 9.15	53.43 ± 3.19	n.d.[b]	1.50 ± 2.60	n.d.
	Blank	Orn.HCl	55.82 ± 4.79	18.17 ± 2.02	98.36 ± 121.88	14.92 ± 1.99	n.d.	n.d.
	B. cereus ATCC 27522	Lys.HCl	61.17 ± 31.98	20.72 ± 18.42	68.64 ± 40.02	n.d.	5.19 ± 4.71	56.50 ± 25.87
	B. cereus ATCC 27522	Orn.HCl	50.90 ± 24.13	21.41 ± 20.41	72.80 ± 38.34	14.85 ± 1.61	8.94 ± 9.66	66.08 ± 68.90
Hsp 2	Blank	Lys.HCl	68.14 ± 26.28	12.73 ± 3.48	94.64 ± 19.12	n.d.	2.70 ± 2.53	n.d.
	Blank	Orn.HCl	45.55 ± 8.15	11.25 ± 3.73	69.45 ± 12.35	3.69 ± 1.14	2.90 ± 1.11	n.d.
	B. cereus ATCC 27522	Lys.HCl	62.34 ± 13.49	33.08 ± 7.25	167.69 ± 57.78	n.d.	18.49 ± 4.59	70.52 ± 54.42
	B. cereus ATCC 27522	Orn.HCl	27.84 ± 7.58	16.23 ± 12.42	107.50 ± 96.41	5.14 ± 4.55	17.11 ± 24.76	64.61 ± 40.08
Hsp 3	Blank	Lys.HCl	26.14 ± 3.10	4.29 ± 0.89	63.56 ± 17.12	n.d.	1.98 ± 0.22	n.d.
	Blank	Orn.HCl	18.04 ± 1.84	3.45 ± 1.05	39.08 ± 4.24	0.78 ± 0.68	1.45 ± 0.06	n.d.
	B. cereus ATCC 27522	Lys.HCl	20.05 ± 6.86	14.06 ± 1.77	103.78 ± 41.27	n.d.	5.84 ± 0.85	25.98 ± 19.79
	B. cereus ATCC 27522	Orn.HCl	11.89 ± 7.58	9.83 ± 6.86	82.70 ± 75.04	2.60 ± 2.58	9.60 ± 7.44	28.28 ± 18.02

Results of three consecutive headspace samples, each of one week duration started at day 1 after inoculation ($n = 3$).
[a] Pyr, pyrazine; MP, methylpyrazine; DMP, 2,5-dimethylpyrazine; 2-AP, 2-acetyl-1-pyrroline; TriMP, trimethylpyrazine; EDMP, 3-ethyl-2,5-dimethylpyrazine.
[b] n.d., not detected.

flasks, where this compound was not detected. The formation of 2-acetyl-1-pyrroline was detected in the cultures supplemented with ornithine hydrochloride, both in the bacterial cultures as in the control flasks in comparable amounts, but not in the cultures supplemented with lysine hydrochloride. As compared to the previous experiment with the addition of ornithine hydrochloride after medium sterilisation (Table 4), the amounts of pyrazines found in the control flasks enriched with ornithine hydrochloride before medium sterilisation were considerably higher. Heat treatment of ornithine hydrochloride apparently enhances the formation of pyrazines. The bacterial activity seems to have a catalytic effect that is comparable in effect to the heat treatment of the precursor.

3.4. Influence of starch as a precursor

A similar experiment was conducted to study the influence of starch supplementation (1%) on the flavour production. The yields of methylpyrazine and 2,5-dimethylpyrazine for three consecutive headspace samples are displayed in Table 6. Only traces of the other pyrazines were detected and no 2-acetyl-1-pyrroline was found. The high standard deviations may result from a heterogeneous distribution of the supplemented starch in the medium. No consistent difference in the flavour production by *B. cereus* strain ATCC 27522 and 14737 was found. In later stages of the experiment (after one week), considerable amounts of pyrazines were also detected in the headspace of non-inoculated medium. Therefore, also in this case, an enzymatic pyrazine formation by the bacteria is not probable.

3.5. Influence of 1-pyrroline as a precursor for the production of 2-acetyl-1-pyrroline

As compared to the formation of pyrazines, the biological production of the rice flavour compound 2-acetyl-1-pyrroline **1** is of special interest because of the extraordinary flavour properties of this compound (Demyttenaere et al., 2002). In the preceding experiments, 2-acetyl-1-pyrroline was only detected in trace amounts in some cultures, hampering a detailed study of its production. Previous experiments by Romanczyk et al. (1995) showed an enhancement of the production of 2-acetyl-1-pyrroline by *B. cereus* by the addition of proline, ornithine hydrochloride, glutamic acid, glucose and amylose to the cultivation medium.

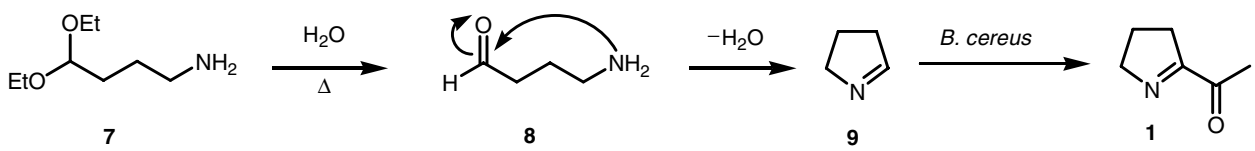
In thermal Maillard reactions, detailed precursor studies have shown that the mechanism of formation of 2-acetyl-1-pyrroline **1** from proline or ornithine proceeds via 1-pyrroline **9** as the key intermediate (Hofmann & Schieberle, 1998). To investigate the influence of this key intermediate, 1-pyrroline, on the bacterial production of 2-acetyl-1-pyrroline by *B. cereus*, the precursor 4-aminobutanal **8** was used in first instance, since 1-pyrroline is difficult to obtain in pure and stable form. 4-Aminobutanal diethyl acetal **7** is a stable form of 4-aminobutanal, from which the free aldehyde can be liberated by hydrolysis (Scheme 1). In boiled or autoclaved aqueous standard solutions of 4-aminobutanal diethyl acetal **7** low amounts of 1-pyrroline **9** were detected (0.5% by GC–MS), which may contribute to the formation of 2-acetyl-1-pyrroline **1** by *B. cereus*.

4-Aminobutanal diethyl acetal was added to the culture medium prior to sterilization in different concentrations, namely 0.1%, 0.2% and 0.4%. Dynamic headspace analysis of the volatiles revealed no significant formation of pyrazines, but 2-acetyl-1-pyrroline was the most important compound in the headspace profile of all cultures, contrary to the previously analysed samples. The amounts of 2-acetyl-1-pyrroline detected in the different headspace samples are displayed in Table 7 and amount up to 23 mg/kg, much higher than the highest amounts of pyrazines detected thus far. At 0.4% of 4-aminobutanal diethyl acetal, an intense browning of the plate count agar was noted after sterilization and bacterial growth was inhibited. The formation of 2-acetyl-1-pyrroline in these cultures should therefore be considered as thermal and not bacterial in origin.

Table 6
Amounts of methylpyrazine and 2,5-dimethylpyrazine (μg/kg) detected in the headspace of blank and *B. cereus* inoculated PCA, supplemented with 1% starch (added after medium sterilization)

Culture/control	Hsp 1		Hsp 2		Hsp 3	
	MP[a]	DMP	MP	DMP	MP	DMP
Blank	0.13 ± 0.25	0.60 ± 0.70	43.00 ± 80.77	17.60 ± 28.71	8.17 ± 7.54	5.26 ± 4.81
B. cereus ATCC 14737	18.74 ± 33.22	35.40 ± 13.39	33.48 ± 31.98	45.44 ± 42.29	1.73	4.20
B. cereus ATCC 27522	0.69 ± 0.26	15.94 ± 8.18	8.93 ± 2.52	92.57 ± 18.43	1.10 ± 0.76	13.94 ± 5.13

Results of the 1st (6-day dynamic headspace started on the 1st day after inoculation), 2nd (7 days started on the 7th day) and 3rd (7 days started on the 14nd day) headspace samples ($n = 3$).
[a] MP, methylpyrazine; DMP, 2,5-dimethylpyrazine.



7 **8** **9** **1**

Scheme 1. Hypothesis of the formation of 2-acetyl-1-pyrroline **1**, catalyzed by *B. cereus* strains, from 4-aminobutanal diethyl acetal **7** as precursor.

Table 7
Amounts of 2-acetyl-1-pyrroline (mg/kg) detected in the headspace of blank and *B. cereus* ATCC 27522 inoculated PCA, supplemented with 0.1%, 0.2% or 0.4% 4-aminobutanal diethyl acetal (added prior to medium sterilization)

Culture/control	Conc. 4-aminobutanal diethyl acetal (%)	Hsp			
		1(5d)	2(6d)	3(7d)	4(7d)
Blank	0.1	1.29	n.d.[a]	n.d.	n.d.
B. cereus ATCC 27522	0.1	6.86	5.88	n.d.	n.d.
B. cereus ATCC 27522	0.2	23.08	16.63	n.d.	n.d.
B. cereus ATCC 27522	0.4	2.94	0.65	0.55	1.63

Results of four consecutive headspace samples of different duration.
[a] n.d., not detected.

From these results, it can be concluded that the addition of 4-aminobutanal diethyl acetal to the plate count agar (prior to sterilization) substantially increased the formation of 2-acetyl-1-pyrroline by the bacterial cultures. The production of 2-acetyl-1-pyrroline increased with higher amounts of the added precursor as long as bacterial growth was not inhibited (at concentrations of 4-aminobutanal diethyl acetal <0.4%). The 2-acetyl-1-pyrroline production was the highest the first days after inoculation and continuously decreased with time. In the control flasks, 2-acetyl-1-pyrroline was only detected in the first headspace sample and in considerably lower amounts than in the bacterial cultures.

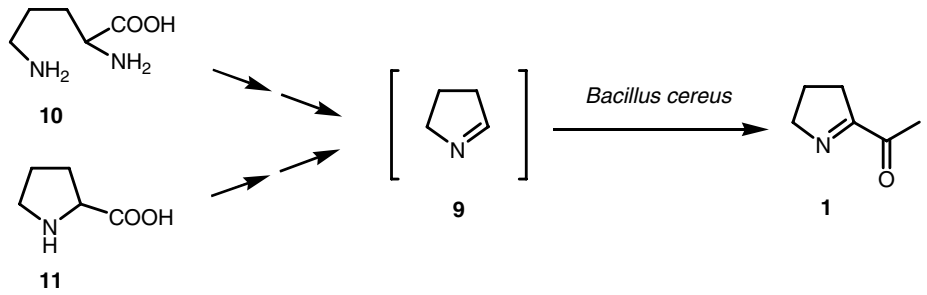
To further investigate the influence of 1-pyrroline as a precursor, the production of 2-acetyl-1-pyrroline by *B. cereus* ATCC 27522 was compared when supplemented with 4-aminobutanal diethyl acetal **7** (0.2%) added before and after medium sterilization, and with 1-pyrroline **9** (0.1%) added after medium sterilization. 1-Pyrroline **9** is an unstable compound since it trimerizes readily. Therefore, it was synthesized from pyrrolidine, distilled and used immediately at a low concentration of 0.1% in the culture medium. After three consecutive headspace samples of five days, the remaining volatiles were analysed by Likens–Nickerson extraction. 2-Acetyl-1-pyrroline was the most important headspace constituent in all the cultures and control flasks. Methylpyrazine and 2,5-dimethylpyrazine were detected in trace amounts, especially in those cultures where the production of 2-acetyl-1-pyrroline was lower. The results of the production of 2-acetyl-1-pyrroline are shown in Table 8. The results of the first headspace samples showed a significant deviation, but the second and the third headspace samples yielded more reproducible amounts of 2-acetyl-1-pyrroline. A significant production of 2-acetyl-1-pyrroline was measured in the *B. cereus* ATCC 27522 cultures supplemented with 4-aminobutanal diethyl acetal prior to medium sterilization and especially in the cultures supplemented with 1-pyrroline. No 2-acetyl-1-pyrroline was formed in the cultures supplemented with 4-aminobutanal diethyl acetal after medium sterilization, except for the first headspace sample where a trace of 2-acetyl-1-pyrroline was detected, comparable with the amounts found in the control flasks. These results indicate that a heat-induced transformation of 4-aminobutanal diethyl acetal is necessary before it can be efficiently converted to 2-acetyl-1-pyrroline by *B. cereus* ATCC 27522. In the control flasks supplemented with 4-aminobutanal diethyl acetal prior to autoclaving 1-pyrroline was detected but only traces of 2-acetyl-1-pyrroline were formed, indicating that bacterial enzymes are essential for the acetylation step. Only those bacterial cultures where 4-aminobutanal diethyl acetal was converted to 1-pyrroline by heat-induced hydrolysis, or where 1-pyrroline was added as such, produced 2-acetyl-1-pyrroline. These findings strongly suggest that the production of 2-acetyl-1-pyrroline occurs via acetylation of 1-pyrroline **9** (which can be deduced from ornithine **10** or proline **11** in common culture media) (Scheme 2). This pathway is similar to the one described for the production of 2-acetyl-1-pyrroline and 6-acetyl-1,2,3,4-tetrahydropyridine by *Lactobacillus* sp. in wine (Costello & Henschke, 2002). Although the actual acetylating C-2 intermediate is not known, a potential candidate is the acetylating cofactor acetyl-CoA.

The results of the final Likens–Nickerson extraction suggest different conclusions and are very irreproducible.

Table 8
Amounts of 2-acetyl-1-pyrroline 1 (μg/kg) detected in the headspace of blank and *B. cereus* ATCC 27522 inoculated PCA. Supplemented with 0.2% 4-aminobutanal diethyl acetal or with 0.1% 1-pyrroline

Culture/control	Supplementation			Hsp 1	Hsp 2	Hsp 3	LN
	Compound	Conc. (%)	Before/after sterilisation				
Blank	4-Aminobutanal diethyl acetal	0.2	Before	2.2 ± 3.2	8.2 ± 11.7	n.d.[a]	50.4 ± 71.3
Blank	4-Aminobutanal diethyl acetal	0.2	After	42.6 ± 19.7	n.d.	n.d.	n.d.
Blank	1-Pyrroline	0.1	After	n.d.	n.d.	n.d.	21.0 ± 8.2
B. cereus ATCC 27522	4-Aminobutanal diethyl acetal	0.2	Before	198.0 ± 280.0	263.6 ± 10.1	239.5 ± 4.7	403.3 ± 570.4
B. cereus ATCC 27522	4-Aminobutanal diethyl acetal	0.2	After	4.2 ± 6.0	n.d.	n.d.	106.2 ± 148.1
B. cereus ATCC 27522	1-Pyrroline	0.1	After	957.7 ± 326.7	513.5 ± 75.6	480.8 ± 131.1	227.1 ± 295.5

Results of three consecutive 5-day headspace samples and of final Likens–Nickerson extraction (LN) ($n = 2$).
[a] n.d., not detected.



Scheme 2. Proposed pathway for the formation of 2-acetyl-1-pyrroline **1** from ornithine **10** and proline **11** via 1-pyrroline **9** by *Bacillus cereus* ATCC 27522 and ATCC 14737.

The heat treatment during this analytical procedure induces additional reactions, such as possibly the hydrolysis of 4-aminobutanal diethyl acetal and the acetylation of 1-pyrroline by Maillard-type reactions. This experiment therefore also shows that the use of simultaneous steam distillation – solvent extraction for the isolation of these Maillard flavour compounds is prone to artefact formation and therefore unreliable.

4. Conclusion

In conclusion, specific *B. cereus* strains grown on plate count agar produce low and mostly irreproducible amounts of 2-acetyl-1-pyrroline. Precursor studies showed that the formation of 2-acetyl-1-pyrroline most probably proceeds via enzymatic acetylation of 1-pyrroline, a metabolic degradation product of the amino acids ornithine and proline. No clear indications for an enzymatic origin of pyrazines were found, except for 3-ethyl-2,5-dimethylpyrazine, an interesting flavour compound produced in low amounts by *B. cereus*. The results of Romanczyk et al. (1995) had demonstrated the formation of 2-acetyl-1-pyrroline by *B. cereus*, supported by various control experiments and labelling studies, but using a thermal treatment during extraction. Further research of this biotransformation indicated the necessity of a non-thermal extraction method to obtain reliable results and revealed the importance of 1-pyrroline as a key intermediate in the formation of 2-acetyl-1-pyrroline by *B. cereus*. This confirms once more the importance of 1-pyrroline in the formation of the flavour compound 2-acetyl-1-pyrroline, in thermal as well as in biological reactions.

Acknowledgements

The authors are indebted to the Research Foundation – Flanders (Belgium) (F.W.O. Vlaanderen) for financial support and for a postdoctoral fellowship of An Adams.

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