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RESEARCH ARTICLE

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Aroma active alkylated pyrazines are produced by *Basfia succiniciproducens* as by-products of succinic acid production

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Abstract

Culture supernatants of *Basfia succiniciproducens* derived from the industrial production of succinic acid exhibit an intense nutty, root vegetable-like, buttery and sourish smell. By means of headspace-gas chromatography-mass spectrometry-olfactometry (HS-GC-MS-O), 14 odour-active compounds were perceived and identified using two GC-columns of different polarity and comparison of retention indices and mass spectra to those of authentic reference compounds. Several alkylated pyrazines, including 2,3,5-trimethylpyrazine, 5-ethyl-2,3-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethyl-6-propylpyrazine and 2,3,5-trimethyl-6-butylpyrazine were found to contribute to the aroma of the culture supernatant. Quantitation of the pyrazines was performed by means of dynamic headspace-GC-MS after standard addition, and 2,3,5,6-tetramethylpyrazine (9.10 mg/L) was the most abundant compound. Waste streams of the biotechnological production of commodity chemicals may thus represent a sustainable resource for the isolation of aroma compounds.

KEYWORDS
alkylated pyrazines, dynamic headspace extraction, flavour, side stream, sustainability

1 | INTRODUCTION

Major advantages of the use of microorganisms for the industrial production of commodity chemicals include the mild reaction conditions, cheap and non-fossil fuel based reactants and the formation of the desired chirality.[1] Currently, succinic acid is biotechnologically produced with an annual production of several tens of thousands of tonnes, and the market is expected to further grow strongly.[2,3] The fermentative production of succinic acid is carried out with, *for example*, *Escherichia coli*, *Corynebacterium glutamicum* and *Basfia succiniciproducens*, using cheap, non-petroleum based and readily available raw materials, *for example*, glycerol (derived from the production of bio fuels) or glucose.[4-7] Succinic acid naturally occurs as an intermediate metabolite of the citric acid cycle. It is commercially used in food as acidifier and taste modifier. Due to its bifunctionality, it also represents a potential starting material for biodegradable polymers and many other C4 compounds.[2,7] *B succiniciproducens* is a Gram-negative bacterium which was first isolated in 2008 by Scholten and Dägele from bovine rumen.[4]

Abbreviations: CIS, cooled injection system; **DHS**, dynamic headspace; **GC**, gas chromatograph(y); **MS**, mass spectrometry; **NMR**, nuclear magnetic resonance; **O**, olfactometry; **RI**, retention index; **S/SL**, split/splitless; **SIM**, selected ion monitoring; **SPME**, solid phase microextraction; **TDU**, thermal desorption unit.

Surprisingly, industrial fermentation broths of *B succiniciproducens* from succinic acid production were observed to emit an intense, nutty, buttery, sourish and root vegetable-like smell. This odour could be attributed, amongst other compounds, mainly to alkylated pyrazines.

Alkylated pyrazines are broadly found in nature, for example, in bacteria, plants or insects but typically in low concentrations.[8] Some representatives are formed during preparation of food by, *for example*, deep frying or roasting from reducing sugars and amino acids via the Maillard reaction.[9] In thermally processed food, they contribute to their typical roasty and spicy odour properties.[10,11] In some of today's common food preparation methods, such as microwave cooking, these compounds are not formed due to the relatively low temperatures, so the demand for natural flavours with roasty odour properties is correspondingly high.[1] Some alkylated pyrazines show very low odour thresholds (<0.01 ng/L air).[10] Actually, 37 alkylated pyrazines are listed on the FEMA GRAS list.[12] Due to these characteristics, they are highly sought-after by the food industry and natural flavour compounds are preferred. They may be extracted from natural sources such as potatoes, coffee and nuts, but alkylated pyrazines do typically occur in trace amounts only in natural sources. Accordingly, the prices for natural pyrazines are high. For example, 1 kg of synthetically produced 2,5-dimethylpyrazine costs ~200 US$, while its natural counterpart is currently sold for ~3,500 US$/kg.[13]

Because of the immense amounts of fermentation broth resulting from the industrial succinic acid fermentation process, the extraction of valuable natural flavour compounds from this broth might be a promising idea. For the first time, an aroma analysis of a fermentation broth of this microorganism was carried out, whereby compounds of interest were subsequently quantitated.

2 | EXPERIMENTAL

2.1 | Culture broths

Culture supernatants of *B succiniciproducens*, deposited under the Budapest Treaty with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) under the deposit number DSM 18541, derived from the biotechnological succinate production, were provided by BASF SE (Ludwigshafen, Germany). Three batches were provided, and all analyses were performed with a representative sample. The microorganism was cultivated in a medium containing 50 g/L glycerol and 10 g/L glucose as carbon source, 5 g/L $(NH_4)_2SO_4$, 2 g/L Na_2CO_3, 1 g/L KH_2PO_4, vitamins, trace metals and osmolytes. The detailed cultivation conditions and medium composition have been described in the patent literature.[14] Besides the culture supernatant, the autoclaved non-inoculated medium was provided as a control.

2.2 | Chemicals

Petroleum ether (40-60°C; 95%) was purchased from Acros Organics (Geel, Belgium). 2,3-Hexanedione (94%) and 2,4,5-trimethyl oxazole (97%) were obtained from Alfa Aesar (Karlsruhe, Germany). Benzaldehyde (99%) and hydrogen peroxide (30%) were obtained from AppliChem (Darmstadt, Germany). Acetic acid (100%), chloroform-d (99.8 atom% D, with 0.03 vol% TMS, stabilized with Ag), diethyl ether (99.5%), iron(II) sulphate heptahydrate (99.5%), sodium carbonate decahydrate (98%), sodium sulphate (p.a.) and sulfuric acid (96%) were purchased from Carl Roth (Karlsruhe, Germany). Pentanal (97%) was obtained from Fisher Scientific (Darmstadt, Germany), Silica gel 60 was purchased from Macherey-Nagel (Düren, Germany) and 2,3-butanedione (97%) from Merck (Darmstadt, Germany). Acetoin (96%), 2,3-pentanedione (97%), 2,3,5,6-tetramethylpyrazine (98%) and 2,3,5-trimethylpyrazine (99%) were purchased from Sigma-Aldrich (Taufkirchen, Germany). 5-Ethyl-2,3-dimethylpyrazine (98%) was obtained from TCI (Eschborn, Germany). Butanal (97%) and propanal (97%) were purchased from Th. Geyer (Renningen, Germany) and ethyl acetate (98%) from VWR (Darmstadt, Germany). Helium (5.0) was obtained from Praxair (Düsseldorf, Germany) and nitrogen (5.0) from Air Liquide (Düsseldorf, Germany). All numbers given in parentheses represent the minimum purity.

2.3 | Methods

2.3.1 | Identification of odour-active compounds

The determination of odour-active compounds was performed by means of headspace-solid phase microextraction-gas chromatography-mass spectrometry-olfactometry (HS-SPME-GC-MS-O). For HS-SPME, 5 mL samples were added to a 20 mL-HS-vial. The samples were incubated and extracted on an agitator (15 min, 60°C, 250 rpm) of a GERSTEL MPS 2XL autosampler (Mülheim an der Ruhr, Germany), followed by an extraction with an SPME fibre for 30 min. The SPME fibre (Supelco, Steinheim, Germany) was coated with divinylbenzene/carboxen/polydimethylsiloxane (1 cm × 50/30 μm). The analytes were desorbed in the GC's inlet (250°C, splitless time: 1 min). The GC system used was an Agilent (Waldbronn, Germany) 7890A gas chromatograph equipped with either an Agilent VF-WAXms column (30 m × 0.25 mm, 0.25 μm film thickness; temperature programme: 40°C (3 min), 5°C/min to 240°C (12 min), carrier gas: helium, 1.56 mL/min (constant)) or an Agilent DB-5ms (deviating final temperature 300°C) and a split/splitless (S/SL) inlet. After the column, the carrier gas was split 1:1 by a GERSTEL μFlowManager splitter to an Agilent 7000B triple quadrupole detector (ionisation energy: 70 eV, ion source: 230°C, quadrupoles: 150°C, transfer line: 250°C, scan in q1: *m/z* 33-300, He quench gas: 2.25 mL/min, N_2 collision gas: 1.5 mL/min) and a GERSTEL ODP3 olfactory detection port (transfer line: 250°C, mixing chamber: 150°C, make up gas: N_2). Olfactometry was performed by three trained panelists. Compounds were considered odour-active if at least two panelists perceived and described the odour. The provided non-inoculated medium was analysed in the same way. The retention indices (RI) were calculated by linear interpolation from the retention times of *n*-alkanes (C_7-C_{30}).[15] The

odour-active compounds were identified by their mass spectra, their RIs on two columns of different polarities, their odour, and by comparison with authentic standards (commercially available or synthesized).

2.3.2 | Synthesis of 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethyl-6-propylpyrazine and 2,3,5-trimethyl-6-butylpyrazine

The pyrazines were synthesized (Figure 1) based on a method described by Bohman *et al*.[16] One eq. 2,3,5-trimethylpyrazine and 0.4 eq. $FeSO_4$ x $7H_2O$ were solved in water and 4 eq. propanal, butanal or pentanal were added, respectively. At 0°C 50 eq. conc. sulfuric acid and 2.2 eq. H_2O_2 (30%) were added, and the solution was stirred for 3 h at room temperature. After 1 h and after 2 h 1.1 eq. H_2O_2 (30%) and 2 eq. of the respective aldehyde were added. Subsequently, the reaction mixture was washed with diethyl ether, the pH of the aqueous solution was adjusted to 8 with Na_2CO_3, and the mixture was extracted twice with diethyl ether. The combined organic extracts were dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The residue was purified by means of column chromatography with SiO_2 using ethyl acetate and petroleum ether (1:1) as eluent. The structures of the isolated compounds were confirmed by means of nuclear magnetic resonance (NMR). The NMR-experiments were performed on a Bruker (Rheinstetten, Germany) Avance II 400 MHz and a Bruker Avance III HD 400 MHz.

NMR data of 2,3,5-trimethyl-6-ethylpyrazine

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.77 (2 H, q), 2.50 (3 H, s), 2.48 (3 H, s), 2.47 (3 H, s), 1.27 (3 H, t). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 153.0, 148.5, 148.2, 147.6, 28.0, 21.6, 21.5, 21.0, 13.0.

NMR data of 2,3,5-trimethyl-6-propylpyrazine

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.72 (2 H, m), 2.50 (3 H, s), 2.47 (3 H, s), 2.47 (3 H, s), 1.71 (2 H, m), 1.00 (3 H, t). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 151.9, 148.4, 148.1, 147.7, 36.6, 22.4, 22.2, 14.1, 14.1.

NMR data of 2,3,5-trimethyl-6-butylpyrazine

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.73 (2 H, m), 2.49 (3 H, s), 2.47 (3 H, s), 2.46 (3 H, s), 1.63 (2 H, m), 1.27 (2 H, m), 0.95 (3 H, t). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 152.2, 148.5, 148.2, 147.8, 34.6, 31.2, 22.9, 21.6, 21.5, 21.1, 14.1.

These data are in accordance with those described in the literature.[17] The NMR spectra can be found in the Figure SA1-SC2.

2.3.3 | Quantitation of alkylated pyrazines

Five alkylated pyrazines were quantitated by means of standard addition and dynamic headspace (DHS)-GC-MS (Table 3). 2,3,5-Trimethyl-6-butylpyrazine could not be quantitated due to its very low concentration in the culture broth. For quantitation, each standard compound was dissolved separately in water, a mixed stock solution was prepared and five standard solutions with differing concentrations were prepared. 100 μL of the standard solutions were added to 1 mL sample, each. Subsequently, 550 μL of these mixtures were extracted at room temperature (22 $\pm$ 1°C) by means of DHS with 3 L nitrogen and a flow rate of 100 mL/min. The analytes in the effluent were trapped on a TDU tube, filled with Tenax TA (GERSTEL). After DHS extraction, the Tenax tube was dried with 1 L nitrogen (flow rate 100 mL/min). Subsequently, the analytes were desorbed from the Tenax tube by means of a GERSTEL thermal desorption unit (TDU) (temperature programme: 30°C (0.5 min), 100°C/min to 240°C (3 min), transferline temperature: 250°C (fixed), split ratio: splitless, septum purge: 3 mL/min) coupled with a GERSTEL cooled injection system 4 (CIS 4) (temperature programme: 10°C (0.5 min), 12°C/s to 240°C (7 min), CIS glass liner packed with Tenax TA, split ratio: 10:1) and analysed by GC-MS. The GC system used was an Agilent 7890B gas chromatograph equipped with an Agilent VF-WAXms column (*cf*. 2.3.1; carrier gas: helium, 1.2 mL/min (constant)). The MS (Agilent 5877B quadrupole detector, ionisation energy: 70 eV, ion source: 230°C, quadrupole: 150°C, transferline: 250°C) was operated in selected ion monitoring-mode (SIM) (Table 1).

3 | RESULTS AND DISCUSSION

3.1 | Identification of odour-active compounds

The odour of the fermentation broths was perceived as sourish, buttery, nutty and root vegetable-like. By means of HS-SPME-GC-MS-O, 14 odour-active compounds were perceived and identified (Table 2,

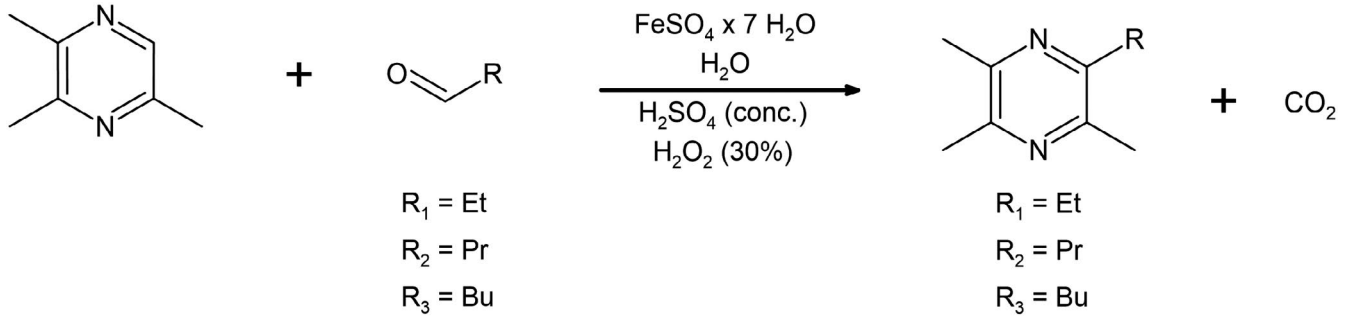

FIGURE 1 Synthesis of alkylated pyrazines *via* Minisci reaction (referring to Bohman *et al*)

TABLE 1 Quantifier ions and qualifiers ions of analytes used for quantitation and starting time of measurement in mass spectrometer

Starting time [min]	*m/z* quantifier ion	*m/z* qualifier ions
14.00	122	42, 81
15.60	135	54, 136, 137
15.60	136	54, 135, 137
17.00	149	122, 150
18.50	136	149, 164

Figure 2). The odour impressions perceived at the olfactory detector port well mirrored the overall impressions of the fermentation broths. Short chain 2,3-diketo compounds, *for example*, 2,3-butanedione and 2,3-pentanedione, are known for their buttery odour.[18] 3-Hydroxy-2-keto compounds (acyloins) are described in the literature as caramel-like, sweetish, buttery (3-hydroxy-2-pentanone) and earthy, mushroom-like (3-hydroxy-2-hexanone).[19] 2,4,5-Trimethyl oxazole is known for its fresh, mustard like[20] and acetic acid for its sourish, vinegar-like odour.[21] Alkylated pyrazines are aroma compounds which are typically formed by thermal food processing, *for example*, by roasting or frying, and exhibit an intense roasty, nutty or root vegetable-like odour.[10,22-24] The biosynthetic production of pyrazines has been described for some bacteria, *for example*, *Pseudomonas* and *Paenibacillus*.[25,26] In addition to their properties as potent aroma compounds, pyrazines are also known for their antimicrobial effects, which may explain their biosynthesis by bacteria to protect themselves from competitors.[27,28]

Diketo compounds, such as diacetyl, are constituents of many dairy products.[29] Diacetyl is produced by many different bacteria, *for example*, *Lactococcus lactis* or *Streptococcus diacetilactis*.[30,31] It is formed from 2-acetolactate, which is an intermediate in the biosynthesis of amino acids, such as valine, leucine, isoleucine, or aspartate.[32,33]

The biosynthesis of valine, leucine and isoleucine starts with the transfer of pyruvate to thiamine pyrophosphate under decarboxylation. In the case of leucine and valine, the resulting acetyl group is transferred to another pyruvate molecule, resulting in 2-acetolactate. In the biosynthesis of isoleucine, the acetyl group is transferred to 2-oxobutanoate instead of pyruvate, resulting in 2-aceto-2-hydroxybutanoate. The next step on the way to the amino acid is catalysed by a ketol-acid reductoisomerase. Dickschat *et al* proposed a pathway for the formation of acyloins in *C glutamicum*.[17] In this proposed pathway, acyloins are formed from 2-acetolactate and 2-aceto-2-hydroxybutanoate by decarboxylation resulting in acetoin or the respective elongated 3-hydroxy-2-keto compounds. By oxidation of the hydroxy group, 2,3-diketo compounds, *for example*, diacetyl, can be formed. A mutant of *C glutamicum* with deleted ketol-acid reductoisomerase activity showed a strongly increased formation of acyloins and 2,3-diketo compounds.[17] The production of acetoin by *C glutamicum* was already observed previously investigating a strain that was unable to form valine, leucine and isoleucine.[34] Furthermore, in both studies not only acyloins and 2,3-keto compounds were found but surprisingly also different alkylated pyrazines. Demain *et al* identified 2,3,5,6-tetramethylpyrazine in the fermentation broth of *C glutamicum*.[34] Dickschat *et al* additionally identified 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethyl-6-prop

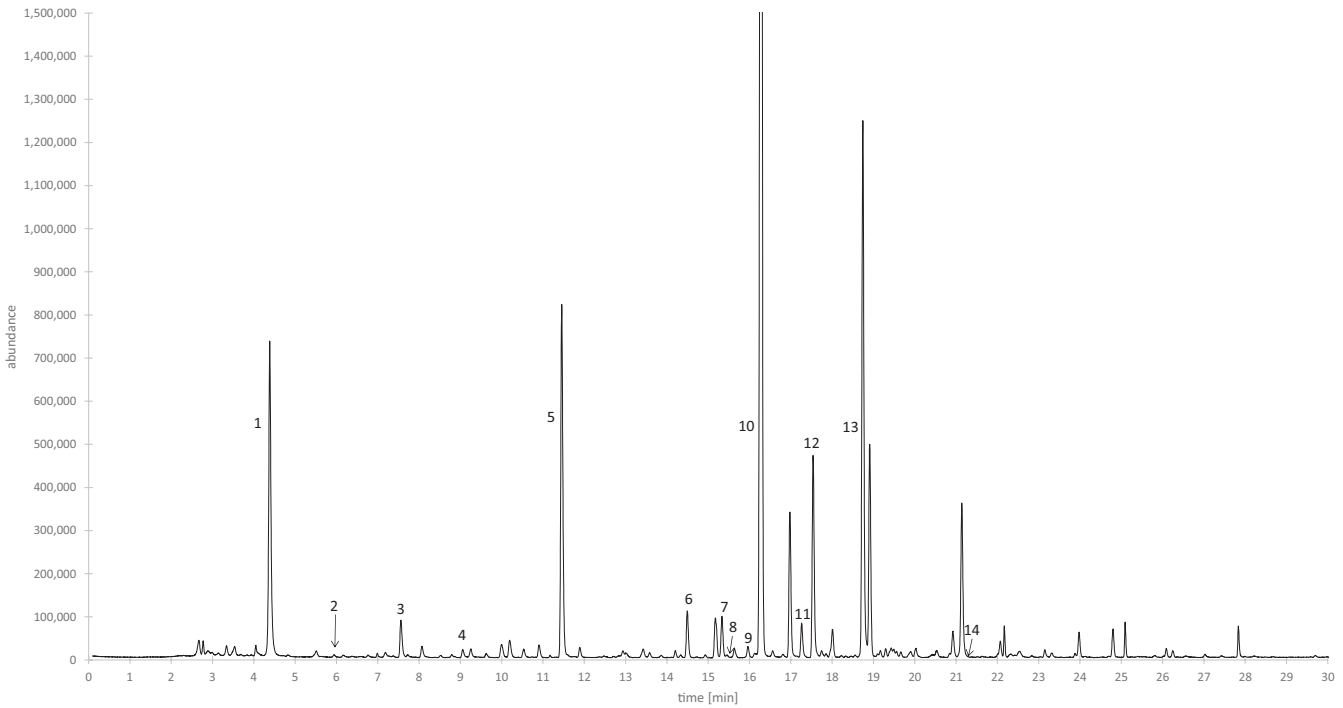

FIGURE 2 HS-SPME-GC-MS chromatogram (determined on a VF-WAXms column) of the analysed fermentation broth of *B succiniciproducens* with perceived and identified odour-active compounds on VF-WAXms column

TABLE 2 Perceived and identified odour-active compounds from the fermentation broth of *B succiniciproducens* analysed by means of HS-SPME-GC-MS-O with their corresponding retention indices (RI) on two columns of different polarity

			RI (VF-WAXms)		RI (DB-5ms)	
#	Compound	Odour impression	Sample	Standard	Sample	Standard
1	2,3-butanedione (diacetyl)	intense buttery	973	977	<700	<700
2	2,3-pentanedione	buttery, fresh	1054	1057	<700	<700
3	2,3-hexanedione	buttery, fruity, sweetish	1131	1131	791	793
4	2,4,5-trimethyl oxazole	green, fresh	1192	1196	833	841
5	3-hydroxybutan-2-one (acetoin)	buttery, green	1285	1284	712	711
6	2,3,5-trimethylpyrazine	nutty, musty	1400	1400	1002	1002
7	3-hydroxyhexan-2-one	roasty, earthy	1438	1434	892	890
8	acetic acid	vinegar	1443	1452	<700	<700
9	5-ethyl-2,3-dimethylpyrazine	cooked potatoes	1458	1458	1088	1087
10	2,3,5,6-tetramethylpyrazine	sweetish, nutty, musty	1470	1469	1085	1085
11	2,3,5-trimethyl-6-ethylpyrazine	spicy, vegetable stock like	1509	1509	1157	1156
12	benzaldehyde	bitter almond	1521	1521	964	964
13	2,3,5-trimethyl-6-propylpyrazine	sweetish, vegetable stock like	1577	1577	1237	1237
14	2,3,5-trimethyl-6-butylpyrazine	nutty, vegetable stock like	1674	1677	1330	1329

ylpyrazine and 2,3,5-trimethyl-6-butylpyrazine.[17] In the proposed pathway by Dickschat *et al*, the alkylated pyrazines are formed from acyloins or 2,3-diketo compounds. The first step is a transamination of these compounds, followed by a condensation reaction of two molecules and oxidation to give the final alkylated pyrazines (Figure 3). However, no quantitative data on the formation of alkylated pyrazines have been reported in this study.

Because of the similarities of the product patterns, it seems likely that *B succiniciproducens* might use analogous pathways to form acyloins, 2,3-diketo compounds and alkylated pyrazines. Therefore, it might be possible to delete the ketol-acid reductoisomerase activity, as described for *C glutamicum*, and to add the required amino acids to the fermentation medium to further increase the production of alkylated pyrazines.

Another *N*-containing heterocyclic compound identified in the samples was 2,4,5-trimethyl oxazole. It is known to be formed from a Schiff base (possibly derived from acyloins or 2,3-diketo compounds) in a condensation reaction with acetic acid.[17,35] Acetic acid was also perceived olfactorily during the analysis by means of GC-MS-O.

All of the described compounds, except for acetoin and acetic acid,[3] have not yet been described previously to be formed by *B succiniciproducens*.

Alkylated pyrazines are known for their intense odour impressions, and some representatives exhibit very low odour thresholds. By investigating the structure-odour-activity of more than 80 pyrazines, Wagner *et al* showed that trifunctionalized pyrazine stereoisomers bearing two methyl groups in *ortho* positions to one nitrogen atom show the lowest odour thresholds compared to isomers bearing two methyl groups in *ortho* and *meta* position.[10] All of the tetra-alkylated pyrazines analysed in this study bear two methyl groups in *ortho* positions. Therefore, it might be possible that the tetra-alkylated pyrazines detected in the fermentation broths of *B succiniciproducens* also show very low odour thresholds. However, the effect of the substitution pattern of tetrafunctionalized pyrazines on the respective odour threshold needs to be investigated to support this assumption.

Due to their desirable odour and low odour thresholds, alkylated pyrazines are important aroma compounds. Nevertheless, they are rarely found in higher amounts in nature and an economical extraction is thus difficult to achieve. 2,3,5-Trimethyl-6-butylpyrazine was only described a few times, *for example*, to be formed by *C glutamicum*,[17] in roasted cocoa,[36] or as a product of the Maillard reaction from wheat protein.[9] 2,3,5-Trimethyl-6-propylpyrazine occurs more often in natural sources. *For example*, it was described to be formed by *Pseudomonas* spp.,[25] in fermented soybean paste,[37] or as an aroma compound of gouda cheese.[22] 2,3,5-Trimethyl-6-ethylpyrazine has also been described as a Maillard product[38] and as an aroma compound found in chocolate.[39] The biosynthetic nature of the formed alkylated pyrazines was clearly shown, as no odour-active alkylated pyrazines could be detected in the non-inoculated media analysed as controls (cf. Figure SD).

3.2 | Quantitation of compounds of interest

Five alkylated pyrazines were quantitated by means of standard addition and DHS-GC-MS (Table 3, Figures SE1 and SE2). Amongst the analysed pyrazines, 2,3,5,6-tetramethylpyrazine showed by

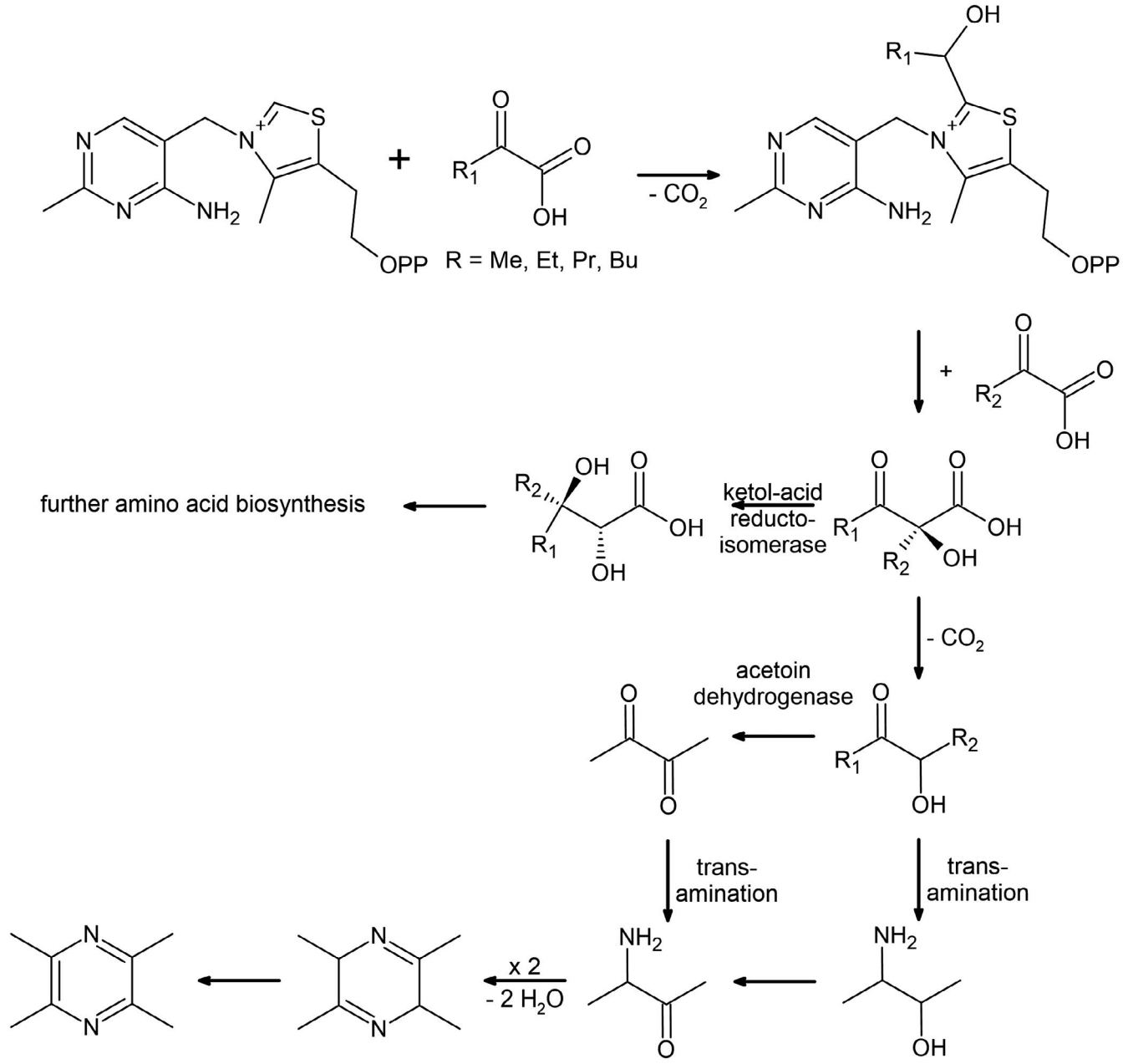

FIGURE 3 Proposed pathway for the formation of alkylated pyrazines according to Dickschat *et al* (here shown for 2,3,5,6-tetramethylpyrazine with $R_1 = R_2 = Me$)

TABLE 3 Quantitated amounts of the five compounds of interest by means of standard addition and DHS-GC-MS

Compound	Amount [μg/L]	R^2
2,3,5-trimethylpyrazine	179	0.998
5-ethyl-2,3-dimethylpyrazine	15	0.999
2,3,5,6-tetramethylpyrazine	9104	0.991
2,3,5-trimethyl-6-ethylpyrazine	36	0.999
2,3,5-trimethyl-6-propylpyrazine	233	0.993

far the highest concentration. The concentrations of the other alkylated pyrazines were much lower. Although the amounts of alkylated pyrazines were far away from other industrial processes for the fermentative production of odour-active compounds, the isolation from the fermentation broth could be achieved by concentration and distillation at reduced pressure or alternatively after acidification by cation exchangers.[14] Currently, natural alkylated pyrazines are typically extracted from food, such as potatoes, coffee or nuts.[13] To the best of our knowledge, there is no industrial production for these alkylated pyrazines based on fermentation. The succinic acid production process has actually neither been designed nor optimized for the production of alkylated pyrazines. Much higher amounts might thus be obtained, for example, by optimization of the fermentation medium or genetic optimization of the *B succiniciproducens* strain.

4 | CONCLUSION

A novel biotechnological access to alkylated pyrazines from culture supernatants of *B succiniciproducens* was investigated. The substrate used for the fermentation process is inexpensive glycerol, which is released in large quantities during the production of biofuels. Isolation of the flavour compounds from culture supernatants, a designated waste product produced during a large-scale process, may allow for a sustainable production of aroma compounds which do not occur in large quantities in nature. Alkylated pyrazines might be separated from the fermentation broth by distillation or cation exchangers. Depending on the cost of downstream processing, it might be economical to monetise the waste stream directly. Alternatively, the strain could be optimised for aroma production, *for example*, by deleting the ketol-acid reductoisomerase activity, or the amino acids needed as precursors could be added to the fermentation medium.

DECLARATION OF INTEREST

A patent application (Pelzer, R., Zorn, H., Birk, F., & Fraatz, M. A. (2019). Fermentative Production of Pyrazines Using Microorganisms of the Genus Pasteurellaceae (EP3680339A1)) has been filed by BASF SE. Ralf Pelzer is an employee of BASF SE. Apart from that, the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Singh P, Pandey A. *Biotechnology for Agro-Industrial Residues Utilisation: Utilisation of Agro-Residues*. Dordrecht, Netherlands: Springer; 2009.
2. Bechthold I, Bretz K, Kabasci S, Kopitzky R, Springer A. Succinic acid: A new platform chemical for biobased polymers from renewable resources. *Chem Eng Technol*. 2008;31(5):647-654.
3. Lange A, Becker J, Schulze D, et al. Bio-based succinate from sucrose: High-resolution 13C metabolic flux analysis and metabolic engineering of the rumen bacterium *Basfia succiniciproducens*. *Metab Eng*. 2017;44:198-212.
4. Scholten E, Dägele D. Succinic acid production by a newly isolated bacterium. *Biotechnol Lett*. 2008;30(12):2143-2146.
5. Scholten E, Renz T, Thomas J. Continuous cultivation approach for fermentative succinic acid production from crude glycerol by *Basfia succiniciproducens* DD1. *Biotechnol Lett*. 2009;31(12):1947-1951.
6. Sánchez AM, Bennett GN, San K-Y. Novel pathway engineering design of the anaerobic central metabolic pathway in Escherichia coli to increase succinate yield and productivity. *Metab Eng*. 2005;7(3):229-239.
7. McKinlay JB, Vieille C, Zeikus JG. Prospects for a bio-based succinate industry. *Appl Microbiol Biot*. 2007;76(4):727-740.
8. Rizzi GP. The biogenesis of food-related Pyrazines. *Food Rev Int*. 1988;4(3):375-400.
9. Lee SE, Chung H, Kim Y-S. Effects of enzymatic modification of wheat protein on the formation of pyrazines and other volatile components in the Maillard reaction. *Food Chem*. 2012;131(4):1248-1254.
10. Wagner R, Czerny M, Bielohradsky J, Grosch W. Structure-odour-activity relationships of alkylpyrazines. *Z Lebensm Unters F A*. 1999;208(5–6):308-316.
11. Rowe DJ. Aroma chemicals for savory flavors. *Perfum Flavor*. 1998;23(4):9-14, 16.
12. Flavor and Extract Manufacturers Association. Entry for pyrazine. Accessed February 21, 2021. https://www.femaflavor.org/flavor-library/search?fulltext=pyrazine
13. Mortzfeld FB, Hashem C, Vranková K, Winkler M, Rudroff F. Pyrazines: synthesis and industrial application of these valuable flavor and fragrance compounds. *Biotech J*. 2020;15(11):2000064.
14. Pelzer R, Zorn H, Birk F, Fraatz MA; BASF SE. Fermentative Production of Pyrazines Using Microorganisms of the Genus Pasteurellaceae. EP3680339A1. January 11, 2019.
15. Van den Dool H, Kratz PD. A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. *J Chromatogr A*. 1963;11:463-471.
16. Bohman B, Berntsson B, Dixon RCM, Stewart CD, Barrow RA. Alkylations and hydroxymethylations of pyrazines via green Minisci-type reactions. *Org Lett*. 2014;16(11):2787-2789.
17. Dickschat JS, Wickel S, Bolten CJ, Nawrath T, Schulz S, Wittmann C. Pyrazine biosynthesis in *Corynebacterium glutamicum*. *Eur J Org Chem*. 2010;13(14):2687-2695.
18. Schmidberger PC, Schieberle P. Characterization of the key aroma compounds in white alba truffle (*Tuber magnatum* pico) and burgundy truffle (*Tuber uncinatum*) by means of the sensomics approach. *J Agr Food Chem*. 2017;65(42):9287-9296.
19. Neuser F, Zorn H, Berger RG. Generation of odorous acyloins by yeast pyruvate decarboxylases and their occurrence in sherry and soy sauce. *J Agr Food Chem*. 2000;48(12):6191-6195.
20. The Good Scents Company. Entry for 2,4,5-trimethyl oxazole. Accessed February 22, 2021. https://www.thegoodscentscompany.com/data/rw1051411.html
21. Blank I, Schieberle P. Analysis of the seasoning-like flavour substances of a commercial lovage extract (Levisticum officinale Koch.). *Flavour Frag J*. 1993;8(4):191-195.
22. Jo Y, Benoist DM, Ameerally A, Drake MA. Sensory and chemical properties of Gouda cheese. *J Dairy Sci*. 2018;101(3):1967-1989.
23. Solina M, Baumgartner P, Johnson RL, Whitfield FB. Volatile aroma components of soy protein isolate and acid-hydrolysed vegetable protein. *Food Chem*. 2005;90(4):861-873.
24. Winter M, Gautschi F, Flament I; Firmenich & Cie. Verfahren zur geschmacksverändernden Behandlung von Nahrungsmitteln und Getränken. Germany DE1695505A1. April 30, 1965.

25. Bañeras L, Trias R, Godayol A, et al. Mass spectrometry identification of alkyl-substituted pyrazines produced by *Pseudomonas* spp. isolates obtained from wine corks. *Food Chem*. 2013;138(4):2382-2389.
26. Rybakova D, Cernava T, Köberl M, Liebminger S, Etemadi M, Berg G. Endophytes-assisted biocontrol: Novel insights in ecology and the mode of action of Paenibacillus. *Plant Soil*. 2016;405(1–2):125-140.
27. Kusstatscher P, Cernava T, Liebminger S, Berg G. Replacing conventional decontamination of hatching eggs with a natural defense strategy based on antimicrobial, volatile pyrazines. *Sci Rep*. 2017;7(1):1-8.
28. Janssens TKS, Tyc O, Besselink H, de Boer W, Garbeva P. Biological activities associated with the volatile compound 2,5-bis(1-methylethyl)-pyrazine. *FEMS Microbiol Lett*. 2019;366(3):1-10.
29. Calbert HE, Price WV. A study of the diacetyl in cheese. *I. diacetyl* content and flavor of cheddar cheese. *J Dairy Sci*. 1949;32(6):515-520.
30. Zuljan FA, Repizo GD, Alarcon SH, Magni C. alpha-Acetolactate synthase of *Lactococcus lactis* contributes to pH homeostasis in acid stress conditions. *Int J Food Microbiol*. 2014;188:99-107.
31. Speckman RA, Collins EB. Diacetyl biosynthesis in *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. *J Bacteriol*. 1968;95(1):174-180.
32. Le Bars D, Yvon M. Formation of diacetyl and acetoin by *Lactococcus lactis* via aspartate catabolism. *J Appl Microbiol*. 2007;104(1):171-177.
33. Yvon M, Rijnen L. Cheese flavour formation by amino acid catabolism. *Int Dairy J*. 2001;11(4–7):185-201.
34. Demain AL, Jackson M, Trenner NR. Thiamine-dependent accumulation of tetramethylpyrazine accompanying a mutation in the isoleucine-valine pathway. *J Bacteriol*. 1967;94(2):323-326.
35. Hwang H-I, Hartman TG, Ho C-T. Relative reactivities of amino acids in the formation of pyridines, pyrroles, and oxazoles. *J Agr Food Chem*. 1995;43(11):2917-2921.
36. Carlin JT, Lee KN, Hsieh OAL, Hwang LS, Ho CT, Chang SS. Comparison of acidic and basic volatile compounds of cocoa butters from roasted and unroasted cocoa beans. *J Am Oil Chem Soc*. 1986;63(8):1031-1036.
37. Jo Y-J, Cho IH, Song CK, Shin HW, Kim Y-S. Comparison of fermented soybean paste (Doenjang) prepared by different methods based on profiling of volatile compounds. *J Food Sci*. 2011;76(3):C368-C379.
38. Scalone GLL, Cucu T, de Kimpe N, de Meulenaer B. Influence of free amino acids, oligopeptides, and polypeptides on the formation of pyrazines in maillard model systems. *J Agr Food Chem*. 2015;63(22):5364-5372.
39. Batista NN, Ramos CL, Dias DR, Pinheiro ACM, Schwan RF. The impact of yeast starter cultures on the microbial communities and volatile compounds in cocoa fermentation and the resulting sensory attributes of chocolate. *J Food Sci Tech*. 2016;53(2):1101-1110.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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