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Metabolite production and kinetics of branched-chain aldehyde oxidation in *Staphylococcus xylosus*

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Abstract

The metabolite production of the gram positive bacterium *Staphylococcus xylosus* when cultivated in a defined medium containing 18 amino acids, 6 vitamins and 2 purines was characterised. Several compounds not previously reported as metabolites of this organism were identified including 2,5-methylpyrazine, 2-phenylethylacetate, 2-methyltetrahydrothiophen-3-one, 3-(methylthio)-propanoic acid and 3-(methylthio)-propanal. The organoleptic metabolites derived from branched-chain amino acid catabolism; 2-methylpropanal from valine, 2-methylbutanal from isoleucine and 3-methylbutanal from leucine were detected at levels ranging from 0.4 to 2.0 μM. The concentrations of the corresponding carboxy acids were 963, 858 and 1486 μM respectively. We demonstrated that α-ketoisocaproic acid was biotransformed to 3-methylbutanal which immediately was oxidised into 3-methylbutanoic acid. Kinetic studies of the conversion of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal into their corresponding acids were also performed and we found K_m values of 0.8, 0.9 and 1.6 μM, and V_{max} values of 9.4, 7.1 and 5.9 μmol/min/10^{12} CFU, respectively.

Keywords: *Staphylococcus xylosus*; 3-Methylbutanal; Kinetics; Branched-chain aldehyde oxidation; Metabolites; Membrane inlet mass spectrometry

1. Introduction

S. xylosus and *S. carnosus*, members of the gram positive Micrococcaceae family, are widely used in combination with lactic acid bacteria as starter cultures in the production of dry fermented sausages. The lactic acid bacteria conserve the product by lowering the pH through lactic acid production, and nitrate reductase of the Micrococcaceae acts on the colour development of the sausage. It is known that staphylococci contribute to the overall flavour of the fermented end product through their lipolytic [1,2] and proteolytic [3] activity. Numerous flavour compounds have been identified in sausages fermented with *S. xylosus* [3–8]. Of these flavour compounds special attention has been paid to the formation of the branched-chain aldehyde, 3-methylbutanal, that improves the sensory quality of sausages [4,9].

It is generally accepted that 3-methylbutanal is derived from the microbial degradation of L-leucine. When L-leucine was added to the growth medium of *S. xylosus* the increased formation of 3-methylbutanal and its corresponding alcohol and acid was observed [10]. Resting cells of *S. carnosus* and *S. xylosus* incubated with L-leucine formed both 3-methylbutanal as well as the corresponding alcohol and acid with 3-methylbutanoic acid as the major product [11–13].

Several catabolic routes for leucine and other branched-chain amino acids may be distinguished [14–16]. The first step, a deamination reaction, may occur through one of three enzymatic reactions: an oxidative deamination of L-amino acids catalysed by leucine dehydrogenase [15] or leucine oxidase [14], or a transamination reaction catalysed by leucine transaminase [16]. The leucine dehydrogenase reaction is NAD(H)-dependent, whereas the leucine oxidase reaction requires FAD. The transaminase uses α-ketoglutarate as acceptor and pyridoxal-5-phosphate as co-factor. All reactions yield the corresponding α-keto acid [14–16]. The α-ketoacid may undergo either oxidative decarboxylation followed by an oxidation to the carboxy acid [17,18], or a dehydrogenation reaction catalysed by a multi-enzyme complex into the carboxy acid via an acyl-CoA complex [19]. Larrouture et al. [11] indicated that both enzymatic reactions may be involved in the deamination of L-leucine and the enzymatic conversion of α-ketoisocaproic acid into 3-methylbutanoic acid in staphylococci. However, none of the enzymes involved in the degradation of the branched-chain amino acids in

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Micrococcaceae has been identified, nor have the major route of leucine catabolism and the regulation of the enzymes involved been elucidated.

In this paper, we have studied the production of volatile and semi-volatile metabolites by *S. xylosus* when grown in a chemically-defined medium. Special attention was paid to the metabolites 2-methylpropanal, 2-methylbutanal and 3-methylbutanal. Their sensory properties make these methyl-branched aldehydes very important and we investigated the kinetics of their oxidation into the corresponding acids.

2. Materials and methods

2.1. Bacterial strain and growth conditions

S. xylosus DD34 was kindly provided by Chr. Hansen A/S, Hørsholm, Denmark. The organism was stored at −80 °C in a basic medium containing 10 g/l casein hydrosylate, 5 g/l yeast extract, 2 g/l NaCl, 1 g/l glucose, 13 g/l K_2HPO_4 [20] and 20% glycerine until use. Inoculum was prepared as follows: the organism was cultured on mannitol salt agar (MSA) (Merck KgaA, Darmstadt, Germany) agar slants and incubated for 48 h at 30 °C. One colony was collected from the MSA agar slant and grown overnight (20 ml medium in 100-ml Erlenmeyer flasks, 30 °C, 110 rpm) in a defined medium (HHW medium) according to Hussain et al. [21]. Cells were then harvested by centrifugation (10,000 × *g*, 10 min, 4 °C) and washed once with sterile 0.9% NaCl solution. Organisms for the experiments were then grown in 250-ml Erlenmeyer flask with 50 ml of HHW medium inoculated with 1×10^6 CFU/ml and incubated at 30 °C with shaking at 150 rpm. All experiments were conducted in duplicate and repeated after a 2–4 month period.

2.2. Characterisation of the microbial flavour formation

Volatile metabolites produced by *S. xylosus* were identified with dynamic headspace GC/MS. Semi-volatile and non-volatile organic acids were identified by their methylesters with static headspace GC/MS. Quantification of both volatile metabolites and the methyl-branched acid 2-methylpropionic acid, 2-methylbutanoic acids and 3-methylbutanoic acid was performed with static headspace gas chromatography and is described as follows.

2.2.1. Dynamic headspace GC/MS

The sample (20 ml) collected from cell suspensions of *S. xylosus* after 24 h of growth was transferred to a 100 ml washing bottle. Tubes, packed with 200 mg Tenax TA® mesh 60–80 (Supelco, Inc., Pennsylvania, USA) were fixed onto the glass tubes of the insert of the washing bottle. The flask was placed in an water bath kept at 50 °C and volatiles were purged onto the trap with nitrogen at a flow rate of 100 ml/min for 30 min. The trapped volatiles were thermally desorbed from the trap in a Perkin Elmer ATD400 thermal desorber (250 °C, 7 min) and trapped by a cold trap (−30 °C) packed with 20 mg Tenax TA® mesh 60–80. The trapped volatiles were injected into the gas chromatograph (HP5890 series II) by flash heating the trap to 300 °C for 1 min with a split flow of 1:20. Transfer-line temperature was 200 °C. Separation was performed on a DP 5 SIL low bleed MS a 60 m × 0.25 mm i.d. a 1 μm film thickness fused silica capillary column (Chrompack, USA) connected to a mass selective detector (HP5971 series II). The GC oven was programmed as follows: 0 °C/2 min, 5 °C/min to 55 °C, 1 °C/min to 75 min, 10 °C/min until the oven reached 280 °C and held for 2 min. Helium grade 5.6 (purity >99.9996%) was used as carrier gas. The column pressure was 90 kPa with a He flow of approximately 1 ml/min. MS settings: ionisation energy 70 eV, scan time 0.47 s, scan range 20–350 amu, solvent delay 7.0 min, interface temperature 280 °C and ion source temperature 180 °C.

2.2.2. Static headspace GC/MS

Samples were prepared in headspace vials as for quantification of the organic acids (described as follows) and placed in an oven at 80 °C for 60 min. One millilitre of the headspace in the sample vial was collected with a gas tight syringe and injected into the split/splitlets injection port of the gas chromatograph. Column, GC oven and MS settings were as described above except for a solvent delay of 10 min instead of 7 min.

2.2.3. Quantification of metabolites

Cells were removed from the culture broth by centrifugation (10,000 × *g*/10 min) and 2 ml of the supernatant was transferred to headspace vials with a volume of 22 ml. Two grams of NaCl was added to the vials to increase the volatility of the analytes of interest and closed with a PTFE faced rubber septum. The sample vials were placed in a Perkin Elmer HS40 automatic headspace sampler (Perkin Elmer, Ltd., Beaconsfield, England) at 2 °C with settings as follows: samples were equilibrated at 70 °C under shaking for 30 min prior to injection on the gas chromatograph (Chrompack CP 9000, Middelburg, The Netherland). Injection time was 0.1 min, pressurised for 1 min, withdrawl time 0.2 min, transfer-line temperature 110 °C. Separation was performed on a CP5 Sil a 60 m × 0.25 mm i.d. a 1 μm film thickness connected to a flame ionisation detector (FID) (280 °C). The GC oven was programmed as described above. Volatiles were quantified by comparing with external calibration curves made from a mixture of standards prepared in 0.1 M sodium phosphate buffer. Semi-volatile organic acids were determined by their methylesters as follows: 1 ml of culture supernatant, 1 ml of a saturated solution of $NaHSO_4$ and 0.5 ml methanol was mixed in a 22 ml HS sample vial. The esterification reaction was performed by placing the closed sample vials in an oven at 80 °C for 2 h prior to analysis. The automatic headspace sampler settings and GC column applied were described as before.

2.2.4. Identification of metabolites

The identification of the detected compounds was based on retention times and mass spectra of authentic material.

2.3. Kinetic experiments

In the kinetic experiments *S. xylosus* was cultivated as described above. Cells were harvested in the late exponential growth phase by centrifugation (10,000 × *g*, 10 min, 4 °C) and washed three times in 0.1 M sodium phosphate buffer (pH 6.0). The cells were re-suspended in 0.1 M sodium phosphate buffer to a cell density of approximately 10^{10} CFU/ml and stored on ice prior to use. A sample containing 39.6 ml 0.1 M phosphate buffer (pH 6.0) was poured into the reacting/measuring cell of a membrane inlet mass spectrometer [22] and substrates of 2-methylpropanal, 2-methylbutanal or 3-methylbutanal were added separately to final concentrations of 0.25–10 μM. The kinetic experiments were carried out at 30 °C.

The membrane inlet mass spectrometer system allowed the concentration of the substrates to be monitored in a continuous manner [23]. Experiments were started by the addition of cells to a density of approximately 10^8 CFU/ml. The exact cell density was determined before each experiment by measuring the absorbance of the suspension at 600 nm. Initial reaction rates of the conversion of aldehydes to corresponding acids were determined by the negative slope of the detected consumption of the aldehydes. The kinetic constants K_m and V_{max} were determined through best fit lines on Hanes-Wolfe plots [24].The acids formed during the kinetic experiments were identified by static headspace GC/MS by their methylesters as described above in samples withdrawn from the cell suspension when the added aldehyde has depleted. Quantification of 3-methylbutanoic acid was performed by static headspace GC–FID as described above.

2.4. Membrane inlet mass spectrometry

A single quadrupole mass spectrometer (Balzers QMG 420, Balzers, Liechtenstein) equipped with a membrane inlet was used for kinetic measurements. A 50 ml stirred sample cell [22] was mounted on the vacuum flange separated from the high vacuum of the mass spectrometer by a 50 μm thick silicone membrane (Sil-Tec Sheeting, Technical Products, Inc., Georgia, USA). The membrane was supported by a porous sinter disc of stainless steel. The membrane interface was connected to the ion source with a 67.5 mm heated (150 °C) stainless steel tube (2.25 mm i.d.). The sample cell was kept at 30 °C and single ion monitoring of the ions of interest was performed with a dwell time of 1 s.

2.5. Trapping experiments

In the trapping experiments cell suspensions were prepared as described for the kinetic experiments. Cells were transferred to the measuring cell of the mass spectrometer and experiments were initiated by supplementing the cell suspension first with 10 μM 3-methylbutanal and then with both 10 μM 3-methylbutanal and 400 μM α-ketoisocaproic acid, sodium salt-methyl-d3 ($(CH_3CD_3)CHCH_2COCOONa$). A shift of 3 Da upwards the mass range for the aldehyde produced from the deuterium-labelled α-ketoisocaproic makes a discrimination between the aldehydes possible by selecting ions that are unique for each aldehyde.

2.6. Chemicals

Amino acids, biotin, nicotinic acid, D-pantothenic acid, pyridoxamine dihydrochloride, adenine sulphate and guanine hydrochloride were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) as well as 3-methylbutanal, 2-methylbutanal and 2-methylpropanal and their corresponding alcohols and acids. Riboflavin, thiamin hydrochloride and pyridoxal hydrochloride was obtained from Merck KgaA (Darmstadt, Germany). α-Ketoisocaproic acid, sodium salt-methyl-d3 (98% purity) was purchased from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA).

3. Results

3.1. Characterisation of the microbial flavour formation

Table 1 lists identified volatile metabolites found in the fermentation broth of *S. xylosus* together with their precursors. A total of 35 compounds was identified. Of the detected metabolites 2,5-methylpyrazine, 2-phenylethylacetate, 2-methyltetrahydrothiophen-3-one, 3-(methylthio)-propionic acid and 3-(methylthio)-propanal have not been previously identified as metabolites of *S. xylosus*. GC/MS mass spectra of these compounds (Fig. 1a–e) were virtually identical to GC/MS spectra of reference materials. Methyl- and dimethylpyrazines have been reported in dry sausage fermented with *S. xylosus* [7], but the exact isomers were not identified. All of the other compounds have previously been identified when *S. xylosus* was cultivated in a model meat substrate [8] or when applied as starter cultures in dry fermented sausages [3–7]. The major part of the metabolites are produced through amino acid catabolism (for references, see Table 1). Although not determined quantitatively, the approximate concentrations of the majority of the detected compounds are in the nanomolar to low micromolar range.

3.2. Quantification of branched-chain amino acid derived metabolites

Table 2 summarises the concentrations of the branched-chain amino acid derived aldehydes, 2-methylpropanal, 2-methylbutanal and 3-methylbutanal, and their correspond-

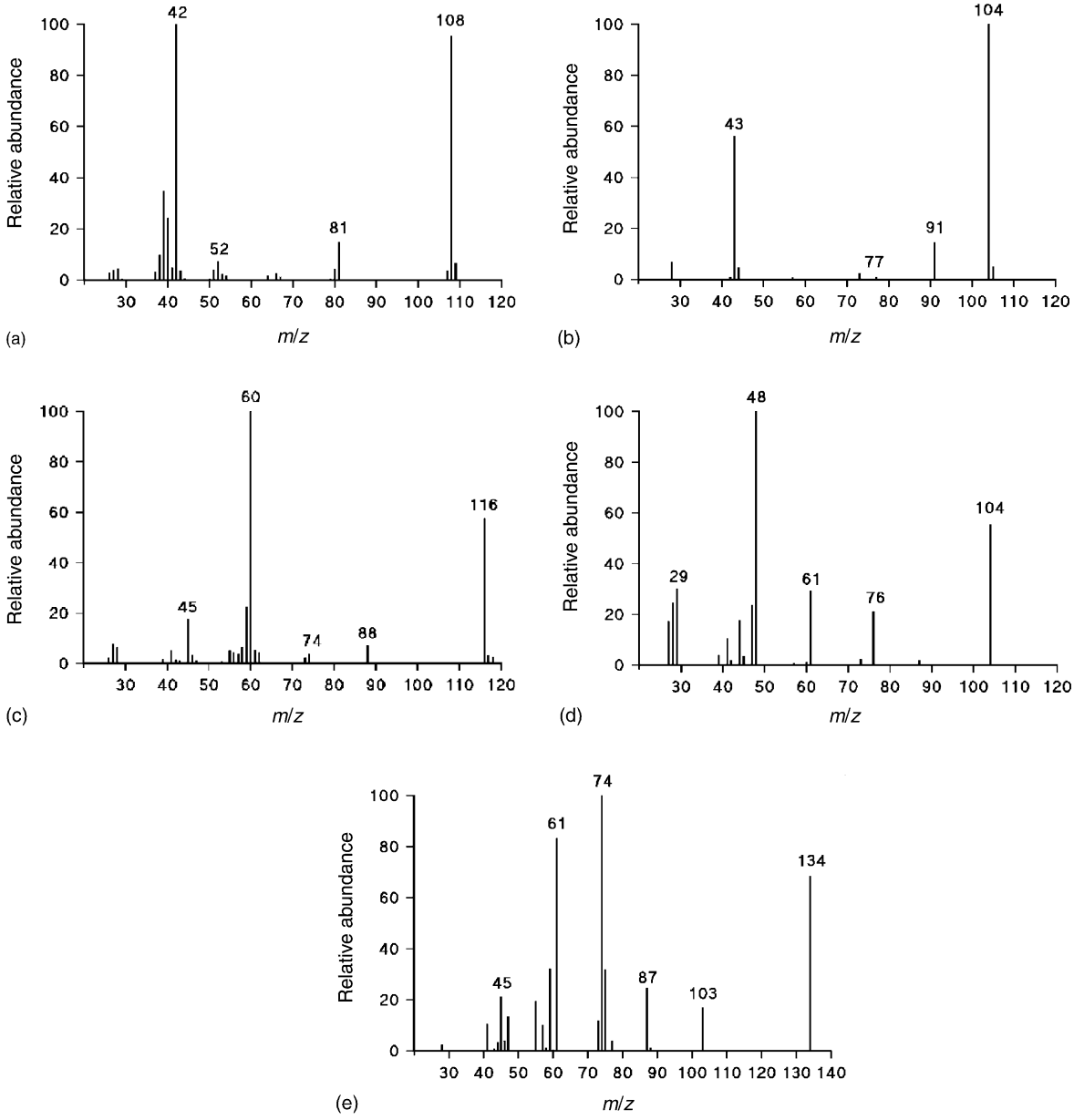

Fig. 1. Electron impact mass spectra of (a) 2,5-dimethylpyrazine, (b) 2-phenylethylacetate, (c) 2-methyltetrahydrothiophen-3-one, (d) 3-(methylthio)-propanal and (e) methylester of 3-(methylthio)-propionic acid detected in the culture fluid of *S. xylosus*.

ing alcohols and carboxy acids in the culture supernatant from cultures of *S. xylosus* grown for 24 h in HHW medium. The levels of 2-methylbutanal and 3-methylbutanal were lower than the corresponding alcohols, whereas that of 2-methylpropanal was higher. However, in all cases the concentrations of the acids were considerably higher.

3.3. Kinetics of branched-chain aldehyde oxidation

The kinetics of the oxidation of the aldehydes, 2-methylpropanal, 2-methylbutanal and 3-methylbutanal, into their corresponding acids was investigated by measuring the decrease in the concentration of the supplemented aldehyde in suspensions of resting cells of *S. xylosus*. Fig. 2 shows the EI mass spectrum of 3-methylbutanoic acid by its methylester detected in a sample withdrawn from the reaction mixture 20 min after the supplementation of 3-methylbutanal. The 3-methylbutanal was not detected in this sample. Similar experiments where suspensions of *S. xylosus* were supplemented with 2-methylpropanal or 2-methylbutanal also showed a conversion of the added aldehyde into the corresponding acid.

Fig. 3 shows the single ion monitoring data of the microbial oxidation of 3-methylbutanal ($m/z = 86$) into 3-methylbutanoic acid. Although not stoichometrically converted the initial rate of consumption of 3-methylbutanal

Table 1
Metabolites identified in the fermentation broth of *S. xylosus*

Compound	Origin of compound	Reference
Alcohols		
Ethanol	Pyruvate	[32]
2-Propanol	Pyruvate	[33]
2-Methylpropanol	Val	[34]
3-Methyl-3-butene-1-ol	Leu	[8]
3-Methyl-2-butene-1-ol	Leu	[8]
2-Methylbutanol	Ile	[35]
3-Methylbutanol	Leu	[10–13]
2-Hydroxy-3-butanone	Pyruvate	[37]
2-Phenylethanol	Phe	[30,31]
Aldehydes		
Acetaldehyde	Pyruvate	[32]
2-Methylpropanal	Val	[28,36]
2-Methylbutanal	Ile	[28]
3-Methylbutanal	Leu	[10,12,13,28,32]
Benzaldehyde	Phe	[29–31]
Benzeneacetaldehyde	Phe	[9,30]
Sulphides		
Dimethyldisulphide	Met	[26]
3-(Methylthio)-propanal	Met	[26]
3-(Methylthio)-propanoic acid[a]	–[b]	–
2-Methyltetrahydrothiophen-3-one	–	–
Acids		
Acetic acid	Pyruvate	[32]
Propanoic acid[a]	Pyruvate	[33]
Butanoic acid[a]	Pyruvate	[33]
2-Methylpropanoic acid	Val	[34]
2-Methylbutanoic acid	Ile	[35]
3-Methylbutanoic acid	Leu	[12,13,36,37]
Lactic acid[a]	Pyruvate	[32]
Benzeneacetic acid[a]	Phe	[29,31]
Ketones		
Acetone	Pyruvate	[33]
2-Butanone	Pyruvate	[37]
2,3-Butanedione	Pyruvate	[32]
2,3-Pentanedione	Thr/Pyruvate	[38]
Acetophenone	Phe	[31]
Esters		
2-Phenylethylacetate	Phe/Pyruvate[c]	[2]
3-Methyl-1-butylacetate	Leu/Pyruvate	[13]
Others		
2,5-Dimethylpyrazine	Thr	[25]

[a] Identified as its corresponding methylester as described in the Section 2.
[b] We were unable to find literature data on the microbial formation of this compound.
[c] Derived from the esterification of 2-phenylethanol and acetic acid.

corresponds very well with the initial rate of 3-methylbutanoic acid formation as determined by off-line GC analysis. When incubating, resting cells of *S. xylosus* with different concentrations of 2-methylpropanal, 2-methylbutanal or 3-methylbutanal, similar curves were observed; however, the initial reaction rates differed. Fig. 4 shows a Hanes-Wolfe plot, in which the initial velocity divided by

Table 2
Concentrations of branched-chain amino acid derived metabolites detected in the culture supernatant of *S. xylosus*

Compound	Concentration (μM)
2-Methylpropanol	1.2 ± 0.2[a]
2-Methylpropanal	2.0 ± 0.1
2-Methylpropanoic acid	963 ± 17
2-Methylbutanol	2.2 ± 0.1
2-Methylbutanal	0.8 ± 0.2
2-Methylbutanoic acid	858 ± 49
3-Methylbutanol	9.3 ± 0.7
3-Methylbutanal	0.4 ± 0.03
3-Methylbutanoic acid	1486 ± 5

[a] Data are means ± S.D. based on two determinations.

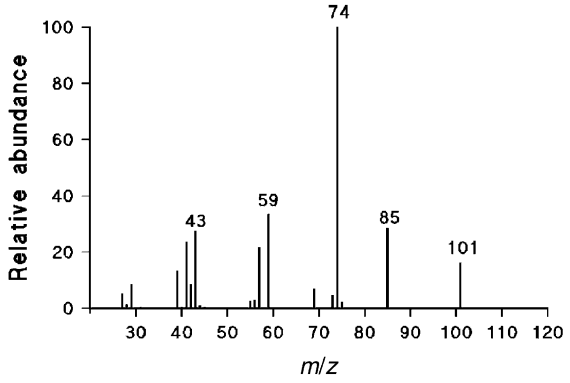

Fig. 2. Electron impact mass spectrum of the methylester of 3-methylbutanoic acid detected in the cell suspension after the depletion of 3-methylbutanal.

the initial substrate concentration is plotted as a function of the initial substrate concentration. Table 3 summarises the measured K_m and V_{max} values determined from the plot as well as the cal culated catalytical constant V_{max}/K_m. Resting cells showed high affinity (low K_m) for all substrates tested ranging from 0.8 μM for 2-methylpropanal to 1.6 μM for 3-methylbutanal and V_{max} values ranging

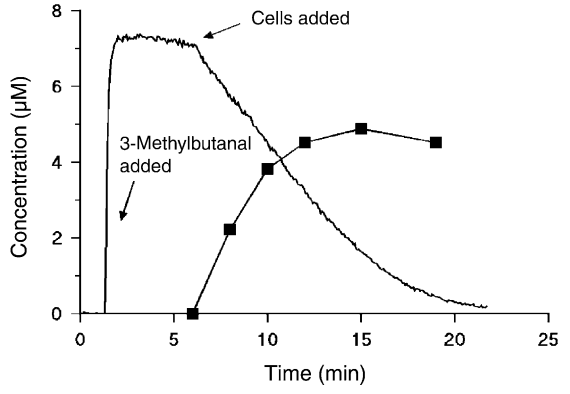

Fig. 3. Microbial oxidation of 3-methylbutanal (–) into 3-methylbutanoic acid (■).

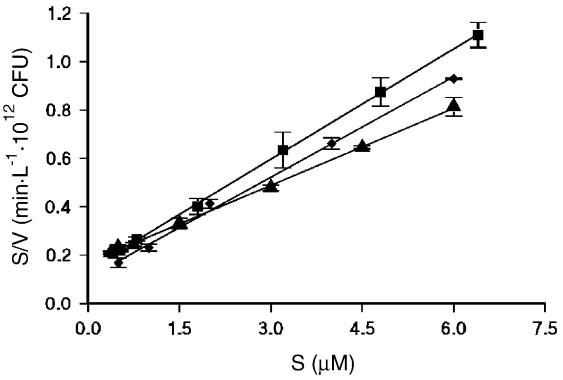

Fig. 4. Activities of oxidation of 2-methylpropanal (◆), 2-methylbutanal (■) and 3-methylbutanal (▲) plotted as initial substrate concentrations divided by initial reaction velocities against the initial substrate concentrations (Hanes-Wolfe plot). The data are means and standard deviations based on results from two experiments performed with cells from individual cultures.

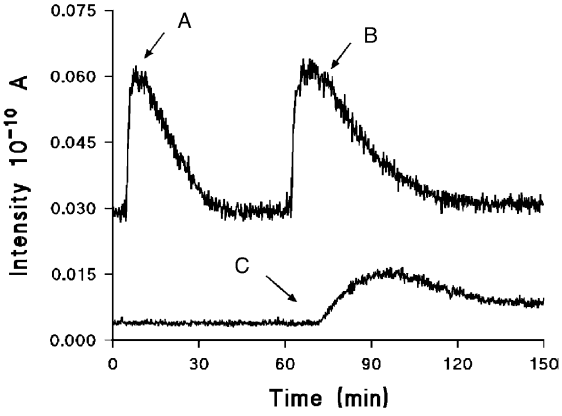

Fig. 5. Formation of 3-methylbutanal from α-ketoisocaproic acid. (A) Supplementation of 10 μM 3-methylbutanal, (B) 10 μM 3-methylbutanal and 400 μM deuterium labelled α-ketoisocaproic acid (C) with the formation of labelled 3-methylbutanal ($(CH_3CD_3)CHCH_2CHO$).

from 5.9 μmol/min/10^{12} CFU for 3-methylbutanal to 9.4 μmol/min/10^{12} CFU for 2-methylpropanal. The catalytic constant for the conversion of 2-methylpropanal to the corresponding acid was two-fold higher than the reaction with 2-methylbutanal and three-fold that of 3-methylbutanal.

3.4. Formation of 3-methylbutanal from α-ketoisocaproic acid

The presence of the methyl-branched aldehydes in the fermentation broth of *S. xylosus* indicate that the acid is formed from α-keto acid via the aldehyde. To investigate this further trapping experiments were carried out as shown in Fig. 5, were a suspension of resting cells of *S. xylosus* was supplemented first with 10 μM 3-methylbutanal and then with both 3-methylbutanal and 400 μM deuterium-labelled α-ketoisocaproic acid (the leucine derived α-keto acid). The α-ketoisocaproic acid was labelled at the 4′-position, where the $–CH_3$ substituent was replaced by $–CD_3$. If the deuterium-labelled α-ketoisocaproic acid is decarboxylated the mass of the resulting 3-methylbutanal will be shifted 3 Da upwards to a molecular weight of 89 Da. The concentration chosen for the trapping experiment was 10 μM (six-fold greater than the determined K_m value) which was sufficient for an almost complete saturation of the enzyme as indicated by the linearity of the decrease in aldehyde content of the suspension (Fig. 5). When adding the unlabelled aldehyde together with the labelled α-ketoisocaproic acid, a reduced oxidation rate of the unlabelled 3-methylbutanal is observed together with an accumulation of the deuterium-labelled 3-methylbutanal.

4. Discussion

Several volatile and semi-volatile metabolites were produced by *S. xylosus*, many of them previously identified as important for the flavour of fermented dry sausages including the methyl-branched aldehydes, 2-methylpropanal, 2-methylbutanal and 3-methylbutanal [3–8]. In addition five metabolites not previously reported as metabolites of *S. xylosus* were identified.

Alkylpyrazines are generally considered to be important flavour compounds in foods and may be synthesised by micro-organisms [25], or through chemical reactions, even at room temperature [26]. The detection of 2,5-dimethylpyrazine in the culture fluid of *S. xylosus* indicated the microbial production of this compound. In *Bacillus subtilis* threonine is considered to be a precursor for 2,5-dimethylpyrazine biosynthesis [27] and a similar pathway may exist in *S. xylosus*.

Engels et al. [28] found that in *Lactococcus lactis* methionine was converted to 3-(methylthio)-propanal through

Table 3
Substrate affinities, reaction velocities and catalytic constants for the conversion of branched-chain aldehydes into corresponding acids by *S. xylosus*

Aldehyde	K_m (μM)	V_{max} (μmol min^{-1} 10^{-12} CFU)	V_{max}/K_m (l min^{-1} 10^{-12} CFU)
2-Methylpropanal	0.8	9.4	11.75
2-Methylbutanal	0.9	7.1	7.89
3-Methylbutanal	1.6	5.9	3.69

4-methyl-thio-α-ketobutyric acid and also proposed a pathway from 3-(methylthio)-propanal through methanethiol to dimethyldisulphide and dimethyltrisulphide. Both dimethyldisulphide and 3-(methylthio)-propanal were found in the fermentation broth of *S. xylosus*, hence a similar catabolic scheme for methionine could very well be active in this organism. In addition 3-(methylthio)-propanal is further oxidised to 3-(methylthio)-propionic acid by *S. xylosus*.

The aromatic compounds produced by *S. xylosus* may be formed through phenylalanine metabolism. Phenylalanine metabolism has been studied extensively in white rot fungi and other organisms [16,29–31], and several catabolic routes of phenylalanine have been described. One route suggested involves a transaminase catalysed deamination of phenylalanine yielding phenylpyruvic acid [30], which was decarboxylated to phenylacetaldehyde, and then reduced to 2-phenylethanol or oxidised to phenylacetic acid and finally converted to benzaldehyde through 2-hydroxy-2-phenylacetic acid and 2-oxo-2-phenylacetic acid. However, Groot et al. [29] demonstrated that the transformation of phenylpyruvic acid to phenylacetic acid or benzaldehyde can be a chemical transformation catalysed by metal ions and O_2. Even though the intermediates of the oxidative metabolic pathway 2-hydroxy-2-phenylacetic acid and 2-oxo-2-phenylacetic acid were not detected, both pathways may be involved in phenylalanine catabolism by *S. xylosus*. Lipase activity has been demonstrated in *S. xylosus* [2] and phenylethylacetate is formed from the esterification of 2-phenylethanol and acetic acid.

The major compounds produced through catabolism of the branched-chain amino acids valine, isoleucine and leucine were 2-methylpropionic acid, 2-methylbutanoic acid and 3-methylbutanoic acid, respectively. The concentrations of the corresponding alcohols and aldehydes detected were small compared with the amount of branched-chain acids.

Different routes may lead from the branched-chain α-keto acids (α-ketoisocaproic acid, α-ketoisovaleric or α-keto-β-methylvaleric acid) to the corresponding carboxy acids in *S. xylosus*. The α-keto acids could be decarboxylated to the aldehyde [17] and then oxidised into the acid by an aldehyde dehydrogenase [18], or they could be dehydrogenated by a branched-chain keto acid dehydrogenase multi-enzyme complex yielding the acid though an acyl-CoA [19].

The observation that labelled 3-methylbutanal was produced together with labelled 3-methylbutanoic acid after the supplementation of *S. xylosus* with deuterium-labelled α-ketoisocaproic acid (Fig. 5) shows that the former pathway is active. Because only 3-methylbutanal, produced from the corresponding keto acid, was detectable when the oxidising enzyme was saturated with substrate this experiment indicated that the reaction rate of the oxidation of 3-methylbutanal to 3-methylbutanoic acid was faster than the rate of formation of 3-methylbutanal from α-ketoisocaproic acid.

The oxidising enzyme exhibited high affinity for all of the branched-chain amino acid derived aldehydes, with the highest affinity observed for the valine derivative, 2-methylpropanal (K_m 0.8 μM, Table 3), and the lowest for 3-methylbutanal from leucine (K_m 1.6 μM). A similar pathway was also active in *Proteus vulgaris*, where a NADP-dependent aldehyde dehydrogenase catalysed the oxidation of 3-methylbutanal to 3-methylbutanoic acid [18]. The K_m value determined for 3-methylbutanal was 30 μM.

In *Enterococcus faecalis*, a branched-chain α-keto acid dehydrogenase catalyses the conversion of α-ketoisocaproic acid to 3-methylbutanoic acid [19]. In this reaction 3-methylbutanal is bypassed and ATP is formed by substrate level phosphorylation by the combined action of a phosphotransbutyrylase and butyrate kinase. However, in *S. xylosus* we found that α-ketoisocaproic acid is decarboxylated into 3-methylbutanal, which then is rapidly oxidised to its corresponding acid. This suggests a different physiological role for the pathway in *S. xylosus*.

5. Conclusions

We identified several important flavour compounds derived from amino acid metabolism, five of them not previously reported as metabolites of *S. xylosus*. We also demonstrated that 3-methylbutanal can be formed from α-ketoisocaproic acid. However, *S. xylosus* exhibited high affinity towards the organoleptic methyl-branched aldehydes and rapidly oxidised them into their corresponding carboxy acids. Therefore, low concentrations of these metabolites were expected. This indicate the importance of controlling this reaction step in *S. xylosus* in order to achieve sensorially significant levels of these metabolites during meat fermentation processes.

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