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ORIGINAL PAPER

Reductive degradation of pyrazine-2-carboxylate by a newly isolated *Stenotrophomonas* sp. HCU1

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Abstract A bacterium growing on pyrazine-2-carboxylate broth was isolated, purified and identified as a strain of *Stenotrophomonas* sp. based on polyphasic taxonomic analyses and designated as strain HCU1. 16S rRNA gene sequence of strain HCU1 showed 98.7% sequence similarity with the type strain of *Stenotrophomonas maltophilia* belonging to *Gammaproteobacteria*. Growth of strain HCU1 was demonstrated when pyrazine-2-carboxylate was used as a sole source of nitrogen. Ring reduction of pyrazine-2-carboxylate was shown as increase in absorbance at 268 nm and the reduced product was confirmed as 1,2,5,6-tetrahydropyrazine-2-carboxylate, while a ring opened product, 2-amino-2-hydroxy-3-(methylamino) propanoic acid (with a loss in carbon atom), indicated a reductive degradation of pyrazine-2-carboxylate by strain HCU1.

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Introduction

Pyrazines are a group of N-heterocyclic aromatic compounds with 1,4-nitrogen substitutions in the benzene ring. They are widely distributed in nature and occur both naturally and anthropogenically (Woolfson and Rothschild 1990; Schulz et al. 2004; Robert et al. 1988). They are used as chemical building blocks for a series of industrial products (Kurniadi et al. 2003; Tinschert et al. 2000; Moser 2008) and are also constituents of biologically active compounds such as antiseptics, disinfectants, herbicides, fungicides, insecticides and pharmaceuticals with different fields of application (Edmondson et al. 2006; Brown, 2002; Weiser et al. 1997). Substituted pyrazines are used as cytotoxic drugs to treat hypoxia cells (Hartman et al. 1984), anti-HIV drug (Prochaska et al. 1993) and in the treatment of breast cancer (Cristofanilli 2005). They contribute to the flavour of roasted and cooked foods (Maga and Sizer 1973; Vasundhara and Parihar 2006). Pyrazine-2-carboxylate is used as a catalyst in oxidation of aromatic hydrocarbons and alcohols (Shulpin et al. 1993).

While much of the work on the microbial metabolism of pyrazines was on their transformations (modification of ring substituents) by bacteria;

Pseudomonas putida, *Pseudomonas acidovorans* (Kiener 1992), *Alcaligenes* sp. (Kiener et al. 1994), *Rhodopseudomonas palustris* (Sasikala et al. 1994) and *Ralstonia* sp. (Tinschert et al. 2000), not much work was done on their degradations (Muller and Rappert 2010). Degradation of aspergillic acid (a naturally existing pyrazine derivative) by *Trichoderma koningii* was recorded (Nishimura et al. 1997). Degradation of 2,3-diethyl-5-methylpyrazine by a newly discovered *Mycobacterium* and degradation of 2,5-dimethylpyrazine by *Rhodococcus erythropolis* were reported (Rappert et al. 2006, 2007). Except for the degradation of hydroxypyrazine to N-formylglycinamide by *Pseudomonas* sp. (Mattey and Harle 1976), there was no other report of the degradative products of pyrazines by bacteria.

Pyrazine-2-carboxylic acid is the central intermediate in metabolism of pyrazine group of compounds by purple bacteria (Sasikala et al. 1994) and also the product of antituberculotic drug, pyrazinamide, transformed by bacterial encoded pyrazinamidase (Zhang and Mitchison 2003). In either case, further metabolism of pyrazine-2-carboxylate was not demonstrated. In the present communication, for the first time, we report the degradative product of pyrazine-2-carboxylate from a newly isolated *Stenotrophomonas* sp. HCU1 which is formed through a reductive pathway.

Materials and methods

Organism and growth conditions

Strain HCU1 was serendipitously identified from a pyrazine-2-carboxylate contaminated broth growing in our lab. Strain HCU1 was characterized based on polyphasic taxonomy as previously described (Anil Kumar et al. 2008). Strain HCU1 was grown in 50 ml nutrient broth (g l^{-1}; peptone (10), yeast extract (3), NaCl (5), pH 7.0) in 250 ml Erlenmayer flasks under agitation (150 rpm) at 30 ± 2°C. For growth on pyrazine-2-carboxylate, 0.1% (v/v) culture from the nutrient broth (grown for 6 h) was inoculated into Biebl and Pfennig's (1981) mineral broth supplemented with pyrazine-2-carboxylate (1.5 mM), as an additional supplement while malate (22 mM) and ammonium chloride (7 mM) served as sole source of carbon, electron donor and nitrogen respectively for chemoheterotrophic growth. For growth on pyrazine-2-carboxylate as sole source of carbon, malate was replaced with pyrazine-2-carboxylate and ammonium chloride served as nitrogen source. Ammonium chloride was replaced with pyrazine-2-carboxylate when used as sole source of nitrogen, both malate and ammonium chloride were replaced with pyrazine-2-carboxylate when used as a sole source of carbon and nitrogen. All the cultures were grown in 50 ml media taken in 250 ml Erlenmayer flasks at 30 ± 2°C in an incubator under agitation at 150 rpm.

Bulk cultivation

Two percent (v/v) of 4–6 h culture of strain HCU1 adapted to pyrazine-2-carboxylate as a supplement was transferred to 1.5 l fresh media in 3 l Erlenmeyer flask and agitated using a magnetic stirrer (~150 rpm) at room temperature.

Isolation and purification of the metabolites

Mid-logarithmic phase culture (8 h) of strain HCU1 was harvested by centrifugation (12,000×*g* for 20 min) and the supernatant was directly concentrated in flash evaporator to dryness. The concentrate was extracted with 50 ml methanol and the precipitated impurities were removed by centrifugation (12,000×*g* for 15 min). The methanolic supernatant was diluted with methanol, water (1:0.2) where further precipitation occurred, which was separated by centrifugation (12,000×*g* for 15 min). The precipitate was redissolved in methanol (fraction A) and the methanolic water supernatant was concentrated to dryness (fraction B). Both fractions were used for further metabolite purification using semi-preparative HPLC.

Preparation of resting cells and assay

Cells of *Stenotrophomonas* sp. HCU1 grown chemoheterotrophically on Biebl and Pfennig's medium supplemented with pyrazine-2-carboxylate (1.5 mM) till late logarithmic phase were harvested by centrifugation (12,000×*g* for 15 min). The pellet was washed twice and resuspended in basal salts medium supplemented with pyrazine-2-carboxylate (1.5 mM). Assay was done in 250 ml conical flasks with 50 ml media and incubated at 30 ± 2°C.

Preparation of cell free extracts and assay

Pyrazine-2-carboxylate (used as an additional supplement) adapted strain HCU1 was harvested by centrifugation (16,000×*g* for 10 min) and the pellet was washed (twice) with 0.05 M Tris–HCl buffer (pH 7.4) and resuspended in 1 ml of the same buffer. Cells were sonicated with MS-72 probe (Bandelin, model-UW 2070) to complete cell lysis after 8–9 cycles. The sonicated cell lysate was used for assay of pyrazine-2-carboxylate reduction and degradation. The assay mixture (1 ml) contained; 200 µM of pyrazine-2-carboxylate, 200 µM of NADPH, 5 mM $MgCl_2$ and 40 µl (130–140 µg ml^{-1} protein) of the cell free extract in Tris HCl (pH 7.4). The reaction was stopped at an appropriate time by adding 10 µl of 5 N HCl and filtered through 0.22 µm polyvinyldienefluoride membrane. The samples were stored at 4°C and were analyzed in HPLC.

Purification and characterization of pyrazine-2-carboxylate reductase

Cell free extract was subjected to 0–30, 30–60 and 60–90% ammonium sulphate saturation and the resulting precipitate was dialyzed. Dialyzed fractions were assayed for reductase activity. The 30% ammonium sulphate precipitated fraction was chosen for enzyme purification as reductase activity was high. The 30% enzyme active fraction was loaded onto a DEAE-cellulose column of 5 × 1.5 cm length with a bed volume of 40 ml and eluted using a sodium chloride step gradient of 0–1 M. The active fractions eluted in the 0.4 M sodium chloride gradient were pooled, concentrated and reloaded on DEAE-cellulose column of 5 × 1.5 cm in length and eluted with 0.3–0.5 M sodium chloride. The eluted fractions were assayed for activity and homogeneity was checked using PAGE electrophoresis. The electrophoresis of the proteins was done on 10 and 12% SDS-PAGE and 8% native PAGE and stained using coomassie blue and silver stain (Laemmeli, 1970). Pyrazine-2-carboxylate reductase activity was studied with time using HPLC. The activity of the enzyme was checked at varying concentrations of pyrazine-2-carboxylate. Ring reduction of pyrazine-2-carboxylate was enzymatically assayed using 1 mM PCA, 200 µM NADPH and increase in absorbance of PCA was calculated in terms of % peak height increase/microgram gram protein/minute. *Km* and *Vmax* of the enzyme were calculated using the software graph pad prism 5.0.

Analytical methods

Growth was measured turbidometrically using a Biochrome spectrophotometer (Libra 12) at 540 nm. Analysis of substrates and products was performed in HPLC at room temperature using a Shimadzu SPD-10AT isocratic or gradient system. Solvent system used for the analysis is given in the foot notes of the respective figures. HPLC instrument details are: solvent flow rate—1.5 ml min^{-1}, Luna 5 μ C_{18} (2) 100A column (250 × 4.6 mm) and the heterocyclic compounds were detected using PDA detector at 268 nm. Pyrazine-2-carboxylate, (t_R = 1.77 min), pyrazine (t_R = 1.27), uracil (t_R = 1.29), pyrazinamide (t_R = 2.02), dimethylaminopyridine (t_R = 4.203), guanine (t_R = 1.60), nicotinic acid (t_R = 1.46) and imidazole (t_R = 1.27) were identified using standards. Increase in the absorbance (m AU) at 268 nm in HPLC was attributed to aromatic ring reduction of the compounds and loss as its utilization (Chia and Trimble 1971). Purity of the metabolites were confirmed using HPLC with three different solvent systems (methanol:water [1:1]; methanol:water:acetonitrile [1:1: 0.25]; water:acetonitrile [7:3]) at 230, 268, 280 and 350 nm for independent analysis.

Semi-preparative HPLC

The methanolic precipitate (fraction A) and methanolic supernatant (fraction B) was purified using semi-preparative column Luna 5 μ, C8 (2) 100A column (250 × 10 mm) using UV-Visible detector at 268 nm. Methanol, water, acetonitrile (1:1:0.25) was used as a solvent at 1.0 ml min^{-1} in isocratic mode and acetonitrile (1:1) water in gradient mode.

FT-IR analysis

The HPLC purified compounds were concentrated and the KBr pellets were used for FT-IR analysis using a Shimadzu make FT-IR 8300.

LC-MS analysis

was performed on a Shimadzu LC-MS (LCMS 2010A) at 40°C (LC column oven) and 85°C (MS ionization chamber). Methanol, water (1:1) was used as solvent at 0.2 ml min^{-1}, Luna 5 μ C_{18} (2) 100A column (250 × 4.6 mm) and the compounds were detected (LC) at 268 nm. The column effluent from the LC was nebulized into an Atmospheric Pressure Chemical Ionization (APCI), Electrospray Ionization (ESI) region under N_2 gas for generating molecular masses in both negative and positive modes.

NMR analysis

Nuclear magnetic resonance (NMR) spectra were recorded with an AC400 spectrometer (Bruker, Rheinstetten, Germany) at a nominal frequency of 400.13 MHz (^{1}H) and 100.62 MHz (^{13}C), respectively. Samples were dissolved in D_2O. The spectrometer settings are: relaxation delay—1 s (^{1}H), 6 s (^{13}C); pulse width-13 µs (1H), 10.5 µs (^{13}C), 90° pulse on ^{1}H; spectral width −8278 Hz (^{1}H), 23980 Hz (^{13}C); pre-scan delay = 6 s (^{1}H, ^{13}C) and 1D sequence power gated decoupling Waltz-16 was employed for ^{13}C. NMR predictions were done form web site: www.nmrdb.org; www.ebi.ac.uk/nmrshiftdb/

Gel exclusion chromatography

Molecular weight of the pyrazine-2-carboxylate reductase was estimated using gel exclusion chromatography in FPLC (Fast Protein Liquid Chromatography) using Sepharose G150 matrix of 150 cm × 3 cm length with standard protein markers catalase (240 kDa), glucose oxidase (186 kDa), bovine serum albumin (67 kDa) and peroxidase (40 kDa).

MALDI TOF analyses

Protein was digested with trypsin with 1:10 ratio overnight at 37°C. The mixture was lyophilized and dissolved in 5 µl of acetonitrile:0.1% trifluroacetate (1:1). 2 µl of which was mixed with 2 µl of 10 mg/ml CHCA (4-cyanohydroxy cinnamic acid) in 5% acetonitrile:TFA (1:1) and spotted onto target plate for MALDI TOF–TOF. m/z values from 500 to 3500 were recorded. The source voltage was 19 kV and matrix was suppression up to 500 Da. Calibration was done using peptich Calibstandard II mono (Bruker). Proteins were identified using the public domain Mascot search engine by incorporating the standard parameters (http://www.matrixscience.com). The database used was Swissport and trypsin was used as proteolytic enzyme for limiting the miscleavages to one. Carbamidomethyl (C) and oxidation (M) of methionine were taken as fixed and variable modifications, respectively.

Results and discussion

Taxonomic affiliation of strain HCU1

Strain HCU1 forms yellow translucent colonies on nutrient agar plates. Cells of strain HCU1 are rod shaped with 1.5 µm in length and 0.5 µm in width and reproduce by binary fission. Strain HCU1 is an obligate aerobe and requires yeast extract for growth. Growth was supported by glucose, fructose, sucrose, acetate, citrate, fumarate, oxaloacetate, succinate, pyruvate, 2-oxoglutarate, malate, lactose and glutamate. Starch was not hydrolyzed, gelatin not liquefied; urease activity, indole production from tryptophan, acid production from glucose were negative, while ammonification was positive. The phylogenetic relationship of strain HCU1 was examined on the basis of 16S rRNA gene sequences. The data obtained revealed that the new isolate clustered with the type strain of *Stenotrophomonas maltophilia* (98.7% similarity; Fig. 1). The EMBL accession number for the 16S rRNA gene sequence of strain HCU1 is AM159126. The strain differs from the type strain of *Stenotrophomonas maltophilia* in lacking methionine requirement, gelatin liquefaction and amylase activities.

Utilization of pyrazine-2-carboxylate by growing cells of *Stenotrophomonas* sp. HCU1

Pyrazine-2-carboxylate supported good growth (with a lag of 2 h) of strain HCU1 when used as nitrogen compared to poor growth (with a lag of 4 h) when used as carbon or carbon and nitrogen (Fig. 2A), while growth was absent without added carbon or nitrogen (Data not shown in Fig. 2A). Pyrazine 2-carboxylate had no effect on growth yield and rate

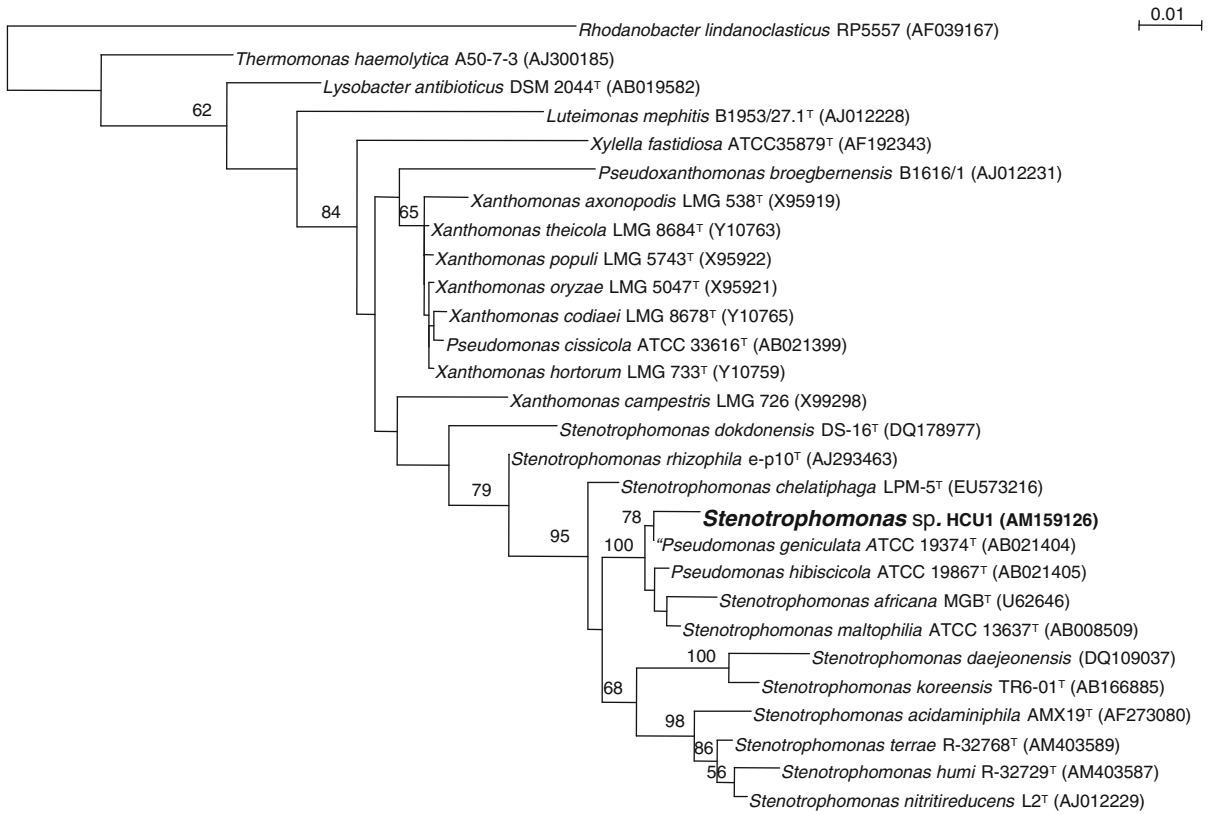
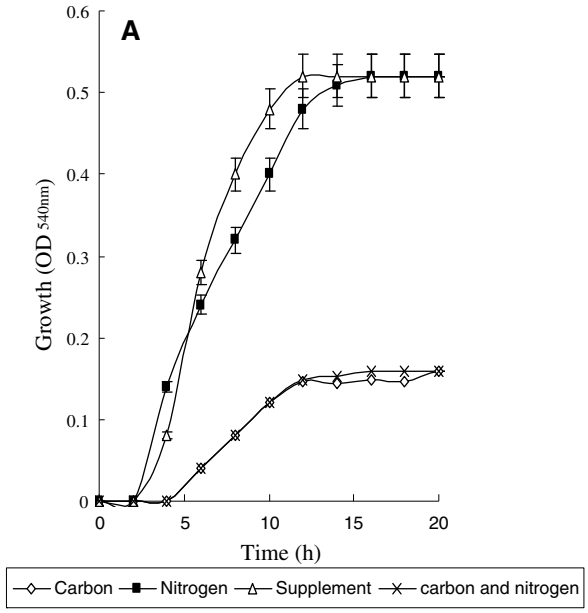
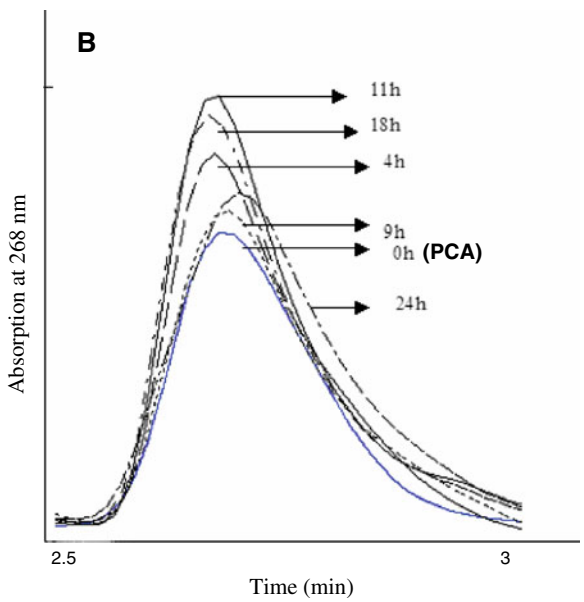


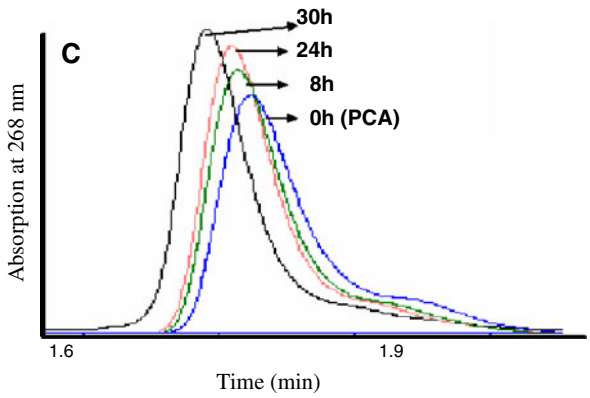
Fig. 1 16S rRNA gene based dendrogram showing the phylogenetic position of *Stenotrophomonas* sp. HCU1 among the *Stenotrophomonas* and *Xanthomonas* representatives of *Gammaproteobacteria*. Dendrogram was constructed using the phyML method depicting the phylogenetic relationship of the strain HCU1 with in the order *Xanthomonadales* using the 16S rRNA gene sequence analysis. Bar represents 1 nucleotide substitution per 100 nucleotides. Bootstrap values (expressed as percentages of 1000 replicates) above 50% are shown at branch points

of strain HCU1 when used as supplement (had a lag of 2 h) to the mineral medium with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively. We are unable to show simultaneous utilization of pyrazine-2-carboxylate during growth of strain HCU1 due to an increase and decrease in absorbance at its maxima (268 nm; Fig. 2B) making it difficult to calculate extinction coefficients. Such phenomena of increase and decrease of pyrazine-2-carboxylate at its absorption maxima (268 nm) was also observed with resting cells of strain HCU1 (Fig. 2C) and with cell free extracts (Fig. 2D). Though we could not observe any notable peaks other than pyrazine-2-carboxylate in the HPLC chromatograms of growing cells (Fig. 2B) and with resting cells (Fig. 2C), at least three chromatographically distinct peaks (presumably the products of the metabolism) together with increase and decrease in pyrazine-2-carboxylate peak was observed in assay using cell free extracts (Fig. 2D).

The phenomena of increase in peak height of pyrazine-2-carboxylate at its absorption maxima compared to uninoculated blank may be attributed to aromatic ring saturation, mimicking reduction of NAD/NADP/FAD to NADH/NADPH/FADH whose conversions are measured spectrophotometrically (Yu and Lee 2001). Pyrazines are structural analogues of pyridines and also integral part of flavins and thus can act as electron acceptors. Ring saturation during bacterial metabolism of aromatic compounds is not restricted to heterocyclics alone (Rhee et al. 1997; Lee et al. 2001), but was also observed with homocyclics (Meckenstock et al. 2000; Mouttaki et al. 2008).







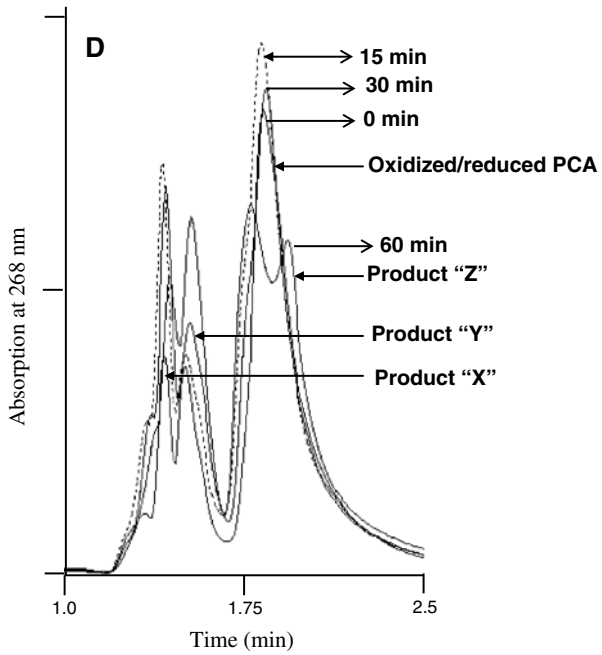


Fig. 2 **A** Growth of *Stenotrophomonas* sp. HCU1 on pyrazine-2-carboxylate when used as carbon, nitrogen, carbon and nitrogen and as supplement to the mineral medium with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen source, respectively. **B** HPLC chromatogram of growing cells culture supernatant of strain HCU1 showing increase, decrease and shift in the peaks corresponding to pyrazine-2-carboxylate (t_R 2.7 min) metabolism. Gradient HPLC was used for the analysis using Tris–HCl (50 mM; pH 7.4) and acetonitrile system (for more experimental details see "Materials and methods" section). *PCA* pyrazine 2-carboxylate. **C** HPLC chromatogram of resting cell suspension of strain HCU1 showing increase, decrease and shift in peaks corresponding to pyrazine-2-carboxylate (t_R 1.7 min) metabolism. Methanol, water, acetonitrile (1:1: 0.25) solvent system with isocratic mode was used. The minor shifts in the t_R are due to the pressure variations of HPLC. **D** HPLC chromatogram of cell free extracts of strain HCU1 showing increase, decrease of pyrazine-2-carboxylate (PCA) and appearance of new peaks (X,Y,Z). Tris–HCl (50 mM, pH 7.4) and acetonitrile (7:3) were used in isocratic mode of HPLC

Growth of *Stenotrophomonas* sp. HCU1 on N-heterocyclic aromatic compounds

Strain HCU1 was tested for its ability to use pyrazine, pyrazine-2-amide, pyrazine-2-carboxylate, nicotinic acid, uracil, imidazole and dipicolinic acid as sole source of nitrogen. Growth could not be demonstrated when used as a nitrogen source except for pyrazine-2-carboxylate however, when used as an additional supplement, reduction of these N-heterocyclic compounds was observed. The compounds which were reduced (% reduction) by strain HCU1 include pyrazine (20%), pyrazine-2-carboxylate (83%), nicotinic acid (20%), uracil (18%), imidazole (15%) and guanine (20%), while pyrazinamide, dimethylaminopyridine and dipicolinic acid could not be reduced.

Characterization of major metabolite isolated from fraction A

One metabolite was isolated through semi-preparative HPLC from fraction A. This metabolite had a t_R of 1.55 min. The metabolite was characterized based on IR, ^{1}H, ^{13}C NMR and mass spectroscopic analyses (Table 1). The metabolite is a white amorphous solid and has absorption maximum at 268 nm. The IR spectral analysis (KBr pellet) indicated NH-stretching (3445 cm^{-1}) and bending (1595 cm^{-1}), secondary amine stretching (1319 cm^{-1}), $-CH_2-$ bending (1408 cm^{-1}) and =CH–bending vibrations (675 cm^{-1}). The ^{1}H NMR spectrum shows (Supplementary Fig. 1A) a singlet at δ 3.3 ppm, triplet at 3.7 ppm, doublets at 4.28 ppm, triplet at 2.6 ppm, quartet at 2.4 ppm and singlet at 9.03 ppm. The resonance at δ 3.3 and 3.7 ppm are assigned to the singlet of triplet –N<u>H</u>–C<u>H</u>–C<u>H</u>–group. At δ 4.28 ppm, doublet corresponds to =C<u>H</u>–CH–bonding and at δ 2.6 and 2.4 ppm, triplet of quartet corresponds to –C<u>H</u>$_2$ –C<u>H</u>$_2$–NH bonding. Singlet δ 9.0 ppm correspond to –COO<u>H</u> proton. The ^{13}C (broad band) spectrum shows (Supplementary Fig. 1B) peaks at δ 42.32 ppm corresponding to –<u>C</u>H$_2$– group, δ 70.22 ppm corresponding to –NH–<u>C</u>H–COOH, δ144.54 ppm corresponding to –N=<u>C</u>H–group. Resonance at δ180.87 ppm corresponds to –<u>C</u>OOH carbon. The experimental NMR shift values matched with the predicted (Table 1) and such comparison was made since no reference match was available. The mass of the metabolite is 128 (M^+). Based on the spectral data the metabolite is proposed as 1,2,5,6-tetrahydropyrazine-2-carboxylate. The ring saturation of the metabolite was confirmed through positive reaction to cyclic imino acid using ninhydrin reagent (Piez et al. 1956).

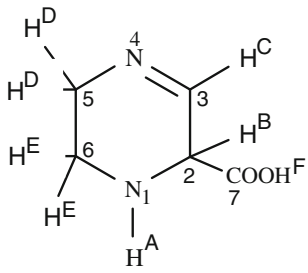
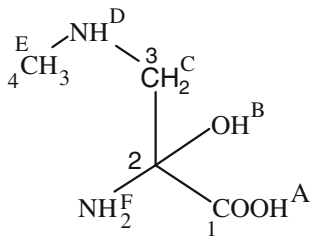
Characterization of major metabolite isolated from fraction B

Eight chromatographically (HPLC) distinct peaks were observed from fraction B (Data not shown). The major metabolite (B1) resolving at 1.5 min was isolated through semi preparative HPLC. The metabolite was characterized based on IR, ^{1}H, ^{13}C NMR and mass spectroscopic analyses (Table 1). The metabolite is an orangish sticky compound and has an absorption maximum at 400 nm. The IR spectral analysis (KBr pellet) indicated OH– and –COOH group stretching (3416, 1711, 1095 cm^{-1}), –NH stretching (3416 cm^{-1}), –NH bending (1614 cm^{-1}), $-CH_3$ bending (1398 cm^{-1}) and –CN group bending (1022 cm^{-1}). The ^{1}H NMR spectrum shows (Fig. 2A, B) a singlet at δ 8.7 ppm corresponding to –COO<u>H</u> proton. Resonance at δ 4.7 ppm singlet corresponds to –O<u>H</u> group, doublet at δ 4.3 ppm, quartet at δ 2.4 ppm and doublet at δ 2.7 ppm corresponds to –C<u>H</u>$_2$–N<u>H</u>–C<u>H</u>$_3$ bonding connectivity. The ^{13}C (broad band) spectrum shows (Supplementary Fig. 2C) peaks at δ 42.5 ppm corresponding to <u>C</u>H$_3$–NH–CH$_2$–group, δ 70.2 ppm corresponding to NH$_2$–<u>C</u>(OH)–COOH and δ 49.5 ppm corresponding to CH$_3$–NH–<u>C</u>H$_2$–C–COOH group. Resonance at δ 181.05 ppm corresponds to –<u>C</u>OOH carbon. The experimental NMR shift values matched with the predicted (Table 1) and such comparison was made since no reference match was available. The mass of the metabolite is 134 (Molecular ion) and the mass fragments (MH^{-1}) (*m/z)* are; 133, 119, 115, 96 and 89. Based on the spectral data analyses, the metabolite is proposed as 2-amino-2-hydroxy-3-(methylamino) propanoic acid. The metabolite showed negative reaction to cyclic imino acid assay.

Isolation and characterization of other metabolites

Two more metabolites (B2 and B3) were isolated from fraction B through semi preparative HPLC, however, these could not be completely characterized owing to their low concentrations. From the molecular mass analysis of the metabolites (Fig. 3A, B), we

Table 1 FT-IR, ^{1}H, ^{13}C and mass spectral data of the purified metabolites

Parameter	Proposed structure and data for			
	Metabolite A1		Metabolite B1	
				
	Experimental	Predict	Experimental	Predict
$\delta(^{1}H)$ (ppm)[b]				
1-H^{A}	3.3 (s,1H)	3.5	8.7 (s,1H)	8.6
2-H^{B}	3.7 (t, 1H)	3.6	4.7 (s,1H)	4.5
3-H^{C}	4.28 (d,1H)	4.2	4.3 (d,2H)	4.5
5-H^{D}	2.6 (t,2H)	3.0	2.4 (q,1H)	3.0
6-H^{E}	2.4 (q,2H)	2.8	2.7 (d,3H)	2.6
7-H^{F}	9.0 (s,1H)	7.4	9.2 (bds, 2H)	8.1
δ (^{13}C) (ppm)[C]				
C-5,6	42.32	47	C-4; 42.5	36
C-2	70.22	74	70.2	73
C-3	144.54	146	49.5	50
C-7	180.87	184	C1; 181.05	179
FT-IR (cm^{-1})	3445, 1730, 1595, 1408, 1310, 1188, 1093, 675		3416, 1711, 1614, 1398, 1095, 1022	
Molecular mass (m/z)	128		134	
Mass fragmentation (M^{+}-1) (m/z)	128 [100%], 102 [5%]		133 [6%], 118 [7%], 115 [100%], 95 [18%], 89 [5%]	
IUPAC name	1,2,5,6-tetrahydropyrazine-2-carboxylic acid		2-amino-2-hydroxy-3-(methylamino) propanoic acid	

[b] ^{1}H chemical shifts are converted to the TMS scale with δ (D_2O, 400 MHz, solvent residual peak referenced at 4.7 ppm at pH7; Jeús et al. 2007) equal to 4.7 ppm. *bds* broad diffused peak

[C] ^{13}C chemical shifts assigned based on TMS as external standard

predicted them to be 1,4-dihydropyrazine-2-carboxylic acid and piperazine-2-carboxylic acid.

Isolation and characterization of an enzyme involved in the ring reduction of pyrazine-2-carboxylate

Pyrazine-2-carboxylate ring reduction was observed by growing, resting and by using cell free extracts (Fig. 2B, C, D). The data indicated a strong aromatic ring reductase involved in the pyrazine-2-carboxylate reduction, which was mined using cell free extracts of pyrazine-2-carboxylate adapted culture. The protocol used for purification of enzyme is given in materials and methods. The enzyme is a ~65 kDa heterodimer of ~43 and 22 kDa subunits (Fig. 4A). It is NADPH dependent reductase, catalyzing the partial reduction of pyrazine-2-carboxylate. Figure 5 shows the reduction of pyrazine-2-carboxylate and simultaneous oxidation of NADPH by the purified enzyme. The

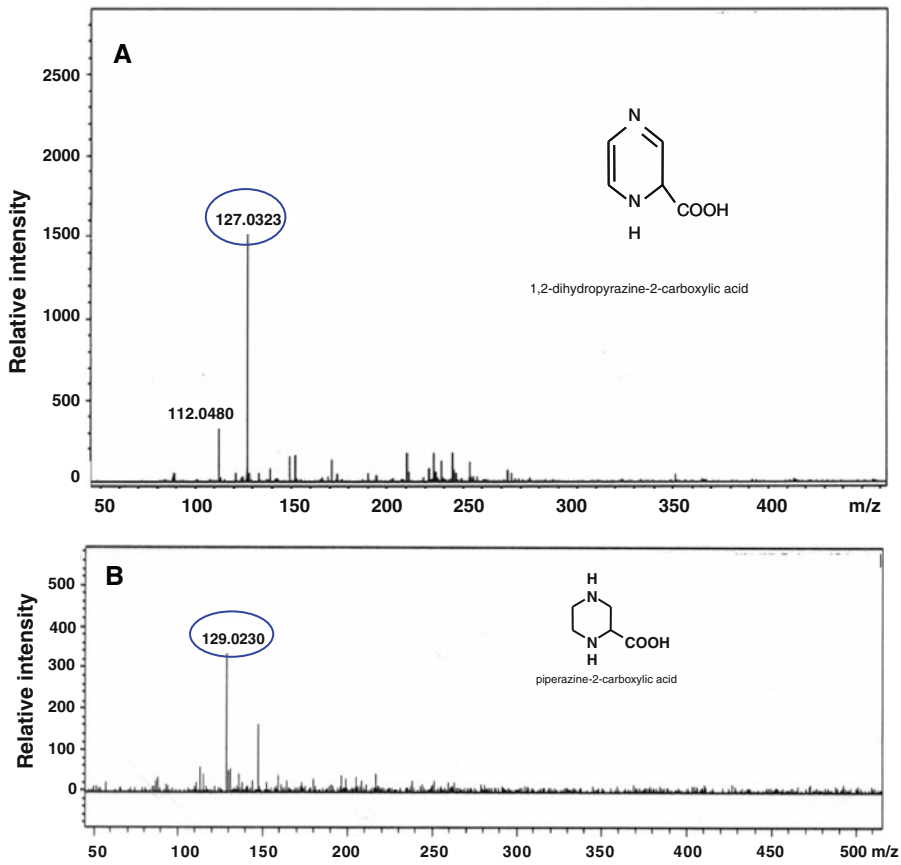


Fig. 3 Identification of two (**A**, **B**) ring saturated metabolites (1,4-dihydropyrazine-2-carboxylic acid and piperazine-2-carboxylic acid) from the culture supernatant (fraction B2 and B3, respectively) of strain HCU1 based on LC-MS analysis. Spectral data was taken in negative mode

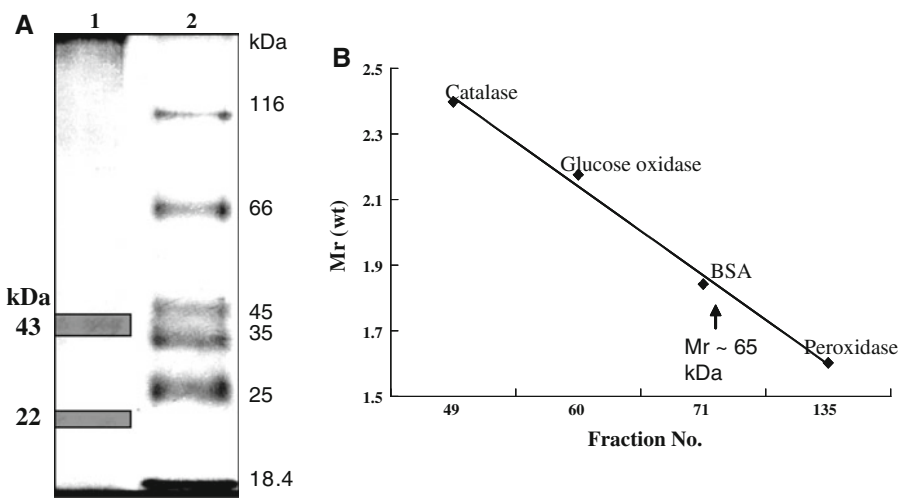


Fig. 4 SDS-PAGE (10%; **A**) and molecular mass analyses (using FPLC; **B**) of the purified pyrazine-2-carboxylate reductase. *Lane 1* subunits of reductase; *Lane 2* protein markers

assay supernatant has a molecular mass of 128 (Fig. 6A) corresponded to the reduced product, which was not observed in the enzyme assayed control (Fig. 6B). Apart from pyrazine-2-carboxylate ring reduction (46%), enzyme also catalyzed the ring reduction of pyrazine (7%), nicotinic acid (10%), guanine (18%), uracil (24%) and imidazole (3.6%). However, the activity was not observed with

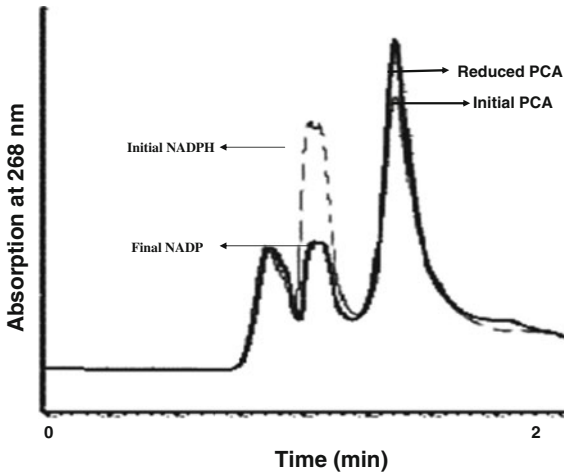


Fig. 5 HPLC chromatogram of enzyme (pyrazine-2-carboxylate reductase) assayed supernatant showing reduction of pyrazine-2-carboxylate and oxidation of NADPH

pyrazinamide, piperazine, dimethylaminopyridine and dipicolinic acid. The enzyme kinetics is shown in Fig. 7A and has a *Km* of 0.475 mmoles and *Vmax* of 18.2 (m AU/μg min^{-1}; Fig. 7B) with a pH and temperature optimum of 7.4 and 30°C.

Total peptide finger printing pattern of the reductase (Table 2) showed the fragments of 0.8, 1.042, 1.363, 1.564 and 2.053 kDa. Mascot search (http://www.matrixscience.com) of the peptide finger printing analysis indicated 59.80 and 43.60 score with the 4-hydroxyphenylpyruvate dioxygenases of *Bacillus* sp. Sg-1 and aspartate semialdehyde dehydrogenase of *Thermoanaerobacter tengcongensis* MB4, respectively. MS–MS fragmentation of the major peptide fragment of mass 1.564 kDa matched with 2-methylthioadenine synthetase of *Vibrio vulnificus* with a score of 16.80. 1.363 kDa peptide fragmentation matched with aromatic amino acid aminotransferase of *Xylella fastidiosa* Temicula 1 with 6.79 score and 1.042 kDa peptide mass fragmentation pattern matched with oxidoreductase 2OG-Fe(II) oxygenase family protein [*Burkholderia thailandensis* MSMB43] with a score of 24.54 and peptide mass fragmentation of 0.882 kDa matched with soluble pyridine nucleotide transhydrogenase of *Mycobacterium abscessus* with a score of 9.80. The reductase isolated from *Stenotrophomonas* sp. HCU1 differs from aromatic ring reductase of *Nocardiodes simplex* (Ebert et al. 1999), *Xen* B reductase of *Pseudomonas fluorescens* (Pak et al. 2000) and 2-aminobenzoyl-CoA monooxygenase/reductase (110 kDa; a tetramer of 42, 30 and 29 kDa) of a denitrifying *Pseudomonas* sp. (Altenschmidt et al. 1992) in the absence of F_{420} or FAD requirement. F_{420} dependent NADPH reductase catalyzing the aromatic ring reduction of

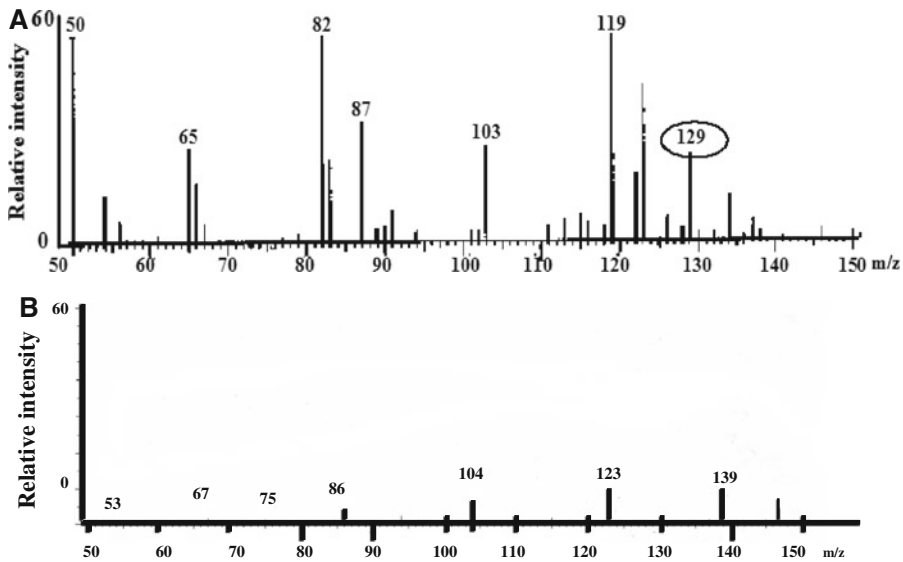


Fig. 6 LC-MS chromatogram of enzyme assay (**A**) and blank (**B**) samples. Encircled mass in spectrum **A** indicates reduced metabolite m/z 129 (MH^{+1}) which is absent in blank **B**

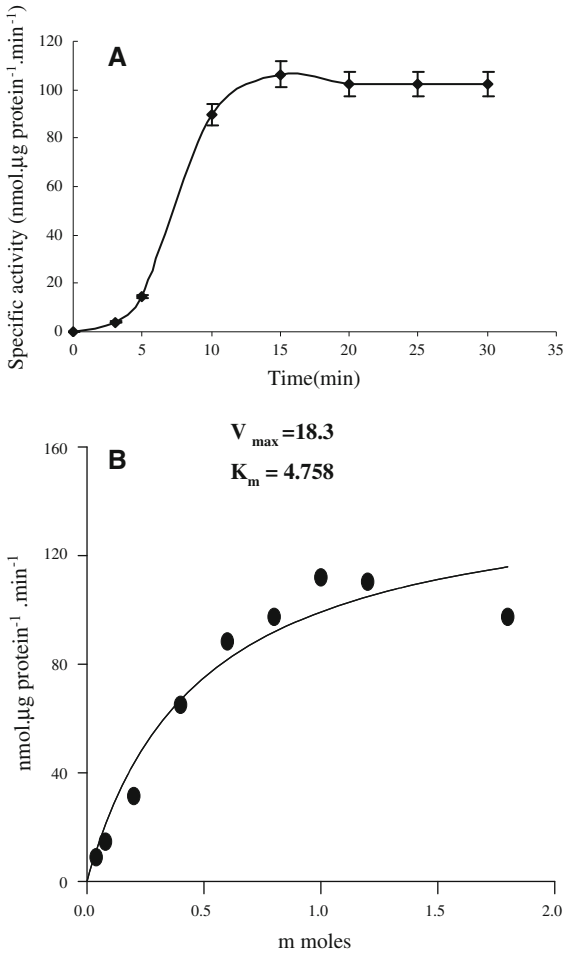


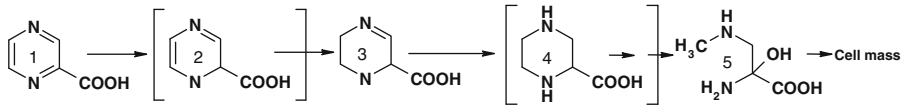
Fig. 7 Kinetics (**A**), *Km* (mmoles) and *Vmax* (min^{-1}) (**B**) of pyrazine-2-carboxylate reductase

2,4,6-trinitrophenol is a two component enzyme system containing NADPH dependent F_{420} reductase (component A) of 30 kDa and hydride transferase (component B) of 38 kDa (Ebert et al. 1999). The pyrazine-2-carboxylate reductase of *Stenotrophomonas* sp. HCU1 also differs from dihydropicolinic acid reductase of *Escherichia coli* (Tamir and Gilvarg 1974) and pentaerythritoltetranitrate reductase (French et al. 1998) of *Enterobacter cloaceae* which are flavin independent enzymes.

Degradation of aspergillic acid in *Trichoderma koningii* was catalyzed by an unnamed 112 kDa enzyme to 2-hydroxyimino-3-methyl-1-pentanal (Nishimura et al. 1997). On the other hand, enzymes with broad substrate specificity like nitrilase, dehydrogenase and 6-methylnicotinate-2-oxidoreductase are also known for transformation of pyrazines (Weiser et al. 1997; Tinschert et al. 2000). Our study indicates a novel degradative pathway (Fig. 8) of pyrazine-2-carboxylate, which proceeds through an initial ring reduction followed by hydroxylation and ring cleavage with a loss of carbon. The initial step of ring reduction in this pathway is catalyzed by an enzyme which is novel as evidenced by MALDI analysis. Anaerobic bacteria metabolize aromatic compounds preferably through a reductive pathway (Carmona et al. 2009), while such an aromatic ring reduction by aerobic bacteria was reported with *Pseudomonas* (Pak et al. 2000), *Nocardiodes* (Ebert et al. 1999) and in the present study with strain HCU1.

Table 2 Amino acid sequences found during MALDI-TOF MS/MS analyses of peptides along with the probable protein and sequence coverage

Peptide mass (kDa) MS/MS	Probable protein	Sequence coverage (%)	Database accession no.	Amino acid sequences	Organism
2.053	–	–	–	–	–
1.564	2-methylthioadenylate synthetase	16.80	gi27365365	QSRLAYDLIEEVK	*Vibrio vulnificus* CMCP6
1.363	Dehydrogenase(putative), aromatic amino acid amino transferase	6.79	gi149915814	GAAPRRPILAEGR	*Roseobacter* sp. AZWK-3B
		6.79	gi28197970	ALGAPEFDFIQR	*Xylella fastidiosa* temicula1
1.042	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	24.54	gi167838986	KTALDELPR	*Burkholderia thailandensis* MSMB43
0.882	Soluble pyridine nucleotide transhydrogenase	9.80	gi169630115	TEHVIRK	*Mycobacterium abscessus*



1. Pyrazine-2-carboxylic acid (m/z)
2. 1,2-Dihydropyrazine-2-carboxylic acid [B2] (m/z)
3. 1,2,5,6-Tetrahydropyrazine-2-carboxylic acid [A1] (m/z 128)
4. Piperazine-2-carboxylic acid [B3] (m/z 130)
5. 2-Amino-2-hydroxy-3-(methyl amino)propanoic acid [B1] (m/z 134)

Fig. 8 Putative reductive pathway of pyrazine-2-carboxylate catabolism in *Stenotrophomonas* sp. HCU1 proposed based on the metabolites identified. While 2 metabolites (A1 and B1) were confirmed, metabolites B2 and B3 are the putative metabolites proposed based on the mass spectral analysis and hence given in the parenthesis

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