Catabolism of pyrazine-2-carboxylate by a newly isolated strain of *Stenotrophomonas* sp. HCU1

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DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that Ms K.S. Rajini has carried out the research work embodied in the present thesis under the supervision and guidance of Dr. Ch. Venkata Ramana for a full period prescribed under the Ph.D ordinance of this University. We recommend her thesis **"Catabolism of pyrazine-2-carboxylate by a newly isolated strain of *Stenotrophomonas* sp. HCU1"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Catabolism of pyrazine-2-carboxylate by a newly isolated strain of *Stenotrophomonas* sp. HCU1"** has been carried out by me under the supervision of Dr. Ch. Venkata Ramana and this has not been submitted to any other Institute or University for the award of any degree or diploma.

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Rajini

Dedicated to my
beloved parents
& husband

LIST OF ABBREVIATIONS

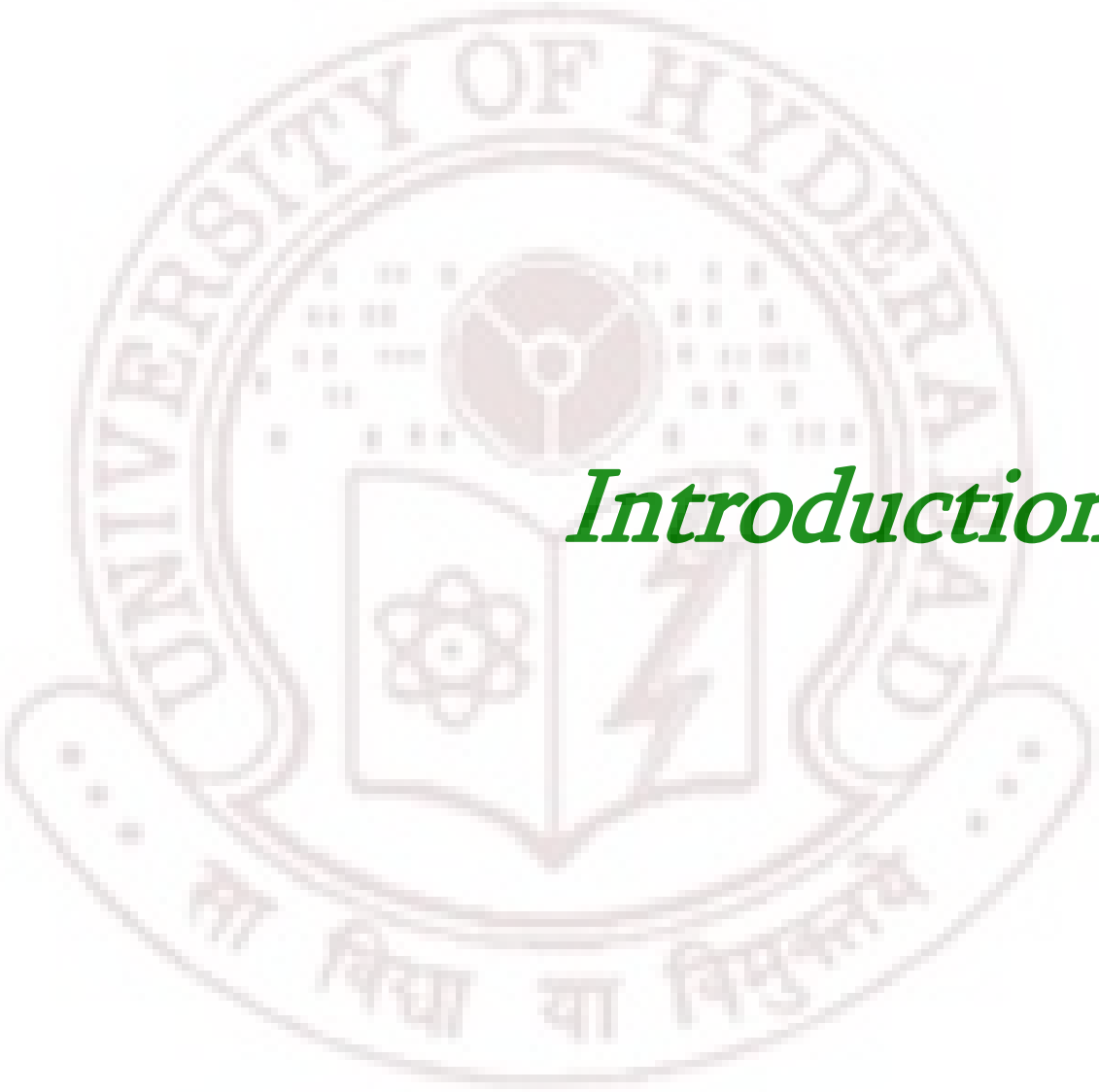
ATP	: Adenosine triphosphate
Da	: Dalton
DAD	: Diode array detector
DEAE Cellulose	: Diethyl aminoethyl cellulose
ESI	: Electron spray ionization
$FADH_2$	: (Reduced) flavin adenine dinucleotide
FT-IR	: Fourier transform infra red spectroscopy
GC-MS	: Gas chromatography mass spectroscopy
HCCA	: 4-cyano hydroxycinammic acid
HPLC	: High pressure liquid chromatography
LC-MS	: Liquid chromatography mass spectroscopy
m mol	: Milli moles
mM	: Milli molar
MALDI TOF	: Matrix associated laser desorption ionization time of flight
MS/MS	: Mass fragmentation of the fragmented mass
NADPH	: (Reduced) nicotinamide adenine dinucleotide phosphate
NMR	: Nuclear magnetic resonance spectroscopy
PAGE	: Polyacryl amide gel electrophoresis
PAH	: Polycyclic Aromatic Hydrocarbons
PCA	: Pyrazine-2-carboxylate
PCR	: Polymerase chain reaction
PDAB	: Para dimethyl amino benzaldehyde
TFA	: Trifluro acetic acid
t_R	: Time of retention
UV	: Ultra violet
µg	: Micro gram

CONTENTS

Abstract

Pyrazines (1,4-nitrogen substituted benzenes) have near ubiquitous biological distribution and are also anthropogenic. Despite their wide occurrence and vast applications, their bacterial degradation studies are limited and hence, it was of interest to isolate a bacterium and study its potential to catabolize pyrazine-2-carboxylate, which was the objective of the thesis.

A bacterium (strain HCU1) capable of metabolizing pyrazine-2-carboxylate was selected from the 50 screened isolates and its affiliation to the genus *Stenotrophomonas* was indicated by its polyphasic characterization. The 16S rRNA gene sequence of strain HCU1 closely clustered with the type strain of *Stenotrophomonas maltophilia* (98.7% sequence similarity). Growth of *Stenotrophomonas* sp. HCU1 and ring reduction of pyrazine-2-carboxylate was demonstrated when pyrazine-2-carboxylate was used as a sole source of carbon, nitrogen and supplement. Ring reduction of pyrazine-2-carboxylate was observed with both growing and resting cells of *Stenotrophomonas* sp. HCU1. Out of 4 metabolites isolated from the culture supernatant of *Stenotrophomonas* sp. HCU1; two (1,2,5,6-tetrahydropyrazine-2-carboxylate, 2-amino-2-hydroxy-3-(methylamino) propanoic acid were characterized based on UV, IR, ^{1}H, ^{13}C NMR and mass spectroscopic analyses. Based on the masses of the identified metabolites a putative reductive pathway of pyrazine-2-carboxylate catabolism is proposed. A ~65kDa enzyme catalyzed the reduction of pyrazine-2-carboxylate to 1,2,5,6-tetrahydropyrazine-2-carboxylate and the enzyme also had a broad substrate specificity for other N-heterocyclic compounds.

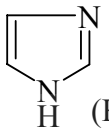


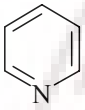
Introduction

1.1 General Introduction

Compounds with oxygen, sulphur, phosphorous and nitrogen substitutions in the aromatic ring are called heterocyclic compounds. Heterocyclic compounds comprise $^{2}/_{3}$ of the known aromatic compounds (Kaiser *et al.,* 1996). They occur naturally and are also anthropogenic. Aromatic compounds exist naturally in biological systems as electron carriers, nucleotides, energy storage molecules, mycotoxins, vitamins and alkaloids. Aromatic compounds are common constituents of geological sources such as petroleum, coal, soil and oil shale and occur through transformations of non-aromatic biological molecules or during burial with effect of physical factors like temperature, pressure and catalytic action of mineral matrix (Barakat *et al.,* 1998). N-heterocycles and S-heterocycles are detected in carbonaceous chondrite meteorites (Sephton, 2002). Anthropogenic heterocyclic aromatic compounds are used as industrial solvents, dyes, explosives, pharmaceuticals and pesticides. Owing to their relatively high water solubility and weak sorption to soil and aquifer materials, heteroaromatic contaminants have the potential to be transported readily to subsurface environments, resulting in the contamination of subsoil regions and groundwater (Hale and Aneiro, 1997). In addition, they are also identified in aquatic sediments, industrial and urban air, seawater and also in marine organisms (Fetzner, 1998). Groundwater contamination by N-heterocyclic compounds has been investigated extensively (Canter and Sabatini, 1994). Contamination of N-heterocycles is of major concern since many of these compounds are toxic, mutagenic and carcinogenic (Fetzner, 1998). Microbial degradation of the aromatic heterocycles is superior to physical and chemical treatment owing to their more expensive way of physical and chemical treatment.

1.2 **Bacterial catabolism of N-heterocyclic compounds**

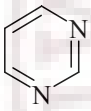
1.2.1 **Imidazoles:** Imidazoles are pentacyclic aromatic rings with nitrogen substitution at  1 and 3 positions. They have antifungal and antimicrobial activities (Kyecuekbay *et al.,* 1995). Biotransformation of imidazoles by *Streptomyces* and biodegradation of histamine (derivative of imidazole) by *Mycobacteria* to imidazolyl ethanol and imidazolyl acetic acid was reported (Tarnok and Tarnok, 1986).

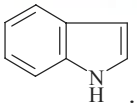
1.2.2 **Pyridines:** Pyridine derivatives are natural constituents of plant alkaloids,  pyridoxyl derivatives and coenzymes such as nicotinamides and nicotines. Anthropogenic derivatives of pyridine include paraquat, diquat and picloram, which are used as herbicides. The simplest of all the pyridines is the pyridine itself whose biodegradation was mostly studied. Aerobic and anaerobic degradation pathways of pyridines by bacteria were proposed and reviewed (Kaiser *et al.,* 1996). *Clostridium* sp. (Holcenberg and Tsai, 1969), *Brevibacterium* sp. (Shukla, 1973), *Corynebacterium* sp. (Shukla and Kaul, 1974), *Nocardia* sp. Z1; *Bacillus* sp. strain 4 (Watson and Cain, 1975), *Micrococcus luteus* (Sims and Oloughlin, 1989), *Rhodococcus opacus* (Brinkman and Babel, 1996), *Gordonia nitida* (Lee *et al.,* 2001), *Pseudomonas* sp. (Mohan *et al.,* 2003; Pandey *et al.,* 2007), *Pseudonocardia* sp. M43 (Lee *et al.,* 2006), *Paracoccus* sp. (Bai *et al.,* 2008), *Shewanella putrefaciens* and *Bacillus sphaericus* (Mathur *et al.,* 2008) were the bacteria reported to degrade pyridines. Several intermediates of the biodegradative pathway were elucidated. Biodegradation of pyridines involves mineralization to glutaric, succinic, maleic and pyruvic acids, (Kaiser *et al.,* 1996) formic acid and ammonia (Lee *et al.,* 2001). Biodegradation of pyridine up to 50 $mg.l^{-1}$ by a bacterial consortium was also reported (Lodha *et al.,* 2008). Biodegradation of pyridoxine in *Arthrobacter* and

Mesorhizobium loti was reported and the gene coding for pyridoxic acid dehydrogenase was characterized (Ge *et al.,* 2008). Gene cluster (*nic* genes) in *Pseudomonas putida* responsible for the aerobic degradation of nicotinic acid was characterized (Jime'nez *et al.,* 2008).

1.2.3 **Pyrazines:** Pyrazines are a group of aromatic compounds with 1, 4-nitrogen  substitutions in the benzene ring. They occur naturally and are also anthropogenic. They are wide spread in nature. Their occurrence, abundance, uses, applications and microbial metabolism are discussed in detail in the section 1.3.

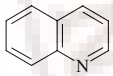
1.2.4 **Pyrimidines:**

 Pyrimidines are major components of nucleic acids. Uracil, a derivative of pyrimidine induced carcinogenicity in rats and mice (Fukushima *et al.,* 1992). Pyrimidine degradation by microorganisms was well reviewed by Vogels and van'der Drift (1976) and Berry *et al.,* (1987) under aerobic and anaerobic conditions. Gene cluster involved in the reductive pathway of pyrimidine catabolism in *Brevibacillus agri* NCHU1002 (Kao and Hsu, 2003) and operon involved in a novel pathway of pyrimidine catabolism in *E.coli* K-12 was characterized (Loh *et al.,* 2006).

1.2.5 **Indoles:** Indole is commonly used name for the benzene ring fused to the 2  and 3 position of pyrrole ring. As anticipated for π-excessive compounds, indole is highly reactive towards electrophilic reagents which include acids and oxidants (Houlihan, 1972) and C3 position of indole is the preferred site for all substitutions (particularly microbial substitutions). Aerobic degradation of indole was reported in *Alcaligenes* (Claus and Kutzer, 1983) *Pseudomonas* sp. (Doukyu and Aono,

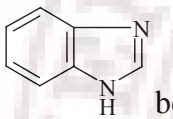
1997) *Acinetobacter* sp. (Kim *et al.,* 2001) and *P. aeruginosa* (Yin *et al.,* 2005). Biodegradative pathway of indole in a gram negative bacterium via indoxyl, 2, 3-dihydroxyindole, isatin, anthranilate, salicylate and catechol was proposed. Direct conversion of 2, 3-dihydroxyindole and further degradation using N-carboxyanthranilate was reported in a gram-positive coccus by Fujiioka and Wada (1968). Claus and Kutzer, (1983) proposed degradative pathway of indole by *Alcaligenes* sp. to fumarate and pyruvate. Anaerobically indole was degraded by methanogens to methane (Wang *et al.,* 1984; Gu and Berry, 1991), and *Desulfobacterium indolicum* (Bak and Widdle, 1986) as a source of carbon and electrons under anaerobic denitrifying conditions (Madsen and Bollag, 1989; Gu and Berry, 1991).

1.2.6 **Quinolines:**

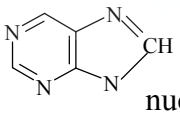


Quinoline contains two fused benzene rings, one of which contains nitrogen atom instead of carbon. Though quinolines are known for their anti microbial activity (Foley and Tilley, 1998), aerobic and anaerobic bacterial degradation and transformation of quinolines were reported (Kaiser *et al.,* 1996). Bacterial strains, which degrade quinolines, include *P. putida, P. fluorescens, Rhodococcus* sp., *Moraxella* sp., *Nocardia* sp., *Desulfobacterium* sp. (Aislabie *et al.,* 1990) and *Burkholderia pickettii* (Jianlong *et al.,* 2002). Four pathways of aerobic degradation of quinoline are known; (1) 5, 6-dihydroxy-2(1H) quinolinate pathway, (2) 7, 8-dihydroxy-2(1H) quinolinate pathway, (3) Anthranilate pathway and (4) 8-hydroxycoumarin pathway (Fetzner, 1998). In both 5, 6-dihydroxy-2(1H) quinolinate and 7, 8-dihydroxy-2(1H) quinolinate pathways, after initial hydroxylation of carbon atom adjacent to N atom, benzene ring is dihydroxylated and subsequently undergoes ring cleavage (this pathway was reported in

Commamonas testesteroni and *Pseudomonas putida*). In contrast, in both anthranilate pathway and 8-hydroxycoumarin pathway, pyridine ring was cleaved prior to benzene ring cleavage. In anthranilate pathway, 2, 4 dioxygenases are rather exceptional as they catalyze the cleavage of two carbon-carbon bonds with the release of carbon monoxide. The molybdenum containing hydroxylases, unusual 2, 4-dioxygenases and *Xen A* reductase of the degradation pathway were discovered (Fetzner, 1998; Griese *et al.,* 2006).

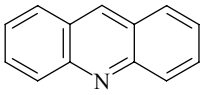
1.2.7 **Benzimidazole:** Benzimidazoles have great biological significance. They are  closely related to purines and 5, 6-dimethyl-1-1-1-(α-D-ribofuranosyl) benzimidazole is an integral part of vitamin B_{12}. Synthetic benzimidazoles are widely used as antihelminthics and fungicides (Preston, 1981). Benzimidazoles have antimicrobial activity against *Helicobacter pylori* and *Fusobacter nucleatum* (Sheng *et al.,* 2006). Benomyl (derivative of benzimidazole) was degraded by *Pseudomonas* strains to 2-aminobenzoate and carbamic acid methyl ester side chain (Fuchs and de Vries, 1978). Carbendazim, a widely used fungicide was degraded by *Rhodococcus erythropolis* (Holtman and Koboyashi, 1997) and *Rhodococcus* sp. djl-6 (Jing-Liang *et al.,* 2006). In this strain, degradation was initiated by the cleavage of methyl carbamate side chain resulting in the formation of 2-aminobenzoate and benzimidazole.

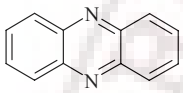
1.2.8 **Purines:**

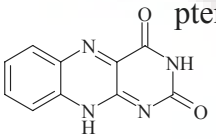
 Purines are major components of nucleic acids. However, purine nucleosides are widely used as antibiotics and chemotherapeutic agents. Purine degradation by microorganisms was well reviewed by Vogels and van'der Drift

(1976) and Berry *et al.,* (1987). Microorganisms capable of metabolizing purines for growth include *Clostridium aciurici*, *Clostridium cylindrosporum* and *Clostridium purinolyticum* (Durre and Andreesen, 1983), *Pseudomonas aeruginosa*, *Ralstonia* sp., *Bacillus subtilis* (Vogels and van'der Drift, 1976) and complete mineralization of these compounds was established (Woolfolk, 1975). Degradation of purine by *Rhodobacter capsulatus* to urea was reported using a mutant. The degradation pattern was identical under aerobic and anaerobic conditions (Busse *et al.,* 1984). Genes and operons that constitute the purine catabolic pathway were characterized (Schulz *et al.,* 2001).

1.2.9 **Carbazoles:** Carbazole is one of the main components of shale oil, crude oil, petroleum products and coal tar. Carbazole used extensively as industrial raw material, is a well-known environmental pollutant. Microbial utilization and degradation of carbazoles were reported (Ouchiyama *et al.,* 1993; Geg *et al.,* 1996; Obata *et al.,* 1997; Li *et al.,* 2006). Alkylcarbazoles are predominant in crude oils and most of the C1, C2, and C3 carbazoles were found to be degraded by a mixed bacterial culture (Fedorak and Westlake, 1984). Carbazole was degraded by *Ralstonia* sp. to anthranilic acid, indole-2-carboxylic acid, indole-3-carboxylic acid, and (1H)-4-quinolinone (Schneider *et al.,* 2000). Carbazole degradation by *Sphingomonas* (Kilbane II *et al.,* 2002) to anthranilic acid, succinic anhydride, succinic acid and fumaric acid was reported. Carbazole catabolic genes (*car* genes) in *Nocardiodes aromaticivorans*, *Pseudomonas resinovorans* and *Sphinogomonas* sp. were studied extensively (Inoue *et al.,* 2006).

1.2.10 **Acridines:** Acridines are three ring N-containing heterocyclic aromatic compounds widely used in the manufacture of dyes and antiseptics. Degradation of  acridines by anaerobic, denitrifying and sulfate reducing microorganisms involves the oxidation of either benzene or pyridine ring and follows common degradation pathway of aerobic aromatic compounds (Knezovich *et al.,* 1990; Bianchi *et al.,* 1997).

1.2.11 **Phenazines:** Phenazines are dibenzopyrazines. They constitute a large family of  heterocyclic nitrogen containing compounds with broad spectrum antibiotic activity. They have antifungal activity and hence used as biocontrol agents. Many strains of the genus *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium* and *Burkholderia* synthesize phenazines. *Pseudomonas aeruginosa* strains produce pyocyanin, phenazine-1-carboxylic acid and phenazine-1-carboxamide (Chin-A-Woeng *et al.,* 2003). Degradation of phenazine-1-carboxylic acid to 4-hydroxy-1-(2-carboxyphenyl) azacyclobut-2-ene-2-carbonitrile and 4-hydroxy-1-(2-carboxyphenyl)-2-azetidinecarbonitrile by *Sphingomonas* sp. DP58 was reported recently (Chen *et al.,* 2008).

1.2.12 **Pteridines and Flavins:** Compounds with fused pyrimidine and pyrazine rings are  pteridines. The yellow-coloured compounds with the basic structure of 7, 8-dimethyl-10-alkylisoalloxazine are generally termed as flavins. Flavins are ubiquitous in nature, and they take part in many biochemical reactions as coenzymes and photoreceptors. Riboflavin is the precursor of all the biologically important reactions. The flavin group is capable of undergoing oxidation-reduction reactions and can accept

either one electron in a two-step process or two electrons at once. Reduction is made with the addition of hydrogen atoms to specific nitrogen atoms on the isoalloxazine ring system. There is a broad distribution of flavins, the majority is found in flavocoenzymes, mainly as flavin adenine dinucleotide (FAD), and in lesser amounts as flavin mononucleotide (FMN), with the common name of riboflavin-5-phosphate. Bacterial degradation of riboflavin by a *Pseudomonas* sp. was known (Harkness and Stadman, 1965).

1.3 **Pyrazines**

1.3 1 **Occurrence of pyrazines**

Pyrazines are widely distributed in nature, occur naturally and are also anthropogenic. Of the pyrazines, alkylated pyrazines are the most abundantly found. They are widely distributed in plants, animals and micro-organisms (Murray and Whitfield, 1975; Woolfson and Rothschild, 1990; Beck *et al.,* 2003). In plants, they occur in leaves, seeds, spices, vegetable tissues, fruits and vegetable oil (Table1). Their concentration was notably increased in species (*Asclepias, Aristolochia*, *Solanum*, *Urticasonchus* and *Aspergillus*) containing toxic material with injurious and urticating properties (Brophy, 1989). The first insects in which pyrazines were discovered were pomerine ants of the genus *Odontomachus*; in which they function as alarming pheromones (Wheeler and Blum, 1973). Their occurrence in wasps, flies, bees, aposematic beetles, butterflies, moths, plant bugs and grasshopper was also reported (Table 2). In some species, they are stored in poison glands, anal glands and mandibular glands and in many; their location in the body was not determined. Pyrazine derivatives exhibit wide aromas in food (Table 3). 2-methoxy-3-isopropylpyrazine, 2-acetylpyrazine

produces a green pea and popcorn like odour respectively. Dimethyl and trimethylpyrazines exhibit the aroma of the roasted nuts and coffee respectively (Rowe, 2005).

Pyrazines (mostly alkylated pyrazines) are synthesized by few of the bacterial and fungal species during their primary or secondary metabolism (Table 4). Aspergillic acid, an important group of naturally occurring pyrazine derivative synthesized by *Aspergillus flavus*, was the first natural product shown to be cyclic hydroxamic acid (Micetich and Mac Donald, 1965). Deoxyaspergillic acid, 3, 6-diisobutyl-2-pyrazinone (flavacol), hydroxyaspergillic acid, neohydroxyspergillic acid, muta-aspergillic acid and 2-hydroxy-3,6-di-sec-butylpyrazine (Buchanan and Houston, 1982) were the other derivatives of aspergillic acid synthesized by *Aspergillus* spp. Hydroxylated pyrazine such as 2-hydroxy-3, 6-diisobutylpyrazine-1, 4-oxide (pulcherriminic acid) was synthesized by *Candida pulcherrima* (Mac Donald, 1965). Volatile pyrazines were formed in the arctic bacteria of cytophaga group (Dickschat *et al.,* 2005b). Dialkyl mixtures of isobutyl and sec-butyl moiety and methyl dialkyl substituted pyrazines were produced by *Paenibacillus polymyxa* (Beck *et al.,* 2003) (Table 6). Pyrazines exhibiting potato like odour were produced by the species of *Enterobacteriaceae* family (Gallois and Grimont, 1985). 2, 5-Dimethylpyrazine, 2, 3, 5-trimethylpyrazine and 2-methyl-5-isopropylpyrazines synthesized by *Klebsiella pneumoniae, Citrobacter freundii* and *Enterobacter agglomerans* were attractive to the Mexican fruit fly, *Anastrepha ludens* (Robacker *et al.,* 2002). Marine bacteria isolated from the North Sea (Germany), *Lokantella* strain BIO-204 and *Dinoroseobacter shibae* DFL-27 synthesize 2-butyl-3,6-

dimethylpyrazine and 2-isopentyl-3, 6-dimethylpyrazine (Dickschat *et al.,* 2005) in addition to 2-ethyl-3, 6-dimethylpyrazine and pyrazine.

1.3.2 **Uses and applications of Pyrazines**

Both naturally occurring and anthropogenic pyrazines have vast applications in agrochemical, pharmaceutical and industrial sectors. Naturally occurring pyrazines serve as alarming/alerting pheromones, trial pheromones, repellants and site markers in bees, insects and moths (Woolfson and Rothschild, 1990). Pyrazines impart flavour to roasted, toasted foods or similarly heated foods (Adams *et al.,* 2002). They are common constituents of foods and were thought to arise from heat induced condensation between amino acids and sugars through Strecker's degradation (their concentration in the foods is in the range of approximately 0.001 to 40 ppm). Many alkylated and methoxylated pyrazines exhibit strong odourous properties and hence used as important flavouring compounds in variety of foods (Table 3). Pyrazines exhibit a wide range of physiological activities compared to pyridine or pyrimidine analogues. They are widely used in the field of medicine as antineoplastics, diuretics, antiinflammatory agents, antidepressants, neuroprotectors and antituberculotics in addition to their use as plant growth regulators, bactericides and fungicides (Table 5).

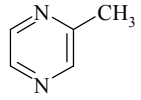
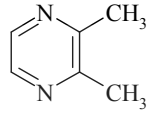
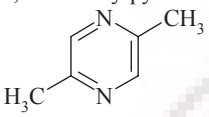
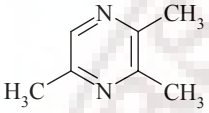
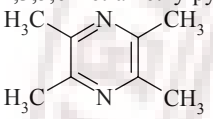
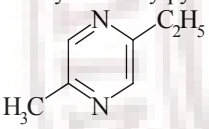
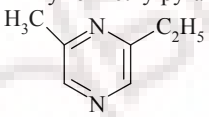
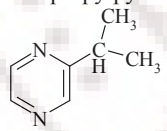
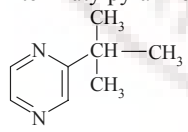
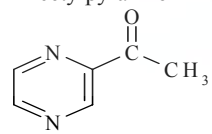
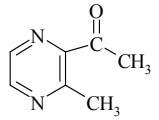
Pyrazines	Source	References
2,5-Dimethylpyrazine 2,5-Diethylpyrazine 2,3,5-Trimethylpyrazine 2,3,5,6-Tetramethylpyrazine	Fusel oil	Cheeseman, 1972
2-Methylpyrazine 2,3-Dimethylpyrazine 2,3,5-Trimethylpyrazine	Chitin pyrolysis at 300-500°C.	Knorr *et al.*, 2006
Alkylpyrazines	Commercial peanut butter preparations	Joo *et al.*, 1997
2-Methoxy-3-isopropylpyrazine 3-Isopropylpyrazine 3-sec-Butylpyrazine 3-Isobutyl-2-methoxypyrazine	Green peas, raw vegetables tissues, leaves of *Lactura sativa*, *Capsicum anuum*	Murray and Whitfield, 1975
X	Roasted spices; Coriandrum	Vasundhara and Parihar, 1987
X	*Asclepia*, *Aristolochia*, *Solanum* and *Urticasonchus*	Brophy, 1989
X	Pepper oil and vetiver oil	Clery *et al.*, 2007

Table 1: Occurrence of pyrazines in plants

X = unidentified pyrazines

Type of pyrazine	Occurrence	Function	References
Palythazine and isopalythazine	*Palythoa tuberculosa*	Alerting its predators	Uemera *et al.,* 1979
2,5-Dimethyl-3-isopentylpyrazines	Ants	Intraspecific signals	Brophy, 1989
Pyrazine-luciferin	Coelenterates, fishes, squids and ostracods	Confers bioluminescence	Mc Capra, 1982; Herring *et al.,* 1982
Dihydroimidopyrazinone	*Cypridina hilgendorfi*	Bioluminiscence	Cheeseman, 1972
Tetrasubstituted alkyl pyrazines	Cephalic secretions of *Mesoponera*	Not specified	Cheeseman, 1972
Pyrazine derivatives	Tiger moth	Attracting its sexual partner and warning off predators	Moore *et al.,* 1990; Baker *et al.,* 1982
Pyrazine derivatives	180 ants, 29 wasps, 7 flies, 2 bees, 30 species of aposematic beetles, butterflies, moths, plant bugs, grasshoppers	Not specified	Moore *et al.,* 1990
Pyrazine derivative	Urine of coyotes and man	Not specified	Albone *et al.,* 1984; Woolfson and Rothschild, 1990
Pyrazine derivative	Nasal mucosa of cow and rabbits, gular glands of alligators and crocodiles	Alerting hypothesis	Weldon and Sampson, 1988
Pyrazine derivative	Faecal pellets of male rabbits and scent glands of Canadian *beaver*	Not specified	Woolfson and Rothschild, 1990
Pyrazines	*Odontomachus hastatus Odontomachus*	Alarming pheromones	Wheeler and Blum, 1973

Table 2: Occurrence of pyrazines in animals

Pyrazine	Odour	Occurrence
2-Methylpyrazine 	Musty, nutty roasted cocoa light	Baked fried and roasted products
2,3-Dimethylpyrazine 	Roasted meat in highest dilution, vanilla and chocolate like	Vegetables, dairy, meat , roasted products, cocoa, coffee, potato chips, shrimp, papaya, asparagus, cabbage and fried potato
2,5-Dimethylpyrazine 	Roasted, cocoa like	In many food flavours, vegetable, dairy, meat and roasted products
2,3,5-Trimethylpyrazine 	Burnt, roasted, earthy and tobacco like	Nuts, meat, roasted products, rum, whisky and popcorn
2,3,5,6-Tetramethylpyrazine 	Musty nutty and burnt flavour	In many flavours, bread, brandy, rum, beef, whisky, coffee and cocoa
2-Ethyl-5-methylpyrazine 	Roasted coffee and cocoa like	In many flavours, vegetables, bread, meat, whisky, coffee and cocoa
2-Ehyl-6-methylpyrazine 	Roasted cocoa and coffee connotations	In many food flavours, fish, meat, roasted products and in alcoholic beverages
2-Isopropylpyrazine 	Dusty roasted nuts	In fried chicken, cocoa filbert, peanut
2-ter-Butylpyrazine 	Green, earthy carrot	-------
2-Acetylpyrazine 	Pop corn, bread crust like, nutty	In roasted flavours, bread, popcorn, cocoa, peanut, filbert
2-Acetyl-3-methylpyrazine 	Roasted potatoes, nutty, vegetable and cereals	In meat, cocoa, coffee and fried potato Contd. in page 14

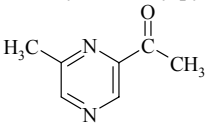
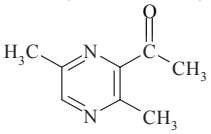
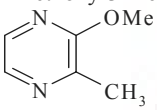
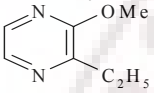
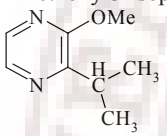
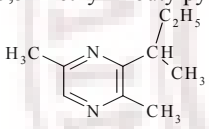
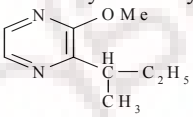
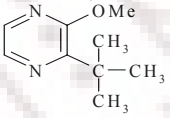
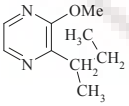
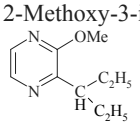
2-Acetyl-6-methylpyrazine 	Aromatic and roasted coffee and cocoa like	In coffee and roasted sesame
2-Acetyl-3,6-dimethylpyrazine 	Roasted, caramellic, hazel nut and popcorn	In coffee
2-Methoxy-3-methylpyrazine 	Roasted peanut, green	In sherry, coffee krill
2-Methoxy-3-ethylpyrazine 	Green earthy and nutty	In sherry
2-Methoxy-3-isopropylpyrazine 	Vegetable, earthy and green	In sherry
3,5-Diethyl-2-butylpyrazine 	Chocolate, raw potato, woody and musc animalic	----------
2-Methoxy-3-isobutylpyrazine 	Green bell pepper and green pea	In green bell pepper, pathegrain oil
2-Methoxy-3-ter-butylpyrazine 	Pea green, earthy and galbanum	In coffee and green peas
2-Methoxy-3-sec-butylpyrazine 	Pea green, earthy and galbanum	In coffee and green peas
2-Methoxy-3-isopentylpyrazine 	Green leafy, lavender at low levels	----

Table 3: Occurrence and odours of pyrazines in foods (Rowe, 2005)

1.4 **Microbial metabolism of pyrazines**

1.4.1 ***De-novo*** **synthesis of pyrazines and its derivatives**

Pyrazines are biosynthesized by some of bacterial and fungal species and occur in nature by Maillard reaction and Strecker's degradation (Hwang *et al.,* 1994). Pyrazine and 2, 5-dimethylpyrazine were formed by the thermal degradation of serine and threonine either at low or high temperatures involving a series of complex reactions (Shu, 1998). Despite wide occurrence of pyrazines; its biosynthesis was reported only in few microorganisms. It was generally believed that pyrazines are not produced by enzymatic reactions in microorganisms, but are thought to arise from the non-enzymatic condensation between the precursors of amino acids. The lower methyl and ethyl pyrazines were formed non-enzymatically by amination of acyloins leading to aminocarbonyl compounds followed by condensation. In the presence of differently substituted acyloins, condensation of amino carbonyl compounds yielded three different dihydropyrazines, which were unstable and were oxidised to 2,5-dimethyl and 2,3,5,6-tetramethylpyrazine with C_{2h} and D_{2h} symmetries respectively (Rizzi, 1988). Diverse alkylated pyrazines were formed by reaction of acyloins with methyl, ethyl and propyl substitutions leading to different alkylated pyrazines (Fig 1). Such a synthesis of pyrazines with different alkylated substitutions occurred in marine artic bacterium of Cytophaga-Flavobacterium Bacteroides group (Schulz and Dickschat, 2007) and bacterial strains; *Bacillus subtilis* and *Bacillus amyloliquefaciens* from rhizosphere region (Schulz *et al.,* 2004). α-aminoketones formed by the degradation of amino acids, undergo alkylation and condensation to pyrazines (Rizzi, 1988). 2, 5-Dimethylpyrazine, 3, 6-dimethylpyrazine and trimethylpyrazines were formed during fermentation of soyabeans by *Bacillus subtilis* (Leejerajumunean *et al.,* 2001). Acetoin, 3-hydroxy-2-butanone

generated during fermentation undergoes condensation yielding tetramethylpyrazine. Another alternative route to formation of pyrazines was condensation of α-aminoketones with α-dicarbonyl compound yielding 2,3-dimethylpyrazine. Formation of higher alkylpyrazines is an enzyme-catalyzed reaction and amino acids serve as precursors for the synthesis. Supplementing valine to culture medium of *Paenibacillus polymyxa* led to increased amounts of 2-isopropylpyrazine and 2, 5-diisopropylpyrazine (Beck *et al.,* 2003). A complex mixture of methyl branched dialkyl-(mixtures of isobutyl and sec-butyl moieties) pyrazines were synthesized by polymyxin producing *Paenibacillus polymyxa*. A total of 19 pyrazine metabolites were identified and of these seven were new natural compounds, predominant being 2, 5-diisopropylpyrazine (Table 6) (Beck *et al.,* 2003). Biosynthesis of 2, 5- and 2, 6-diisopropylpyrazine and 2, 5- and 2, 6-diisobutylpyrazine was by amidation of an amino acid and condensation reaction with α, β-dicarbonyl compound as reported in plants (Murray and Whitfield, 1975). 2-Methoxy-3-isopropylpyrazine was biosynthesized by *Pseudomonas perolens* and *Pseudomonas taetrolens* (Gallois and Grimont, 1985). The metabolic pathway proposed involves condensation of valine and glycine to yield 2, 5-diketopiperazine, which then undergo a series of reactions; enolization and *O*-methylation, proton shift and dehydration yielding 2-methoxy-3-isopropylpyrazine (Cheng *et al.,* 1991).

Pyrazines	Microbial species	References
Aspergillic acid, neo-hydroxyaspergillic acid, Hydroxyaspergillic acid, muta aspergillic acid	*Aspergillus flavus, Aspergillus sclerotorium*	Micetich and Mac Donald, 1965
Deoxyaspergillic acid, flavacol (3,6-diisobutyl-2-pyrazinone),Deoxymutaaspergillic acid, 2-Hydroxy-3,6-di-sec-butylpyrazine	*Aspergillus parasiticus*	Buchanan and Houston, 1982
Emimycin-antibiotic	*Streptomyces*-2020	Cheeseman, 1972
2,3,5,6-Tetramethylpyrazine, 2,5-Dimethylpyrazine,2,3,5-Trimethylpyrazine, 2,3,5,6-Tetramethylpyrazine	*Bacillus subtilis* *Bacillus* sp.	Leejerajumunean *et al.,* 2001
Pulcherriminic acid (2-hydroxy-3,6-diisobutylpyrazine-1,4-oxide)	*Candida pucherrima* *Bacillus subtilis* *Bacillus licheniformis* *Bacillus macerans* *Bacillus cereus* *Micrococcus violagabriellae*	Mac Donald, 1965; Uffen and Canale-Parola, 1972
2-Methoxy-3-isopropylpyrazine, 2-Methoxy-3-sec-butylpyrazine	*Pseudomonas perolens*	Cheng *et al.,* 1991
2,5-Dimethyl-3-(methylsulfanyl)pyrazine	Marine artic alpha proteobacteria of Cytophaga-Flavobacter-Bacteroides group	Dickschat *et al.,* 2005a,b
3-Isopropyl-2-methoxy-5-methylpyrazine, 3-Isopropyl-2-methoxy-methylpyrazine, 3-Isobutylmethoxypyrazine, 3-sec-Butyl-2-methoxy-5-methylpyrazine	*Serratia oderifera, Serratia ficaria, Serratiarubidea, Cedaceae davisae*	Gallois and Grimont, 1985
Pyrazine,2-methylpyrazine,2,5-Dimethylpyrazine, 2,3,5-Trimethylpyrazine, 2-Methyl-5-isopropylpyrazine	*Klebsiella pneumoniae, Citrobacter freundii, Enterobacter agglomerans*	Robacker *et al.,* 1997; Robacker and Lauzon, 2002
Pyrazine, 2-Ethyl-3,6-dimethylpyrazine, 2,3,5,6-Tetramethylpyrazine, 2-Butylpyrazine	*Lokantella* strain BIO-204, *Dinoroseobacter* DFL-27	Dickschat *et al.,* 2005
2,3,5,6-Tetramethylpyrazine	*Corynebacterium glutamicum*	Demain *et al.,* 1967
2,5-Dimethylpyrazine,2,3,5-Trimethylpyrazine, 2,5-Dimethyl-3-(3-methylbutyl)pyrazine	*Microbacterium foliorum*, *Staphylococcus scohnii*, *Staphylococcus equorum*	Deetae *et al.,* 2007
2-Methoxy-3-(1-methylpropyl)pyrazine	*Halomonas venusta*	Bungert *et al.,* 2001

Table 4: Microbial synthesis of pyrazines

Pyrazine	Uses/Application	References
2-Ethyl-3,6-dimethyl-5,6-dihydropyrazine, 2-Ethyl-3,6-dimethyl-5,6-dihydropyrazine, 6-Tetrahydropyrazine, 2-Propyl-3,6-dimethyl-5,6-dihydropyrazin 2-Hexyl-3,6-dimethyl-5,6-dihydropyrazine	Used in beverage and food industry, chocolate confectionery and coffee	Kurniadi *et al.,* 2003
Aspergillic acid,Pyrazinamides 2-(allylthio) Pyrazine	Bactericidal Bactericidal Chemoprotective activities	Mac Donald, 1973; Kim *et al.,* 1997
2-p-Aminobenzenesulfonamido-3-methoxypyrazine	Long acting sulfonamide	Cheeseman, 1972
Methyl-3-piperdiniylpyrazine, Amiloride(N-amidino,3,5-diamino-6-chloropyrazine carboxamide)	Anti-depressant	Cheeseman, 1972
Thionazine(O,O-diethyl-O-pyrazinylphosphorothioate)	Diuretic, Soil insecticide and nematicide	Cheeseman, 1972
Ligustrazine(2,3,5,6-tetramethylpyrazine) 8-Heteroaryl-6-phenylimidazo (1,2-a) pyrazines	Medicinal agent in lung protection and cystic fibrosis, Modulators of kinase activity	Dharmananda, 2005; Pippin *et al.,* 2007
Hydroxylatedpyrazine-2-carboxylic acids	Building blocks for pharmaceutical synthesis of antiinflammmatory, cardiotonic and vasodilator effects	Tinschert *et al.,* 2000
Pyrazine amidines and amidoximes,3,5-Bis-(trifluromethylphenyl)amide of 6-chloropyrazine-2-carboxylic acid, VAPY(*Vibrio alginolyticus* Pyrazine)	Tuberculostatic activity, anti tuberculotic activity cerebral embolism, cerebral infraction and *Diabetes mellitus*	Foks *et al.,* 1977 ; Dolezal *et al.,* 2002; Yanai *et al.,* 1995
Pyrazinamides;Emimycin (Pyrazine-2-one-4-oxide)	Plant growth regulators Antibiotic and antibacterial agent	Griffin *et al.,* 1990; Cheeseman, 1972
2-Sulfanamido-3-methoxypyrazine Pyrazinediazohydroxide	Novel antineoplastic agent	Cheeseman, 1972; Edelman *et al.,* 1998
Oltipraz (pyrazine derivative)	Anti-HIV agent	Prochaska *et al.,* 2001
Cephalostatins(bis-Steroidal pyrazines)	Anti-neoplastic agents	Moser *et al.,* 2008

Table 5: Uses and applications of pyrazines

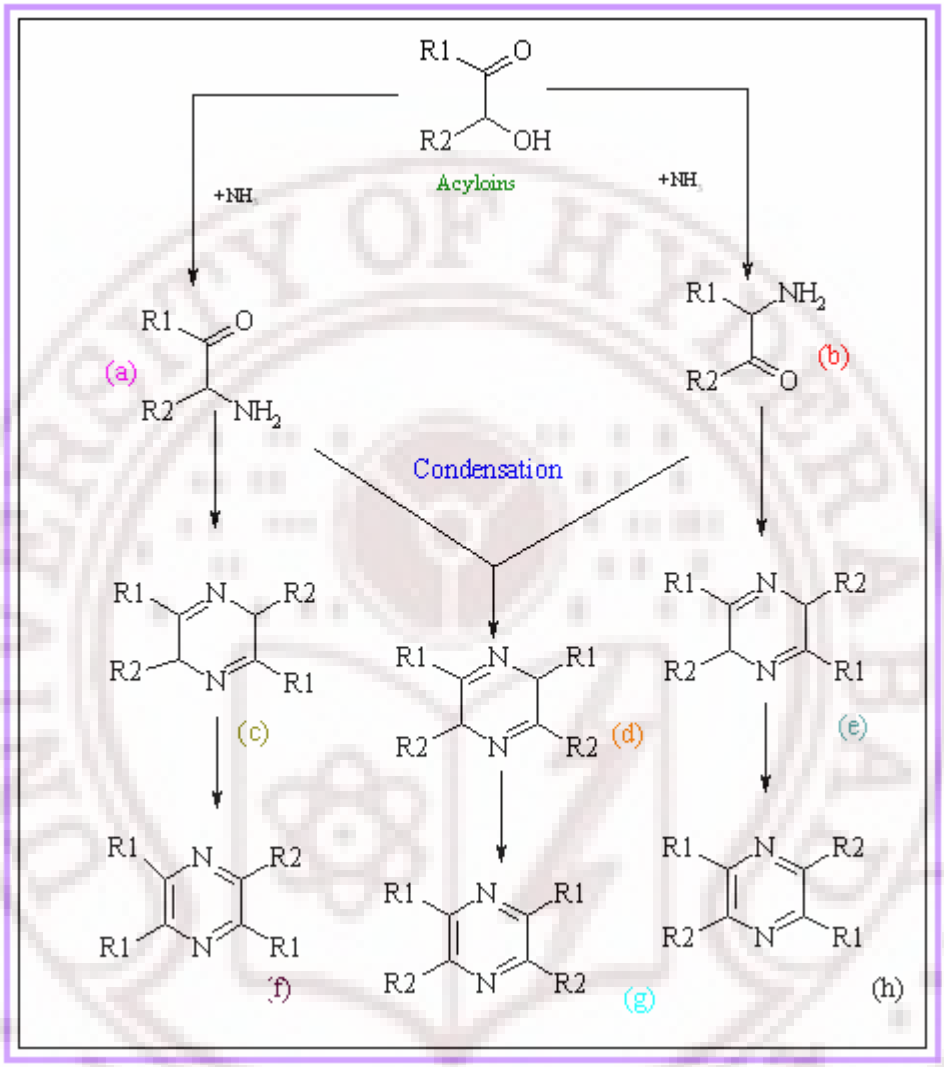


Fig 1: Proposed biosynthetic formation of alkyl pyrazines (Schulz and Dickschat, 2007)

(a) & (b) are regioisomers of aminocarbonyl compounds; (c,d & e) are dihydropyrazines (f & h)-Oxidised pyrazines with C_{2h} symmetry (g)-pyrazine with C_{2v} symmetry.

Chondromyces crocatus	***Paenibacillus polymyxa***
2,5-Dimethylpyrazine 2-Methoxy-3-methylpyrazine (1-Methylethylpyrazine) 2-Methyl-6-(1-methylethyl)pyrazine 3-Methoxy-2,6-dimethylpyrazine 3-Methoxy-2,5-dimethylpyrazine 3-Methoxy-2,5-diisopropylpyrazine 3-Methoxy-2,5-disec-butylpyrazine 3-Methoxy-2,5-diisobutylpyrazine 2-Methyl-5-(1-methylethyl)pyrazine Dimethyl-(1-methylethyl)pyrazine 2,6-Bis(1-methylethyl)pyrazine 2-Methoxy-3- (1-methylpropyl)pyrazine 2-Methoxy-3-(-2-methylpropyl)pyrazine 2,5-Bis (1-methylethyl)pyrazine 2-(1-Methylethyl)-6-(1-methylethyl)pyrazine 2-(1-Methylethyl)-5-(1-methylethyl)pyrazine 2-(1-Hydroxy-1-methylethenyl)-3-methoxypyrazine 2-(1-Methylethenyl)-5-(1-methylethyl)pyrazine 2-(1-Hydroxy-2-methylpropyl)-3-methoxypyrazine 2-(1-Hydroxy-1-methylpropyl)-3-methoxypyrazine 2,5-Bis (2-methylpropyl)pyrazine 2-Benzyl-3-methoxypyrazine 3-Methoxy-2,5-bis(1-methylpropyl)pyrazine 3-Methoxy-2-(1-methylpropyl)-5-(2-methylpropyl)pyrazine	2-Isopropylpyrazine 2-Methyl-6-isopropylpyrazine 2-Methyl-5-isopropylpyrazine 2-Isobutylpyrazine 2,3-Dimethyl-5-isopropylpyrazine 2,5-Dimethyl-3-isopropylpyrazine 2-Methyl-5-isobutylpyrazine 2,6-Dimethyl-5-isopropylpyrazine 2,6-Diisopropylpyrazine 2,5-Diisopropylpyrazine 2,5-Dimethyl-6-isobutylpyrazine 2,6-Dimethyl-5-isobutylpyrazine 2-Isopropyl-6-isobutylpyrazine 2-Isopropyl-5-secbutylpyrazine 2-Isopropyl-5-isobutylpyrazine 2,5-Dimethyl-3, 6-diisopropylpyrazine 2,6-Diisobutylpyrazine 2-Isobutyl-5-sec-butylpyrazine

Table 6: Diversity of pyrazines synthesized by *Chondromyces crocatus* and *Paenibacillus polymyxa* (Beck *et al.,* 2003; Schulz *et al.,* 2004)

An alternate biosynthetic pathway for the formation of 2-methoxy-3-isopropylpyrazine was conversion of 3-isopropyl-2, 5-diketopiperazine to 3-isopropyl-2-(1H) pyrazinone which undergoes *O*-methylation to 2-methoxy-3-isopropylpyrazine (Fig 2). Pyrazine ring formation via enzyme mediated reactions or chemical reactions were not investigated. It was hypothesized that hydroxymethyl group originates from S-adenosyl group (Cheng *et al.,* 1991). Biosynthetic pathway of 2-methoxypyrazines and other substituted pyrazines was proposed that might be functional in plants (Murray and Whitfield, 1975). According to the hypothesis, pyrazines were synthesized from α-amino acids and α, β-dicarbonyls. Another pathway for substituted pyrazine biosynthesis involves oxidation of threonine to 2-aminoacetocetate by threonine dehydrogenase. 2-aminoacetoacetate formed undergoes spontaneous decarboxylation to aminoacetone. Two molecules of aminoacetone undergo self-condensation to 2, 5-dimethylpyrazine (Miller III *et al.*, 1973). This was the possible mechanism for the 2, 5-dimethylpyrazine synthesis in *Pseudomonas taetrolens* provided an active threonine dehydrogenase was produced. The marine bacterium *Halomonas venusta* produced 2-methoxy-3(1-methylpropyl) pyrazine with strong musty odour grown on ZoBell medium with the addition of artificial sea water. The biosynthesis of 2-methoxy-3(1-methylpropyl) pyrazine required glycine and isoleucine and 2, 5-dioxo-3-(1-ethylpropyl) piperazine was the presumed intermediate (Bungert *et al.,* 2001). Aspergillic acids synthesized by growing cells of *Aspergillus flavus* and *Aspergillus oryzae* (Micetich and Mac Donald, 1965) on yeast extract glycerol medium supplemented leucine and isoleucine. Leucine and isoleucine undergo condensation to aspergillic acid, which was then hydroxylated to

hydroxyaspergillic acid (Micetich and Mac Donald, 1965). Pulcherriminic acid (2, 5-diisobutyl-3,6-dihydroxypyrazine-1,4 dioxide) was synthesized by *Micrococcus violagabriellae*, aerobic spore forming bacteria-*Bacillus cereus*, *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus macerans* and yeast, *Candida pulcherrima* (Mac Donald, 1965; Uffen and Canole-Parola, 1972).

In *Candida pulcherrima* and *Bacillus subtilis*, pulcherriminic acid was biosynthesized from the supplemented L-leucine to a cyclic dipeptide intermediate; Cyclo L-leucyl-L-leucyl which was then converted to pulcherriminic acid (Mac Donald, 1965). The enzymes involved in the biosynthesis were not known. It was hypothesized that enzyme associated with the carbohydrate synthesis and extracellular accumulation of the product of carbohydrate metabolism triggered or induced pulcherriminic acid production and plays a role the formation of cyclic acid. Compound has a strong musty potato like odour with an odour threshold of 2 parts of compound per 10^{12} parts of water. *Chondromyces crocatus* was a prolific producer of pyrazines in varying concentrations. 27 different pyrazines were produced depending on the culture medium employed (Schulz and Dickschat, 2007) (Table 6). Alkylated pyrazines like 3-isopropylpyrazine 2, 5-dimethyl-3-isopropylpyrazine were the major constituents. Isopropyl, isobutyl, sec-butyl and methyl pyrazines were derived from valine, leucine, isoleucine and alanine. 2, 5-Disubstituted pyrazines; unusual pyrazines like 2-isopropyl-5-isopropenylpyrazine, α-hydroxylated methoxy alkylated pyrazines, 2-methoxy-3-(1-methylpropylpyrazine) and 3-benzyl-2-methoxypyrazines were formed by the organism.

Several low molecular weight monocarbonyls and dicarbonyls compounds were produced by *Lactobacillus bulgaricus* on amino acid growth media and several alkyl

pyrazines were reported from reaction of lysine-acetoin, lysine-acetol and ornithine-dihydroxy acetone at room temperature in generation of swiss cheese (Leejerajumunean, 2001).

Possible precursor of pyrazine formation in fermented soyabeans by *Bacillus subtilis* and *Bacillus natto* was acetoin. Condensation of two molecules of acetoin yields tetramethylpyrazine and in combination of α-hydroxyketone contributes to the formation of 2, 3-dimethylpyrazines (Leejerajumunean, 2001).

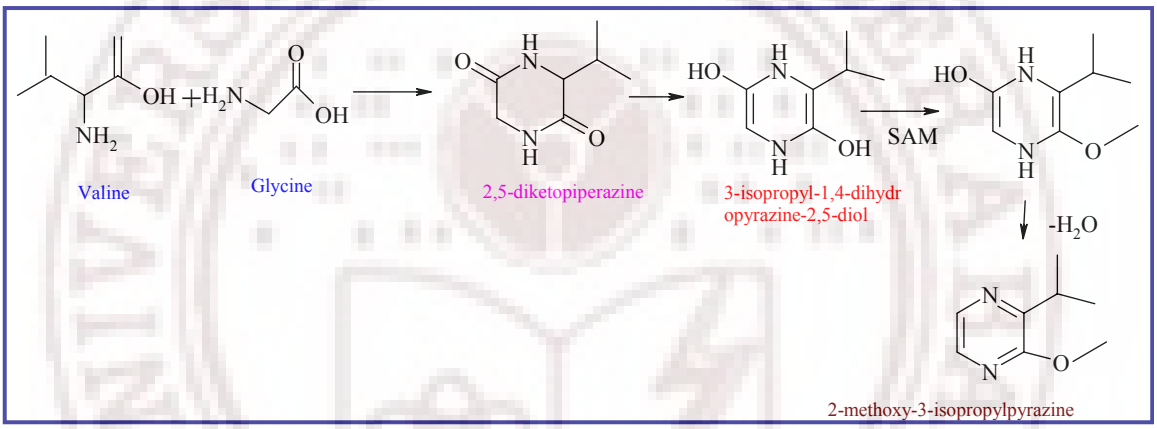


Fig 2: Biosynthesis of 2-methoxy-3-isopropylpyrazine in *Pseudomonas perolens* (Cheng *et al.*, 1991)

1.4.2 **Microbial degradation of pyrazines**

Despite the wide occurrence and vast applications of pyrazines very few studies have been concentrated on the biodegradation of pyrazines and its derivatives.

1.4.2.1 **Photocatalytic and biological degradation of pyrazines**

An integrated approach of solar photocatalysis and biological treatment for the degradation of methylpyrazine, cyanopyrazine and pyrazinamide was reported (Reddy *et al.*, 2004). This approach had showed high efficiency compared to the biological/photocatalytic treatment alone. Pyrazinamide was hydrolyzed to pyrazinoic

acid, which undergoes decarboxylation to pyrazine and hydrolysis to yield acid, alkylated aldehyde, ammonia and carbondioxide. Methylpyrazine was dealkylated and decarboxylated to yield pyrazine and was degraded to acid, aldehyde and ammonia. Prephotocatalytic treatment was followed by biological treatment. The increase BOD/COD to 0.71 confirmed the feasibility of biological degradation after photocatalytic treatment.

1.4.2.2 **Microbial degradation of pyrazines**

Oxidative degradation of hydroxypyrazine was reported in *Pseudomonas* species (Mattey and Harle, 1976). The initial step was hydroxylation to a dihydroxypyrazine, which then undergoes ring cleavage by an oxygenase to N-formylglycinamide; the cleavage product undergoes reduction and decarboxylation to glycine and subsequent utilization of glycine by glycerate pathway (Fig 3). Photometabolism of pyrazine group of compounds by *Rhodopseudomonas palustris* OU11, through a central intermediate pyrazinoic acid was reported (Fig 4) (Sasikala *et al.,* 1994). *Mycobacterium* strain DM11 was reported to degrade 2, 3-diethyl 5-methylpyrazine (Rappert *et al.,* 2006) when used as sole source of carbon, nitrogen and energy through a hydroxylated intermediate, which undergoes ring cleavage with ammonia liberation. The ring cleavage product was not identified. *Rhodococcus erythropolis* strain DP-45 was reported to utilize 2, 5-dimethylpyrazine, 2, 3-dimethylpyrazine, 2, 6-dimethylpyrazine, 2-ethylpyrazine, 2-methylpyrazine 2,3,5-trimethylpyrazine, 2-ethyl-5,6-dimethylpyrazine as a sole source of carbon and nitrogen (Rappert *et al.,* 2007). The basic requirement for the strain is pyrazine ring with one methyl or ethyl substitution and with one free position in the ring. A hypothetical pathway for the degradation of 2, 3-diethyl-5-methylpyrazine (DM) and

2,5-dimethylpyrazine was proposed (Fig 4). According to the proposed pathway (Fig 5), initially 2, 3-diethyl-5-methylpyrazine 2, 5-dimethylpyrazine was hydroxylated to 2, 3-diethyl 6-hydroxy-5-methylpyrazine and 2-hydroxy-3, 6-dimethylpyrazine respectively. The loss of 2, 3-diethyl-6-hydroxy-5-methylpyrazine and 2-hydroxy-3, 6-dimethylpyrazine was accompanied by the release of ammonia. Subsequent steps in the degradation were not reported. In *Rhodococcus erythropolis*, the expression of first enzyme of the pathway was induced by 2, 5-dimethylpyrazine and 2-hydroxy-3, 6-dimethylpyrazine, whereas the second enzyme was constitutively expressed (Rappert *et al.,* 2007). Flavoprotein inhibitors, cytochrome P-450 inhibitors and competitive inhibitors of flavin containing monooxygenases inhibited the activity of first and second enzyme. Biodegradation of aspergillic acid (1-hydroxy-3-isobutyl-6-sec-butyl-2-pyrazinone) by *Trichoderma koningii* ATCC 76666 to leucine and 2-hydroxyimino-3-methyl-1-pentanol was well known (Fig 6) (Nishimura *et al.,* 1991). Aspergillic acid degrading enzyme; catalyzing the cleavage of pyrazine ring was isolated and purified. The enzyme was a homodimer of 112 kDa with an isoelectric point of around 5.3. Aspergillic acid degrading enzyme was active towards the aspergillic acid related compounds having 1-hydroxypyrazinone or 2-hydroxypyrazine oxide structure and having no hydroxylated alkyl group at the carbon 6 in the ring (Nishimura *et al.,* 1997).

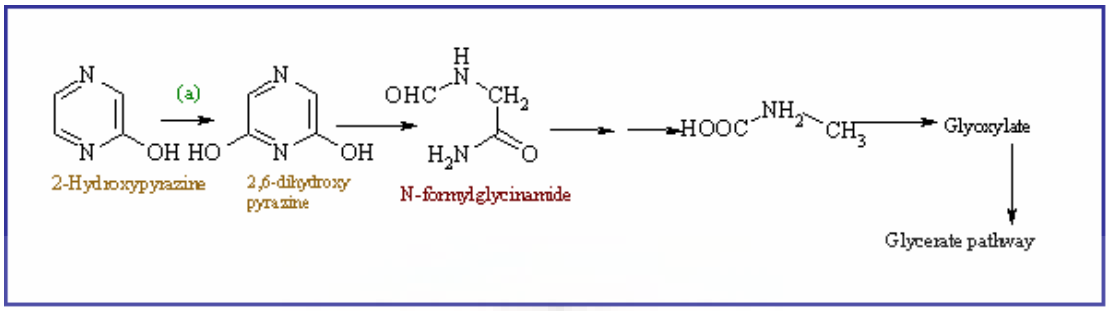


Fig 3: Proposed pathway for degradation of 2-hydroxypyrazine by a *Pseudomonas* sp. PZ3 (Mattey and Harley, 1976)

(a)-Monooxygenase

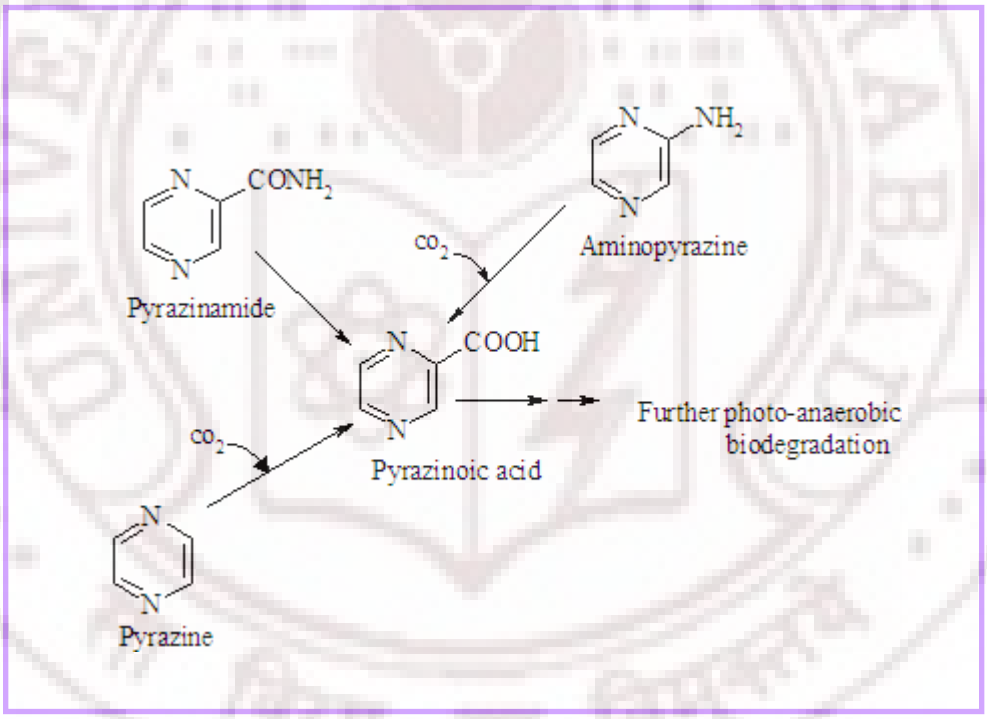


Fig 4: Hypothetical scheme for photodegradation of various pyrazine derivatives by *Rhodopseudomonas palustris* OU11 (Sasikala *et al.,* 1994)

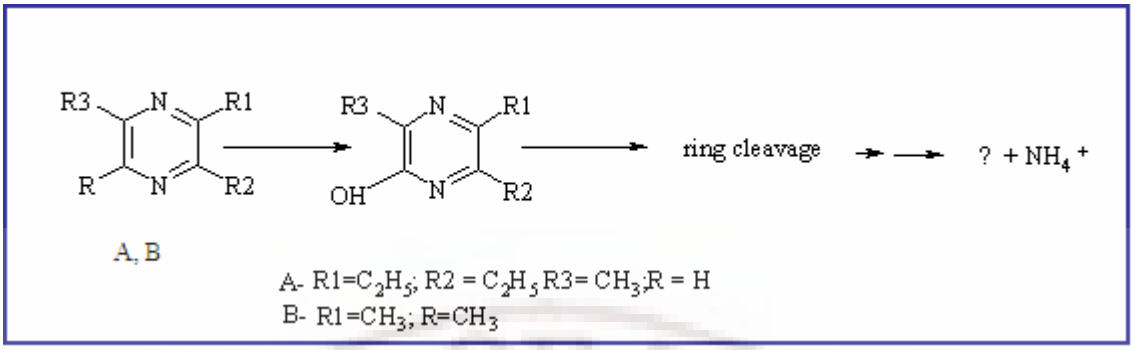


Fig 5: Proposed initial biodegradation of alkylated pyrazines in *Mycobacterium* sp. and *Rhodococcus erythropolis* (Rappert *et al.*, 2006; 2007)

A = 2,3-Diethyl-5-methylpyrazine; B = 2,5-Dimethylpyrazine

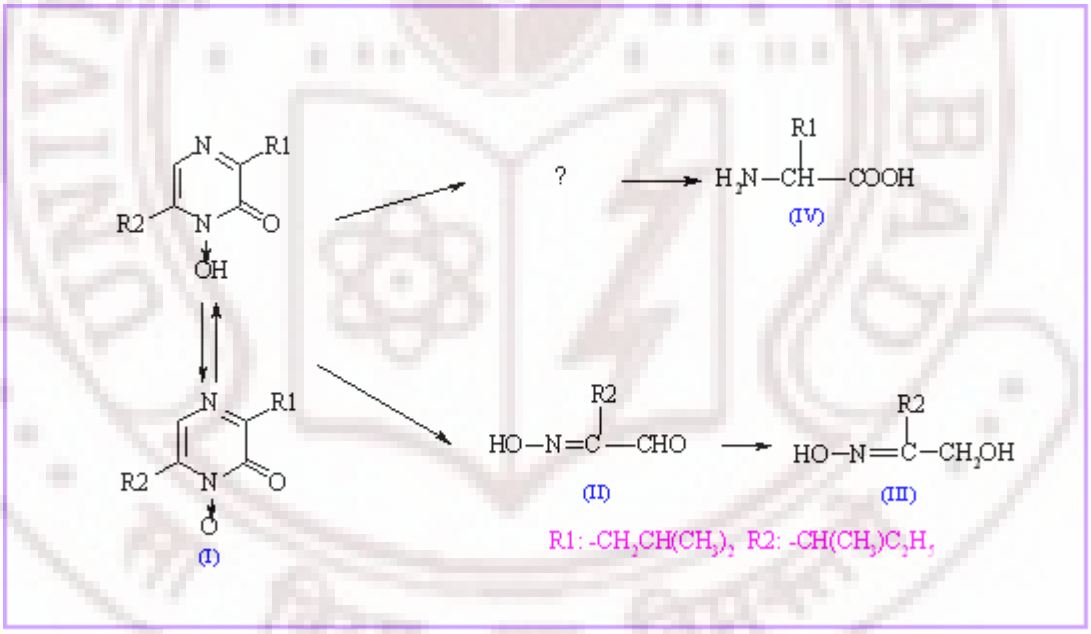


Fig 6: Proposed pathway for the degradation of aspergillic acid by *Trichoderma koningii* ATCC 76666 (Nishimura *et al.*, 1997)

(I)-Aspergillic acid; (II)-2-hydroxyimino-3-methyl-1-pentanal; (III) 2-hydroxyimino-3-methyl-1-pentanol; (IV) leucine

1.4.3 **Biotransformation of pyrazines**

The available literature on microbial metabolism of pyrazines was concentrated on the transformation of pyrazines to its hydroxylated or carboxylated educts. *Pseudomonas putida* metabolizing toluene via benzyl alcohol transformed 2,5-dimethylpyrazine and 2,3,6-trimethylpyrazine to 5-methylpyrazine-2-carboxylic acid and 5,6-dimethylpyrazine-2-carboxylic acid, respectively (Keiner, 1992; Schmid *et al.,* 2001). Resting cells of *Pseudomonas acidovorans* DSM 4746, *Alcaligenes faecalis* DSM 6929 grown on appropriate pyridine carboxylic acids were capable of oxidizing pyrazine carboxylic acids (Kiener *et al.,* 1994). *Ralstonia/Burkholderia* DSM 6920, which grows on 6-methylnicotinate was shown to hydroxylate a series of heterocyclic aromatic compounds by the action of 6-methylnicotinate-2-oxidoreductase at C2 position (Tinschert *et al.,* 2000). Pyrazine-2-carboxylic acid and 5-chloropyrazine-2-carboxylic acid were transformed to 3-hydroxypyrazine-2-carboxylic acid and 3-hydroxy-5-chloropyrazine-2-carboxylic acid (Fig 7a).

Alcaligenes eutrophus, (a quinolinic acid assimilating bacteria) transforms quinolinate to 6-hydroxyquinolinate (Uchida *et al.,* 2003). This strain has the capability to transform pyrazine-2,3-dicarboxylate and pyrazine-2,3-dicarbonitrile to 5-hydroxypyrazine-2,3-dicarboxylic acid and 5-hydroxypyrazine-2,3-dicarbonitrile (Fig 7a). 2-Cyanopyrazine was biotransformed to 5-hydroxypyrazine-2-carboxylic acid by the growing cells of *Agrobacterium* sp. DSM 6336. The biotransformation was a two step process (Fig 7b) which involves the hydrolysis of the nitrile group of 2-cyanopyrazine to pyrazine-2-carboxylic acid by a nitrilase and subsequent hydroxylation to 5-

hydroxypyrazine-2-carboxylic acid catalyzed by a nicotinate dehydrogenase (Wieser *et al.,* 1997) respectively when used as sole source of carbon and energy.

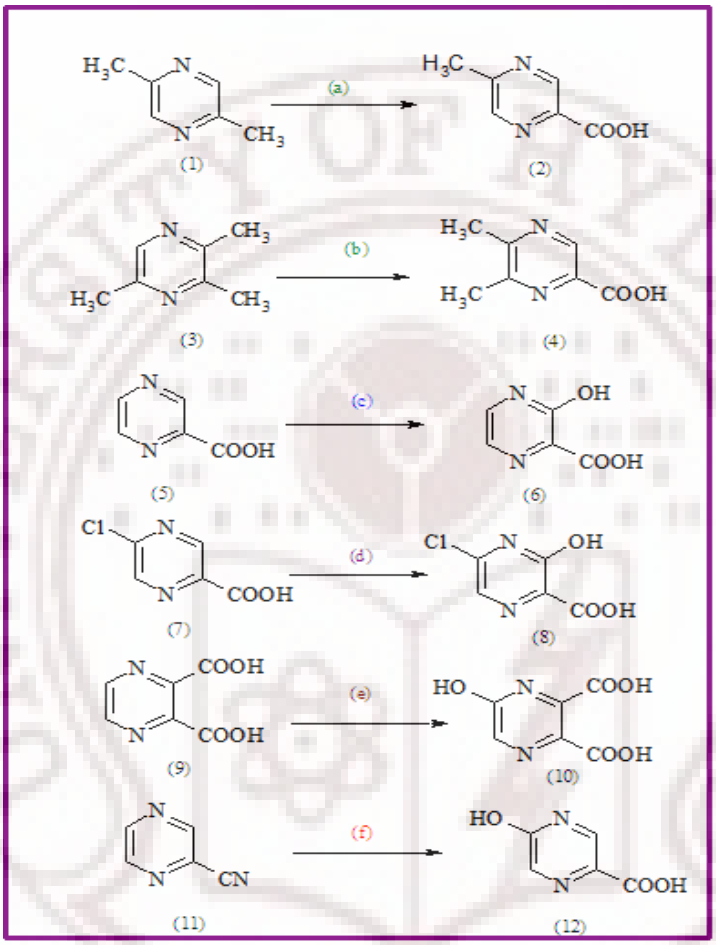


Fig 7a: Transformation of pyrazines by different bacterial strains

(a & b)- *Pseudomonas putida* (Kiener, 1992; Schmid *et al.,* 2001) (c) *Pseudomonas acidovorans* and *Alcaligenes faecalis* (Kiener *et al.,* 1994) (d) *Ralstonia/Burkholderia* sp. (Tinschert *et al.,* 2000) (e) *Alcaligenes eutrophus* (Uchida *et al.,* 2001) (f) *Agrobacterium* sp. (Wieser *et al.,* 1997).
(1)-2,5-Dimethylpyrazine (2)-5-Methylpyrazine-2-carboxylic acid (3)-2,3,6-Trimethylpyrazine (4)-5,6-Dimethylpyrazine-2-carboxylic acid (5)-Pyrazine-2-carboxylic acid (6)-5-hydroxypyrazine-2-carboxylic acid (7)-5-Chloropyrazine-2-carboxylic acid (8)-3-Hydroxy-5-chloropyrazine-2-carboxylic acid (9)-Pyrazine-2,3-dicarboxylic acid (10)-5-hydroxypyrazine-2,3-dicarboxylic acid (11)-Pyrazine-2,3-dicarbonitrile (12)-5-Hydroxypyrazine-2,3-dicarbonitrile.

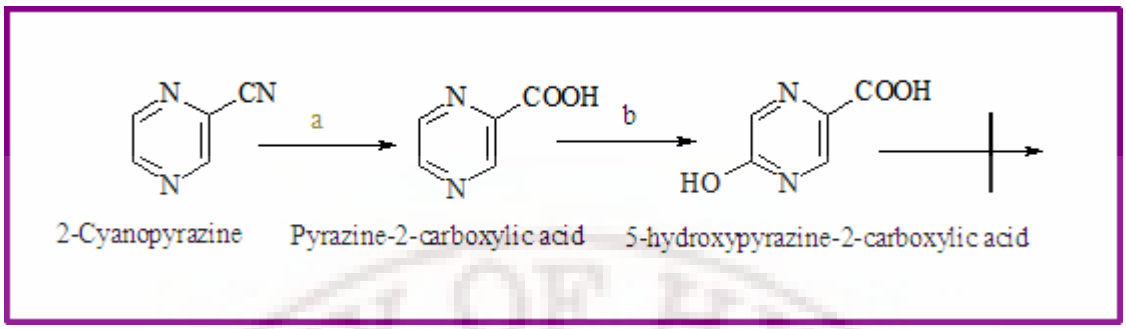


Fig 7b: Proposed biotransformation of 2-cyanopyrazine in *Agrobacterium* sp.

(Wieser *et al.,* 1997), (a)-Nicotinate dehydrogenase (b)-Nitrilase

1.5 **Definition of the Problem**

Review of literature on the microbial metabolism of pyrazines reveals that; despite its structural simplicity, wide occurrence and vast applications very little is known on its microbial degradation; hence a study was initiated with the following objective.

OBJECTIVE

To identify a bacterium and to study for pyrazine-2-carboxylate metabolism

Materials and Methods

2.1.1 **Glassware:** All the glassware used in the present experiments including test tubes, pipettes, measuring cylinders, culturing flasks, reagent bottles, petriplates and screwcap test tubes were of Borosil and Duran brand.

2.1.2 **Cleaning:** The glassware used in the experiments was initially soaked in dilute chromic acid (5 % w/v of potassium dichromate in 10 % v/v sulphuric acid) for 24 h and cleaned in tap water and teepol; a detergent. After removing all the traces of the detergent, the glassware was rinsed with single distilled water and kept in the oven for drying at 100 °C.

2.1.3 **Water:** Single and double distilled water obtained from Milli-Q plant stored in white carboys was used for rinsing of glassware and for preparation of media, stock solutions and chemical analysis.

2.1.4 **HPLC water:** Milli-Q water was used for HPLC analysis.

2.1.5 **Chemicals:** The chemicals used in this study were of analytical grade from Sigma-Aldrich, Lancaster, Ranbaxy, Merck, Qualigens, e-Merck and Himedia.

2.1.6 **Determination of pH**: pH was determined using a digital pH meter (Digisun electronics, India model (DI-707).

2.1.7 **Sterilization:** Sterilization of the culture media and glassware was done by autoclaving at 15 lbs for 15 min.

2.2.1 **Organism and growth conditions:** The chemotrophic bacterium strain HCU1 (ABRC 505) was serendipitous, which was found growing on pyrazine media in our laboratory. The organism was grown chemoorganoheterotrophically on mineral medium (Table 7) with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen

sources respectively, in 250 ml erlenmayer flasks and kept under agitation in an orbitek shaker at 30 ± 2 ºC.

2.2.2 **Maintenance of stock cultures:** Stock culture of strain HCU1 was maintained as agar slants. Slants were prepared by using 2 % (w/v) agar solidified nutrient broth filled to 1/10 volume in test tubes in slanting position. Culture was taken onto the inoculation loop and streaked onto the slant. After 48 h of incubation, slant cultures were preserved under refrigeration at 4 ºC until further use. The slants were sub cultured every 90 days and contamination from other bacteria was checked periodically by streaking onto nutrient agar plates.

2.2.3 **Confirmation of purity of the cultures.** Culture was checked for its purity before and after assay by streaking on nutrient agar plates [($g.l^{-1;}$ peptone-10, NaCl-5, beef extract-3 and agar 20) Difco manual, 1998] and incubating at 30 ± 2 ºC.

2.3 **ASSAYS**

2.3.1 **Assay with growing cells:** Logarithmically grown (12 h) culture of strain HCU1 (0.4 $O.D_{540nm}$) was transferred into 50 ml Biebl and Pfennig's broth in a 250 ml erlenmayer flask. 2.5 ml of 30 mM pyrazine-2-carboxylate stock was added to a final concentration of 1.5 mM and incubated at 30 ± 2 ºC at 150 rpm in an orbitek shaker incubator. After incubation the culture was harvested for analysis.

2.3.2 **Assay with the resting cells:** Cells of strain HCU1(0.4 $O.D_{540nm}$) grown chemoheterotrophically on Biebl and Pfennig's medium supplemented with 1.5 mM pyrazine-2-carboxylate, till late logarithmic phase were harvested by centrifugation (12,000 x g for 15 min). The pellet was washed twice and resuspended in basal salts

medium supplemented with 1.5 mM pyrazine-2-carboxylate; 50 ml assay mixture in 250 ml conical flasks was incubated at 30 ± 2 ºC and 150 rpm.

Ingredients	**$g.l^{-1}$**
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.4
NH_4Cl	0.04
$CaCl_2.2H_2O$	0.05
Organic carbon source/e^- donor	3.0
Yeast extract	0.12
Ferric citrate (0.1 % w/v)	5 ml
Trace elements SL_7	1 ml

Mineral medium used for the growth of chemotrophic bacteria (Biebl and Pfennig, 1981). SL_7 ($mg.ml^{-1}$): HCl (25 % v/v)-1ml; $ZnCl_2$-7; $MnCl_2.4H_2O$-100; H_3BO_3-60; $CoCl_2.6H_2O$-200; $CuCl_2.H_2O$; $NiCl_2.6H_2O$-20; $NaMoO_4.6H_2O$-40

Table 7: The composition of Biebl and Pfennig's medium (Biebl and Pfennig's 1981)

2.3.3 **Assay with cell free extracts:** Pyrazine-2-carboxylate induced, logarithmically growing cells of strain HCU1 were harvested by centrifugation (16,000 x g for 10 min), pellet was washed (twice) with 0.05 M Tris buffer (pH 7.4) and resuspended in 1 ml of the same buffer. Cells were sonicated with MS-72 probe (Bandelin, Germany make, model-UW 2070) to complete cell lysis after 8-9 cycles. The cell suspension was

centrifuged (16,000 x g for 20 min) and the supernatant was used as a source of enzyme. 200 µ moles of pyrazine-2-carboxylate, 200 µ moles of coenzyme, 5 mM $MgCl_2$ and 40 µl of the cell sonicate was added and volume was made up to 1 ml. The reaction was stopped by adding 10 µl of 5N HCl and analyzed by PDA detector in HPLC.

2.4 **Bulk cultivation for transformation studies:** For bulk cultivation, *Stenotrophomonas* sp. was grown in 3 litre erlenmayer flask containing Biebl and Pfennig's medium supplemented with 1.5 mM of pyrazine-2-carboxylic acid. With 2 % (v/v) of initial inoculum, organism was grown for 8 h till two-fold increase in the peak height was observed.

2.5 **Sample collection for isolating pyrazine-2-carboxylate metabolizing bacteria**: Soil samples collected, from (1) Oil contaminated soil dump site of a filling station at Kukatpally, Hyderabad and (2) Organic rich garden soil of the green house at University of Hyderabad, were used for enrichment studies.

2.5.1 **Enrichments and isolation of pyrazine-2-carboxylic acid metabolizing bacteria:**

Both phototrophic and chemotrophic bacterial strains were used for screening of pyrazine-2-carboxylic acid metabolizing bacteria. One gram of soil sample was dissolved in 9 ml of sterile distilled water and shaken well and kept aside till the soil is sedimented. One ml of the supernatant was drawn and added to 49 ml of Biebl and Pfennig's broth with malate as carbon and 1 mM pyrazine-2-carboxylate as nitrogen source and incubated aerobically at 30 ± 2 °C under agitation at 150 rpm. After 24 h of incubation a loopful of the culture was taken and streaked on to Biebl and Pfennig's agar medium with pyrazine-2-carboxylate as nitrogen source and incubated. Different bacterial colonies were picked up and were purified by successive streaking on to agar plates.

2.6 **Physiological and Biochemical characterization**

2.6.1 **Direct microscopic observation and gram staining:** Cells of strain HCU1 were observed using phase contrast microscopy. Gram staining was done using the standard protocol.

2.6.2 **Growth modes**

2.6.2.1 **Chemolithoautotrophy:** The organism was grown chemolithoautotrophically with sodium bicarbonate (0.1 % w/v) as carbon source and sodium thiosulphate (1 mM) as electron donor in Biebl and Pfennig's broth.

2.6.2.2 **Chemoorgano-heterotrophy:** The organism was grown chemoorgano-heterotrophically with malate (0.3 % w/v) as carbon source and electron donor.

2.6.2.3 **Chemolithoheterotrophy**: The organism was grown chemolithoheterotrophically with malate (0.3 % w/v) as carbon source and sodium thiosulphate (1 mM) as electron donor.

2.6.2.4 **Fermentative mode:** In this mode sucrose of 0.3 % w/v was used as carbon source and electron acceptor under anaerobic incubation.

2.6.2.5 **(Anaerobic) Respiratory mode:** In this mode, sucrose (0.3 % w/v) was used as a carbon source and nitrate as electron acceptor under anaerobic condition.

2.6.2.6 **Utilization/growth on organic carbon substrates:** Different organic carbon sources such as TCA cycle intermediates (0.3 % w/v) and carbohydrates and aromatic substrates were supplemented as carbon sources for studying the utilization as growth substrates.

2.6.2.7 **Growth on nitrogen sources:** Inorganic and organic nitrogen compounds (0.12 % w/v) were used as nitrogen sources for studying their utilization by strain HCU1.

2.6.2.8 **Growth at different temperatures:** Growth of the strain at different temperatures (4, 10, 20, 25, 28, 30, 35, and 40 °C) was monitored by incubating the inoculated cultures in the Biebl and Pfennig's broth.

2.7 Biochemical characterization of strain HCU1

2.7.1 **Indole production**: The culture was grown overnight on tryptone medium and harvested. Indole production was detected by PDAB reagent (Kupfer and Atkinson *et al*., 1964). To 1 ml of the culture supernatant 1 ml of PDAB reagent (60 mg of para-dimethyl aminobenzaldehyde to 1 ml of 3N H_2SO_4) was added. Orangish colour of supernatant was considered positive for indole production.

2.7.2 **Methyl red test**: The culture was grown on glucose phosphate broth and after 24 h of incubation 5-6 drops of methyl red indicator was added. Production of acid stable end products in culture medium was detected by the methyl red test. To the culture, methyl red reagent in alcohol was added. The dye is red at acidic pH of 4.4 and yellow at pH above 6 and is orange in colour within a pH range of 4-6. Change in the colour of the medium to red was considered positive for acid production by the culture.

2.7.3 **Voges Proskauer test:** The test was done to detect for the presence of acetoin/2, 3-butanediol production by the bacterial strain. The culture was grown overnight on glucose phosphate broth and acetoin production was detected by Barrit's reagent (5 % (w/v) α-naphthol in absolute alcohol and 0.2 ml of KOH). The change in the colour of the medium to red is considered positive.

2.7.4 **Citrate utilization:** The capability of the strain to utilize citrate was shown by this method. Dye bromothymol blue, used in the medium is green at pH above 6.8 and blue at pH less than 6. The organism was inoculated on Simmon's citrate agar medium and citrate utilization was detected by the change in pH the medium to acidic indicated by the colour of agar medium to blue.

2.7.5 **Ammonification:** The process of forming ammonia from organic nitrogenous matter is called ammonification. Strain HCU1 was inoculated into peptone broth (peptone-10, beef extract-3, and yeast extract-2 $g.l^{-1}$) and incubated for 24 to 48 h. Mineralization of organic nitrogen to ammonia was detected by the formation of brown precipitate using nessler's reagent.

2.7.6 **Amylase activity**: Log phase bacterial culture of strain HCU1 was inoculated on a nutrient agar medium supplemented with 1 % (w/v) starch and after overnight incubation the plate was flooded with 1 % KI to detect the presence or absence of starch. The colourless zone around the colonies indicates presence of amylase.

2.7.7 **Catalase activity:** Logarithmically grown culture was taken on to a slide and a drop of hydrogen peroxide was added. Bubbling of the culture indicates the presence of catalase enzyme and liberation of molecular oxygen.

2.7.8 **Gelatinase activity:** The bacterial culture was stab inoculated and grown overnight on to nutrient gelatin medium (nutrient broth + 13 % w/v gelatin). After overnight incubation, the nutrient gelatin tube was kept at 0 ºC. Gelatin liquefaction was considered positive if the medium remains in liquid state after incubation of the tube at 0 ºC.

2.7.9 **Nitrogenase activity:** Nitrogenase activity was analyzed in terms of acetylene reduction to ethylene using (Shimadzu 14B make) Gas chromatograph (GC) equipped

with Flame Ionization Detector (FID) and poropak-N (stainless steel, 2 metres, 89/100 mesh). The operating parameters of GC were: carrier gas-nitrogen; oven injector and detector temperatures- 90, 70 and 70 ºC respectively. The 5 ml culture is dispensed in to GC tubes (15 x 150 mm) were sealed with suba seals flushed with ultra pure argon (99 % v/v). Ten percent of argon gas phase was replaced with acetylene and incubated at 30 ± 2 ºC. Activity was monitored at an interval of 15 minutes. The concentration of ethylene was calculated from the peak heights resolved from the data station and calibrated against standard ethylene. Positive pressure in the assay tubes was maintained by replacing the sample with drawn with argon.

2.7.10 **Urease activity:** Activity was measured in terms of urea utilization and ammonium liberation using urea agar. The organism was inoculated on to urea agar and checked for growth g/l; peptone-1, dextrose-1, NaCl-5, KH_2PO_4-2, urea-20, and phenol red-0.012 with a pH of 6.8.

2.8 16S rRNA gene amplification and sequencing:

2.8.1 **DNA extraction and purification:** Strain HCU1 was harvested by centrifugation of well grown liquid cultures (10,000 rpm for 15 min) and the genomic DNA was isolated by the method of Marmur (1961) modified as illustrated below. Except the solvents used, glassware, buffers and solutions were all sterilized by autoclaving.

Protocol for DNA extraction and purification

2 to 3 g wet packed cells

↓

Suspended in 50 ml Saline – EDTA (0.15 M NaCl + 0.1 M EDTA, pH 8)

↓

Centrifuged (10,000 rpm, 10 min)

↓

Suspended the cells in a total volume of 25 ml of Saline – EDTA

↓

Lysed the cells
Ten milligrams of lysozyme was added to the cells suspended in 25 ml Saline-EDTA. The mixture was incubated at 37 °C with occasional shaking till increase in viscosity was observed which indicated the lysis of the cells (30–60 min). When lysozyme was used, SDS was also added after the cells had lysed followed by the 60 °C heating and cooling.

↓

Sodium Perchlorate (5 M) was added to a final concentration of
1 M to the viscous, lysed suspension

↓

Equal volume of chloroform – Isoamyl alcohol (24:1 v/v) mixture was added to the lysed cell suspension and shaken for 30 minutes

↓

Centrifuged (10,000 rpm for 15 min)
formation of three layers

↓

Pipetted out upper aqueous layer which contained nucleic acids

↓

Added 95 % (v/v) ethyl alcohol to precipitate nucleic acids

↓

Removed the precipitated nucleic acids by stirring with the help of a glass rod (removed excess alcohol by pressing the glass rod against the container)

↓

Transferred and dissolved the precipitate into 10 – 15 ml
of dilute saline citrate (0.015 M NaCl + 0.015 M tri sodium citrate; pH 7)

Contd. in pg 40

↓

Adjusted the above solution approximately to standard saline citrate concentration by adding concentrated saline citrate solution (1.5 M NaCl + 0.15 M tri sodium citrate pH 7)

↓

Shaken well with an equal volume of chloroform–Isoamyl alcohol (24:1 v/v) for 15 minutes

↓

Centrifuged and removed the supernatant
(Repeated three times to remove all the proteins)

↓

Added 95 % (v/v) ethyl alcohol to the supernatant; dispersed the precipitate in ½ to ¾ of the supernatant volume

↓

Added ribonuclease [(50 μg ml^{-1}) ribonuclease was dissolved in 0.15 M sodium chloride pH 5 and heated at 80 °C for 10 minutes to inactivate any DNase present]

↓

Incubated for 30 minutes at 37 °C

↓

Added chloroform – Isoamyl alcohol (24:1 v/v) and centrifuged (10,000 rpm for 10 minutes)
(Repeat three times to remove all the proteins)

↓

To the supernatant 95 % (v/v) ethyl alcohol was added to precipitate the nucleic acids

↓

Dissolved the precipitate in 9 ml dilute saline citrate

↓

Added 1.0 ml acetate – EDTA solution (3 M sodium acetate + 0.001 M EDTA pH 7)

↓

While stirring the solution rapidly with glass rod isopropyl alcohol was added drop wise into the vortex

↓

DNA as fibrous material was collected on glass rod

↓

Washed the DNA isolated with ethyl alcohol

Contd. in pg 41

↓

DNA was dissolved in dilute saline citrate (0.015 M NaCl + 0.0015 M tri sodium citrate) and then stored at 4°C

Twenty five microlitres of the DNA stock in concentrated buffer solution was diluted to standard saline buffer concentration with diluted saline citrate buffer (0.015 M NaCl + 0.0015 M tri sodium citrate) and the absorption at 260 nm was noted.

2.8.2 **16S rRNA gene sequence analysis:**

(i) DNA extraction

Agarose gel electrophoresis: 10 µl of genomic DNA and 10 µl of standard genomic DNA (as marker) were electrophoresed (Bangalore GENEI) in 0.8 % (w/v) horizontal agarose gel in TAE buffer at 15 V cm^{-1}, stained in 0.5 $\mu g.ml^{-1}$ ethidium bromide and visualized on UV transilluminator (Bangalore GENEI).

(ii) Amplification of 16S rRNA gene: Amplification is routinely performed on 50 µl volumes in 0.2 ml microfuge tubes using a DNA thermal cycler (MJ Mini Personal Thermal Cycler-BIO-RAD). All plastic ware were autoclaved and ultraviolet irradiated. The primers used for the amplification of the 16S rRNA gene are Eub27F (5′GAGTTTGATCCTGGCTCAG-3′) and Univ1492R (5′-GGTTACCTTGTTACGACT T-3′). The concentration and volume of the reaction mixture are as follows.

1. Primers: 2 µl of each primer (10 pmol μl^{-1}),

2. Template: 2 µl of DNA template (25 ng μl^{-1}),

3. Water: 19 µl and

4. Master Mix: 25 µl (Obtained from Bangalore GENEI)

The cycling parameters were given as follows.

No. of cycles	Denaturation		Annealing		Elongation	
	Temp (°C)	Time	Temp (°C)	Time	Temp (°C)	Time
1	96	2 min				
32	96	40 s	50	40 s	72	1 min
1			42	1 min	72	5 min

Agarose gel electrophoresis: 5 µl of amplified DNA and 5 µl of 1 kbp DNA marker were electrophoresed in 2 % (w/v) horizontal agarose gel as described earlier in TAE buffer at 15 V cm^{-1}, stained in 0.5 $\mu g.ml^{-1}$ ethidium bromides and visualized on UV transilluminator.

(iii) PCR amplicon purification: The amplified product was purified by using the QIAquick PCR Purification Kit (Cat. No.28104) and the quality and concentration of the purified product were checked by agarose gel electrophoresis as described previously.

(iv) 16S rRNA gene sequencing and assembling of the 4 partial sequences: The complete length of the 16S rRNA gene sequence was obtained by sequencing with 4 primers Eub27F (5′-GAGTTTGATCCTGGCTCAG-3′), 5′-372F (5′-TACGGGAGGCAGCAG-3′), 5′-790F (5′-GATACCCTGGTAGTCC-3′) and Univ1492R (5′-GGTTACCTTGTTACGACT T-3′). The 16S rRNA gene amplicon was sequenced at MWG, Bangalore, India. The 4 sequences obtained as .scf format were assembled using software SeqMan in the DNA STAR Lasergene 6 package.

2.8.2 **Methods of phylogenetic tree construction:** Character based methods were used for phylogenetic analysis.

(i) Character based methods: Maximum likelihood method in PhyML was used for phylogenetic dendrogram construction.

(ii) Sequence alignment using Clustal X: Sequences were aligned using the Clustal X program (Thompson *et al*., 1997) and the alignment was corrected manually. The alignment file was saved with ".phy" extension.

(iii) Maximum likelihood method in PhyML program: The Clustal X alignment file with ".phy" extension was used as input file in PhyML program. The dendrogram was constructed using PhyML (Guindon and Gascuel, 2003) program using 100 replicates of non parametric bootstrap analysis, GTR model of nucleotide substitution and 4 substitution rate categories.

2.9 **ANALYSIS**

2.9.1 **Growth and biomass**: Growth was measured turbidometrically, in terms of absorbance in a UV spectrophotometer using uninoculated medium as blank at 540 nm.

2.9.2 **Estimation of pyrazine-2-carboxylate:** Pyrazine-2-carboxylate was qualitatively and quantitatively estimated using 1 % w/v ferrous sulphate reagent (Oliveto, 1962). To 1 ml of supernatant 1 ml of reagent was added and orange red colour developed was read at 440 nm in UV spectrophotometer.

2.9.3 **Estimation of cyclic imino acids:** Cyclic imino acids were qualitatively estimated using 1 % (w/v) ninhydrin in glacial acetic acid (Piez *et al.,* 1956). Reagent was added to the test solution and heated in a boiling water bath for 5-10 minutes and colour developed was optically read at 350 nm in UV spectrophotometer.

2.9.4 **Estimation of proteins:** Proteins were estimated using Bradford's method (Bradford, 1976).

2.10 HPLC ANALYSIS

2.10.1**N-heterocyclic compounds:** These compounds were detected in HPLC (Shimadzu LC 20AT Japan) isocratic mode with DAD-SPDM20A and a reverse phase column Luna RP-C_{18} (2) 5 µm (250 x 4.6 mm) using diode array detector at 268 nm with a mobile phase of methanol: water: acetonitrile (1:1:0.25) or with 50 mM Tris-HCl pH 7.4, acetonitrile (70:30) at a flow rate of 1.5 $ml.min^{-1}$), injection volume was 20 µl. The t_R of pyrazine-2-carboxylate is 1.7 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).

2.10.2 **Isolation and purification of metabolites**: The culture supernatant was concentrated under vacuo. The crude concentrate was extracted into methanol and metabolites were isolated from methanol extract. Purification was done using semi preparative HPLC using Luna 5 µ C_8 (2) 100A column (250 x 10 mm) using UV-Visible detector at 268 nm with methanol, water and acetonitrile (1:1:0.25) and water, acetonitrile as a solvent system at 2.0 $ml.min^{-1}$ in isocratic and gradient modes respectively. The HPLC purified compounds were concentrated and purity was confirmed with analytical C_{18} column with three different solvents and different wavelengths and used for elucidation of structure using IR, ^{1}H and ^{13}C NMR and mass spectroscopic analyses.

2.11 **Characterization of metabolites:** Purified metabolites were characterized using UV-Visible spectrophotometer, FT-IR, ^{1}H, ^{13}C NMR and LC-MS and GC-MS analyses.

2.11.1 **UV analysis:** The culture after harvesting was centrifuged at (16,000 x g for 10 min) and supernatant sample was analyzed in a Spectronic Genesys-2 spectrophotometer in the UV range.

2.11.2 **Infra-red (IR) analysis:** Spectrums were recorded on a Shimadzu FT/IR 8300 spectrophotometer. Solid samples were recorded as KBr wafer and liquid sample as thin film between NaCl plates are reported in cm^{-1}.

2.11.3 **NMR Analysis:** Purified compound suspended in 0.4 ml deuterated water in tubes of 5 mm x 12 mm length were used for ^{1}H and ^{13}C NMR analyses in Bruker AC-400 (400 MHz) analyzer.

2.11.4 **LC-MS analysis:** Liquid chromatography (LC) - Mass Spectrometry (MS) analysis was performed with Micro mass (Deltanics Bruker), equipped with an automatic injector. MS was performed using MS-ESI ion source (Nitrogen flow rate 0.5/h). Working conditions were in ESI probe both negative and positive ion mode and the separation was done using Agilent-400 Binary gradient HPLC 1200-series with UV detector on C_{18} column (Luna 5 µ 150 x 4.6 mm) analysis was performed at 30 ºC (LC column oven) and 85 ºC (MS ionization chamber). Mobile phase consisted of acetonitrile, water (50:50) with a flow rate of 0.8 ml $.min^{-1}$ and the column was equilibrated for 10 min prior to each analysis. 10 µl of the sample was injected and compounds were detected (LC) at 268 nm. The column effluent from the LC was nebulized into an ion source (ESI) region with collision energy of 10 eV for generating

molecular masses. MS-MS was done in Bruker Q-TOF source temperature of 100 °C with injection volume 5 µl with methanol water as the solvent.

2.11.5 **GC-MS Analysis:** The metabolites were extracted into methanol and were analyzed on Gas chromatography-Mass spectrometry (GC-MS) Shimadzu GC-MS QP 2010, with an injection volume of 2 µl on EI probe with column temperature of 300 ºC, injection temperature 310 ºC, column flow 5.2 $ml.min^{-1}$, split ratio was 5.0 with helium as the carrier gas and methanol as the solvent and the molecular masses of the column effluent from the GC was analyzed through 2010 mass analyzer.

2.12 **Isolation and electrophoresis of proteins:** Pyrazine-2-carboxylate induced strain HCU1 was harvested by centrifugation (12,000 x g for 10 min) and the pellet was washed twice with 0.05 M Tris buffer (pH 7.4) and resuspended in the same buffer. Cells were sonicated with MS-72 probe (Bandelin, model-UW 2070) to complete lysis after 8-9 cycles. The cell lysate was centrifuged (16000 x g) for 10 min and supernatant was used as a source of enzyme. The crude extract was subjected to 0-30 % ammonium sulphate saturation. The resulting precipitate was dialyzed overnight at 4 °C with the same buffer and dialyzed fraction was loaded onto DEAE-Cellulose column of 40 ml bed volume (18 x 2.5 cm in length). Proteins were eluted using the sodium chloride step gradient from 0-1 M. The active fractions were pooled and reloaded on to DEAE-cellulose column with a step gradient of 0.3-0.5 M NaCl (5 x 0.5 cm in length). The electrophoresis of the proteins was done on the 12 %, 10 % SDS-PAGE and 8 % Native PAGE and stained with coomassie blue or silver stain (Laemmeli, 1970).

2.13 **MALDI TOF analysis:** Protein was digested with trypsin with 1:10 ratio overnight at 37 °C. The mixture was lyophilized and dissolved in 5µl of acetonitrile: 0.1 % TFA

(1:1). 2 µl of which was mixed with 2 µl of 10 mg/ml HCCA in 5 % acetonitrile: TFA (1:1) and spotted onto target plate for MALDI TOF and m/z values from 500-3500 were recorded. The source voltage 19 KV and matrix suppression 500 Da. Calibration was done using peptich Calibstandard II mono (Bruker).

Results

3.1 **Screening for pyrazine-2-carboxylate metabolizing bacteria**

Both phototrophic and chemotrophic bacteria were screened for pyrazine-2-carboxylate utilization. The phototrophic bacteria used for screening include, *Rhodopseudomonas palustris* JA1, *Rhodobacter sphaeroides* JA337, *Rubrivivax* sp. JA2, *Rhodocysta* sp. JA335 and *Rhodocysta centinarium* DSMZ 9894. Apart from the laboratory strains, fifty pure isolates of chemotrophs and two enrichment cultures obtained in the present study were used for pyrazine-2-carboxylate utilization.

3.1.1 **Utilization of pyrazine-2-carboxylate by pure cultures of bacteria**

Among the five phototrophic bacteria screened, 10 % utilization (Table 8) of pyrazine-2-carboxylate was observed with *Rhodopseudomonas palustris* JA1 alone. Among the chemotrophs screened, utilization of pyrazine-2-carboxylate was observed with seven of the fifty bacterial isolates (Table 8). The biomass of the pyrazine-2-carboxylate supplemented cultures was two fold higher than the control cultures (without PCA). The utilization of pyrazine-2-carboxylate by the bacterial isolates observed after 18 h of incubation ranged from 10-37 % (absorbance of pyrazine-2-carboxylate of the uninoculated control as 0 %) (Table 8). Highest pyrazine-2-carboxylate utilization of 37 % was observed with bacterial isolate C6. Utilization of pyrazine-2-carboxylate by the bacterial isolate C6 on repeated batch transfers could not be demonstrated and the trend remained the same with other bacterial isolates C2, C3, C4, C7 and C8 (Fig 8). On the other hand, transformation (shift in absorbance of PCA at 268 nm) of pyrazine-2-carboxylate was observed with isolate C26 alone.

3.1.2 **Reduction of pyrazine-2-carboxylate by mixed and pure bacterial cultures**

Two enrichment cultures were used in addition to the pure cultures while screening for pyrazine-2-carboxylate utilizing bacteria. Increase in absorbance of the compound at 268 nm in the culture supernatant was demonstrated rather than its utilization by bacterial isolates and enrichment cultures. This increase in absorbance of pyrazine-2-carboxylate can be attributed to the pyrazine ring reduction. Ring reduction of pyrazine-2-carboxylate is defined as % increase in the absorbance of pyrazine-2-carboxylate at 268 nm, taking pyrazine-2-carboxylate absorbance of the uninoculated control as 0 %. Increase in absorbance of pyrazine-2-carboxylate by enrichment and pure cultures observed after 18 h of incubation ranged from 2-50 % (Table 9). There was no difference in the biomass of control and pyrazine-2-carboxylate supplemented cultures.

Among the screened bacterial isolates, utilization of pyrazine-2-carboxylate was not observed with many of the bacterial isolates and there was no consistent utilization of pyrazine-2-carboxylate by the bacterial isolates. Apart from utilization of pyrazine-2-carboxylate, ring reduction of pyrazine-2-carboxylate was also observed with few of the bacterial isolates. It was of interest to study the reductive metabolism of pyrazine-2-carboxylate by bacteria. Bacterial isolate C45 (HCU1) showing highest reduction of pyrazine-2-carboxylate was taken up for detailed study.

Bacteria	Growth (OD_{540nm})		Pyrazine-2-carboxylate (% utilization)
	Without PCA	With PCA	
Chemotrophs			
C2	0.18	0.37	26
C3	0.10	0.21	10
C4	0.08	0.13	14
C6	0.28	0.45	37
C7	0.21	0.50	25
C8	0.08	0.12	11
C26	0.21	0.34	T
Phototroph *Rhodopseudomonas palustris* JA1	0.24	0.36	10

Table 8: Screening bacteria for utilization of pyrazine-2-carboxylate (1mM) as nitrogen source

(T= Transformed pyrazine-2-carboxylate; PCA= Pyrazine-2-carboxylate)

Log phase culture of bacterial isolates were inoculated into Biebl and Pfennig's broth with malate as carbon and pyrazine-2-carboxylate as nitrogen source and incubated aerobically at 30 ± 2 ºC under agitation at 150 rpm. Data pertains to the analysis of the pyrazine-2-carboxylate levels in culture supernatants after 18 h.

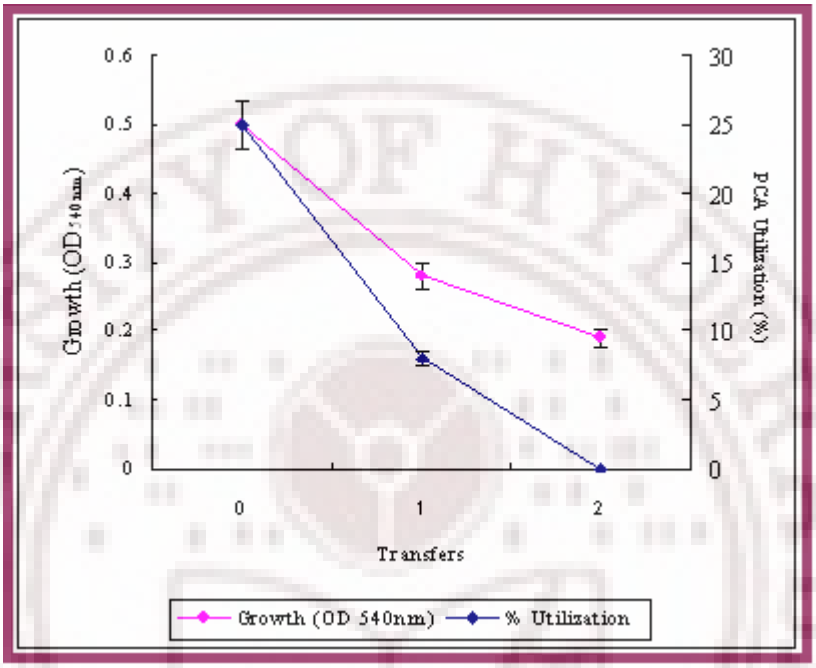


Fig 8: Growth of bacterial isolate C6 and utilization of pyrazine-2-carboxylate on batch transfers

(PCA = Pyrazine-2-carboxylate)

0.1 % w/v of inoculum (0.2 O.D) was batch transferred to Biebl and Pfennig's broth with malate as carbon and pyrazine-2-carboxylate as nitrogen source and incubated aerobically at 30 ± 2 ºC under agitation at 150 rpm. Data pertains to the analysis of the pyrazine-2-carboxylate of the culture supernatant after 18 h.

Mixed/pure bacterial cultures	Growth (OD_{540nm})		Reduction of pyrazine-2-carboxylate (%)
Mixed cultures	Without PCA	With PCA	
Mixed culture (soil enrichment)	0.26	0.28	37
Mixed culture (culture media)	0.27	0.30	50
Pure cultures			
C23	0.22	0.25	30
C 35 (2Y1)	0.22	0.24	8
C37(2Y2)	0.25	0.26	28
C38	0.20	0.20	2
C41	0.19	0.22	14
C 30	0.20	0.20	11
C45	0.23	0.28	40

Table 9: Mixed and pure cultures of bacteria showing reduction of pyrazine-2-carboxylate

Mixed and pure cultures of bacteria were inoculated into Biebl and Pfennig's broth with malate (22 mM) and pyrazine-2-carboxylate (1.5 mM) as carbon and nitrogen sources, respectively and incubated aerobically at 30 ± 2 ºC under agitation at 150 rpm. Data pertains to analysis of the pyrazine-2-carboxylate levels in the culture supernatant after 18 h.

Ring reduction of pyrazine-2-carboxylate is defined as % increase in the absorbance of pyrazine-2-carboxylate at 268 nm over the control (uninoculated blank). 100 % reduction is defined as two fold increase in the absorbance of pyrazine-2-carboxylate over the control (absorbance of pyrazine-2-carboxylate in the uninoculated control).

3.2 **Identification of isolate C45 based on polyphasic taxonomic approach**

Among the screened bacterial isolates showing ring reduction of pyrazine-2-carboxylate, bacterial isolate C45 showing highest reduction of pyrazine-2-carboxylate was chosen for further studies. Bacterial isolate C45 (strain HCU1) was characterized based on polyphasic taxonomic approach.

3.2.1 **Source** Bacterial isolate C45 was serendipitous of the contaminated culture media, which was found growing in our lab.

3.2.2 **Microscopic characteristics** Cells of strain HCU1 are motile rods of 0.5µ diameter and 1.5 µm in length; cell division was by binary fission

3.2.3 **Cultural characteristics** Twenty-four hour incubated colonies on nutrient agar were 1-2 mm in diameter, circular, convex and translucent with entire margin and golden yellow colour. Growth on nutrient broth was uniformly suspended and the broth was colourless, while on Biebl and Pfennig's broth, growth appears in the form of uniform suspension with deep yellow colour with malate as carbon source.

3.2.4 **Physiological characteristics**

Strain HCU1 is an obligate aerobe. Aerobic growth (Table 10) occurs as chemoorganoheterotrophy (organic substrate as source of carbon, electrons and energy). Fermentative and anaerobic growth could not be demonstrated. The organic substrates that were utilized as carbon sources include (Table 11A) citrate, fumarate, L-malate, 2-oxoglutarate, oxaloacetate, pyruvate, succinate, benzoate, D-fructose, D-glucose, D-lactose, D-sucrose, glycerol, hexonoate, propionate and sorbitol. Growth of strain HCU1 could not be demonstrated on mannitol, starch and butyrate. In addition to ammonium

chloride (Table 11B) as nitrogen source, growth was also demonstrated on nitrogen substrates viz., DL-alanine, L-glutamate, L-glycine, and nitrate but not on nitrite. FT-IR analyses of whole cell transmission spectra of strain HCU1 (Fig 9B) with the strain *Stenotrophomonas maltophilia* K279A (Fig 9A) differs in amine formation at wave number (cm^{-1}) of 2400. The pH range for strain HCU1 is 6.0–10, with an optimum at pH range of 6-7. The temperature range was 8-40 °C and the optimum is at 30 °C. The organism has the capability to tolerate high concentrations of the salt to 4 % (w/v).

3.2.5 **Biochemical characterization** Strain HCU1 showed negative for indole, methyl red and Voges Proskauer tests (Table 12). The strain showed positive for citrate utilization, ammonification and catalase activities, while negative for gelatinase, urease, amylase and nitrogenase activities.

3.2.6 **16S rRNA gene sequence analysis of strain HCU1**

3.2.6.1 **16S rRNA gene amplification and sequencing:** The 16S rRNA gene of the bacterial strain HCU1 was amplified from the genomic DNA using the protocol mentioned in the materials and methods and sequenced. The pair wise sequence similarities of strain HCU1 with the nearest type strains were found using EzTaxon server. 16S rRNA gene sequence of the strain HCU1 was deposited in the Gen Bank with the EMBL/DDBJ accession number of AM159126.

3.2.6.2 **Phylogenetic analysis-Dendrogram:** Dendrogram was constructed based on 16S rRNA gene sequence using the software as mentioned in materials and methods. NCBI BLAST analysis of 16S rRNA gene sequence of strain HCU1 showed 98.7 % similarity with the 16S rRNA gene of *Stenotrophomonas maltophilia* ATCC 13637 and 99.2 % with *Pseudomonas geniculata* ATCC 19374. *Pseudomonas geniculata* is one among the

26 species, which is not included in the *Pseudomonas* cluster. *Pseudomonas geniculata* is closely related to the genus *Stenotrophomonas* rather than to the genus *Pseudomonas* (Anzai *et al.,* 2000). Since the taxonomic position of *Pseudomonas geniculata* is ambiguous, strain HCU1 showing 99.2 % similarity with the type strain *Pseudomonas geniculata* is named as *Stenotrophomonas* sp. HCU1. The strain is out grouped with *Pseudomonas geniculata* (ATCC 19374) and *Stenotrophomonas maltophilia* (ATCC 13637) (Fig 10). DNA-DNA hybridization of the strain HCU1 with the type strain needs to be done to know its exact taxonomic status.

Growth mode	Substrate used (% w/v)	Growth (OD $_{540nm}$)	Growth
Control	-	0.07	
Chemoorganoheterotrophy	Malate	0.45	+
Anaerobic (Respiratory)	Sucrose + NO_3^-	0.07	NG
Chemolithoautotrophy	Dark, $Na_2S_2O_3$ +HCO_3	0.07	NG
Chemolithoheterotrophy	Dark, $Na_2S_2O_3$ + Malate	0.40	+
Fermentative	Sucrose	0.06	NG

Table 10: Growth modes of *Stenotrophomonas* sp. HCU1

(+ = Growth positive; NG = No growth; - = nil; Control = Basal medium without carbon and electron source)

Chemoorganoheterotrophic and chemolithoautotrophic media (Biebl and Pfennig's broth) were inoculated with 0.1 % (v/v of 0.4 $O.D_{540nm}$) log phase culture of strain HCU1 with malate, sucrose (0.3 % w/v) and bicarbonate (0.1 % w/v) as carbon sources and sodium thiosulphate (1 mM) as electron donor and incubated aerobically at 30 ± 2 °C under agitation at 150 rpm. Growth of the strain HCU1 was measured after 18 h.

Carbon source (0.3 % w/v or v/v)	Growth (OD_{540nm})	Growth
Control	0.10	-
Acetate	0.20	+
Citrate	0.32	+
Fumarate	0.40	+
2-oxoglutarate	0.40	+
DL-Malate	0.40	+
Oxaloacetate	0.40	+
Pyruvate	0.35	+
Succinate	0.45	+
Fructose	0.35	+
Glucose	0.55	+
Lactose	0.50	+
Sucrose	0.40	+
*Mannitol	0.05	-
*Sorbitol	0.15	+
*Glycerol	0.24	+
*Butyrate	0.05	-
*Hexonoate	0.20	+
*Propionate	0.30	+
Benzoate	0.15	+
*Benzylamine	0.12	-

Table 11A: Growth of *Stenotrophomonas* sp. HCU1 on different organic substrates as carbon sources

* Compounds used at a concentration of 0.1 % w/v; + = Supported growth; - = Not supported growth

0.5 % v/v (0.4 $O.D_{540nm}$) log phase culture of strain HCU1 was inoculated on to Biebl and Pfennig's broth with different organic substrates as carbon sources and ammonium chloride as nitrogen source and incubated aerobically at 30 ± 2 ºC under agitation at 150 rpm for 24 h. Organic substrates were utilized at a concentration of 0.3 % (w/v) except for the highlighted compounds which were used at a concentration of 0.1 % (w/v). Data values are the average of an experiment repeated thrice.

Nitrogen source (0.1 % w/v)	Growth (OD_{540nm})
Control	0.20
DL-Alanine	0.30
Ammonium chloride	0.40
L-Glutamate	0.65
L-Glycine	0.55
Nitrate	0.60
Nitrite	0.20

Table 11B: Growth of *Stenotrophomonas* sp. HCU1 on different nitrogen sources

Data values are the average of the experiment repeated thrice. Experimental conditions are same as described in table 11 A.

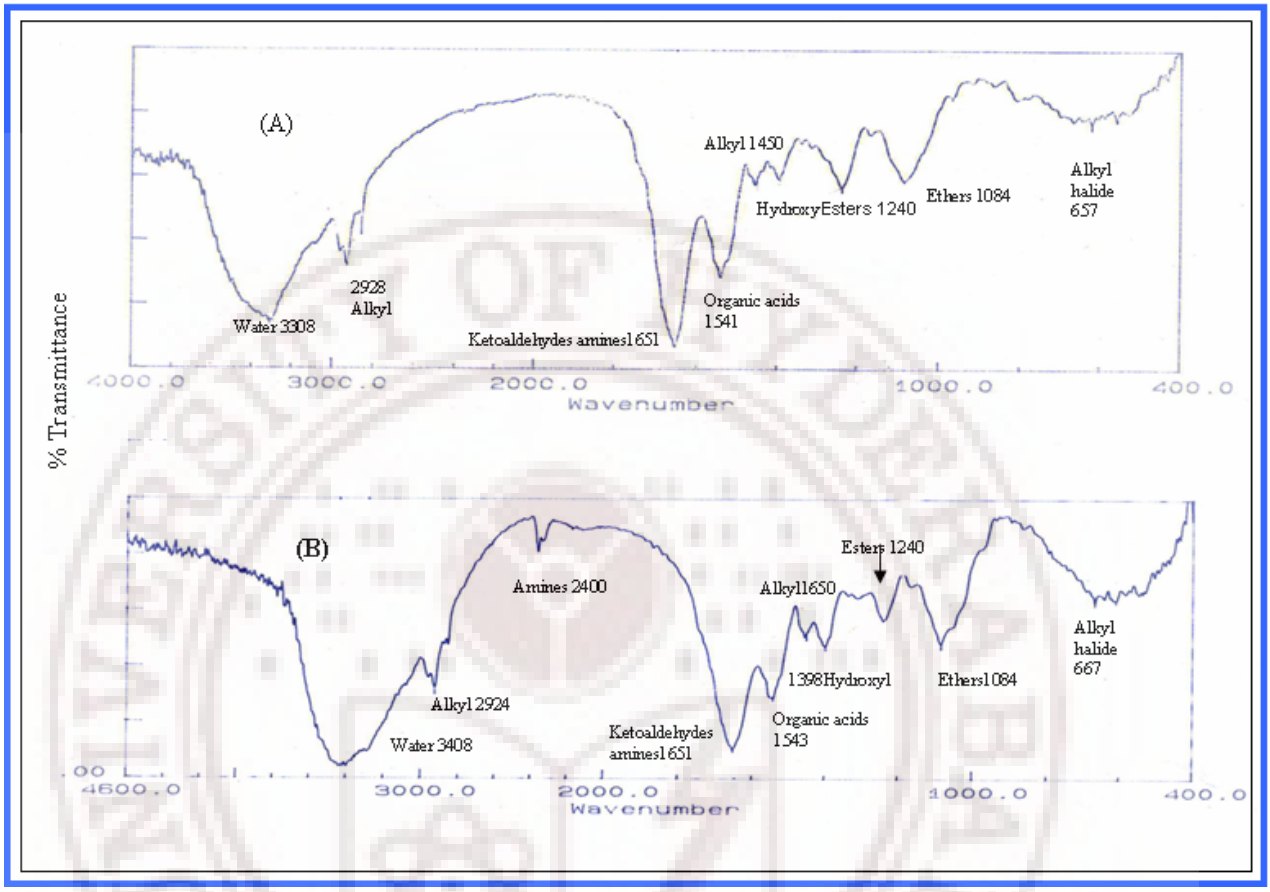


Fig 9: FT-IR typing of (A) type strain *Stenotrophomonas maltophilia* K279A and (B) Strain HCU1

Log phase cultures of *Stenotrophomonas maltophilia* and strain HCU1 were harvested and the culture pellets were lyophilized. Transmission spectrum of the lyophilized pellets were recorded using Shimadzu infrared spectrophotometer.

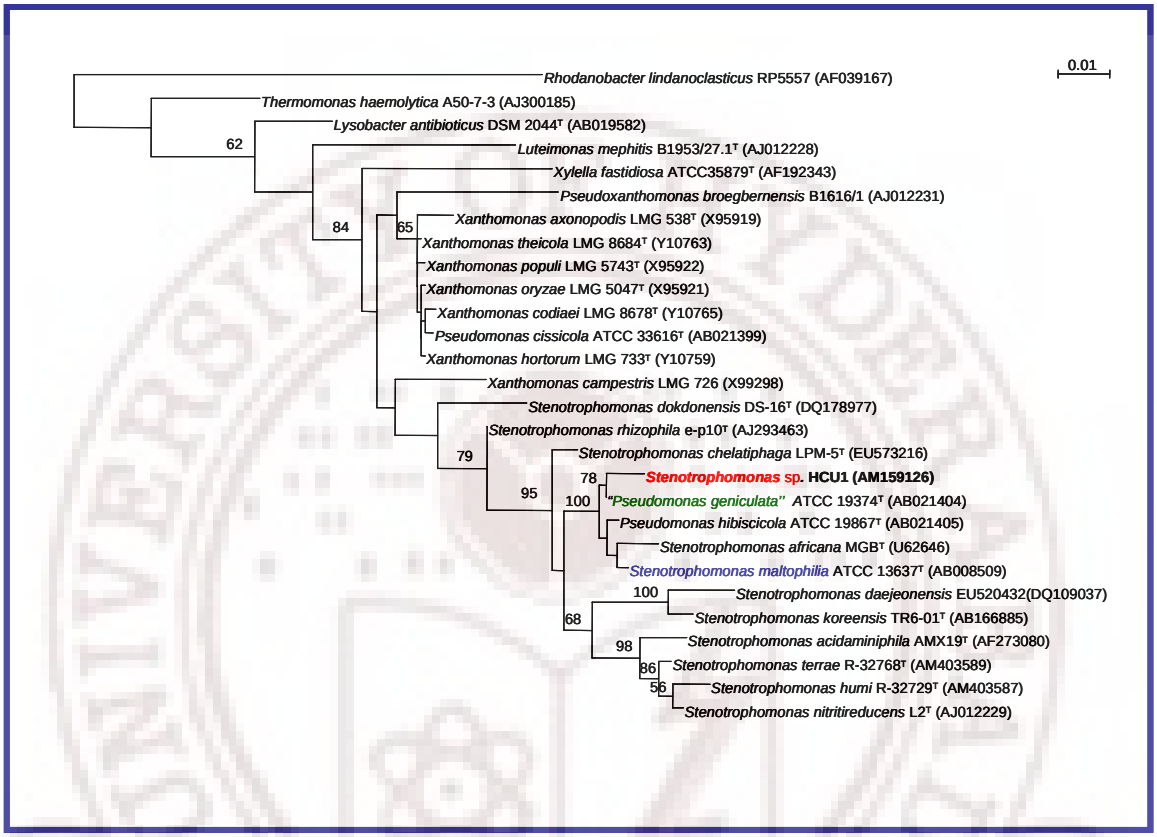


Fig 10: 16S rDNA-based dendrogram showing the phylogenetic position of *Stenotrophomonas* sp. HCU1 among the *Stenotrophomonas* and *Xanthomonas* representatives of *Gammaproteobacteria*

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.

Characters	*Stenotrophomonas maltophilia* (ATCC 13637^T)	*Stenotrophomonas* sp. (strain HCU1)
Cell size (WxL)	0.5x1.5µ	0.5x1.5µ
Colour of the cell suspension	Yellow	Yellow
Methionine requirement	+	-
N- sources		
Nitrate	-	+
Nitrite	-	-
Organic substrates		
L-Glutamate	+	+
L-Glycine	+	+
L-Alanine	+	+
Glucose	+	+
Fructose	+	+
Lactose	+	+
Sucrose	+	+
L-malate	+	+
Fumarate	+	+
Pyruvate	+	+
Citrate	+	+
Succinate	+	+
α-ketoglutarate	+	+
Biochemical tests		
Citrate	+	+
Ammonification	+	+
Nitrite reduction	-	-
Catalase	+	+
Gelatinase	+	-

Table 12: Comparative characters of strain HCU1 with the type strain of *Stenotrophomonas maltophilia*

(+ = Supported/utilized; - = Not supported/not utilized; ND = not detected)

3.3 **Catabolism of pyrazine-2-carboxylate by S*tenotrophomonas* sp. HCU1**

3.3.1 **Growth and utilization of pyrazine-2-carboxylate as carbon, nitrogen and as a supplement by *Stenotrophomonas* sp. HCU1**

Growth of *Stenotrophomonas* sp. HCU1 and utilization of pyrazine-2-carboxylate (1.5 mM) was demonstrated when pyrazine-2-carboxylate was used as a sole source of carbon (Fig 11), nitrogen (Fig 12) replacing malate or ammonium chloride, respectively for chemoorganoheterotrophic growth. There was an initial lag of 4, 2 and 2 h when pyrazine-2-carboxylate was used as sole source of carbon (Fig 11), nitrogen (Fig12) and supplement (Fig 13), respectively. Presence of pyrazine-2-carboxylate did not alter the doubling time of the strain. However, ring reduction of pyrazine-2-carboxylate was exponential without any lag and was simultaneous with the growth of strain HCU1 (Fig 11). Maximum ring reduction of pyrazine-2-carboxylate was 40 %; when used as nitrogen source, 80 %, when used as carbon source and 96 % as a supplement at 8, 6 and 12 h, respectively. There was subsequent loss of reduced pyrazine-2-carboxylate at 14 and 18 h when used as carbon and nitrogen source, respectively. Complete utilization of the reduced pyrazine-2-carboxylate was observed when used as carbon source; such a complete utilization of the reduced pyrazine-2-carboxylate was not observed when used as nitrogen source or supplement. Highest reduction of pyrazine-2-carboxylate was observed when pyrazine-2-carboxylate was used as an additional supplement and hence catabolism of pyrazine-2-carboxylate by strain HCU1 was studied with pyrazine-2-carboxylate as an additional supplement.

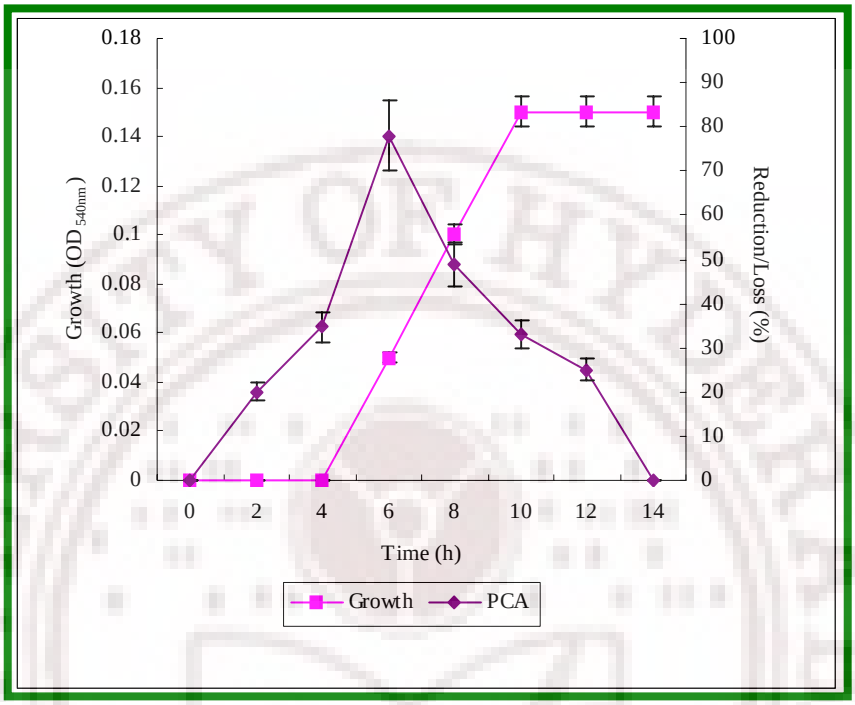


Fig 11: Chemoorganoheterotrophic growth of *Stenotrophomonas* sp. HCU1 on pyrazine-2-carboxylate as sole carbon source

0.5 % v/v (0.2 $O.D_{540nm}$) of pyrazine-2-carboxylate (as a carbon source) induced log phase culture of strain HCU1 was inoculated on to Biebl and Pfennig's broth with pyrazine-2-carboxylate as carbon and ammonium chloride as nitrogen source and incubated aerobically at 30 ± 2 ºC under agitation at 150 rpm. Growth of the culture and reduction of pyrazine-2-carboxylate was monitored at regular intervals of time.

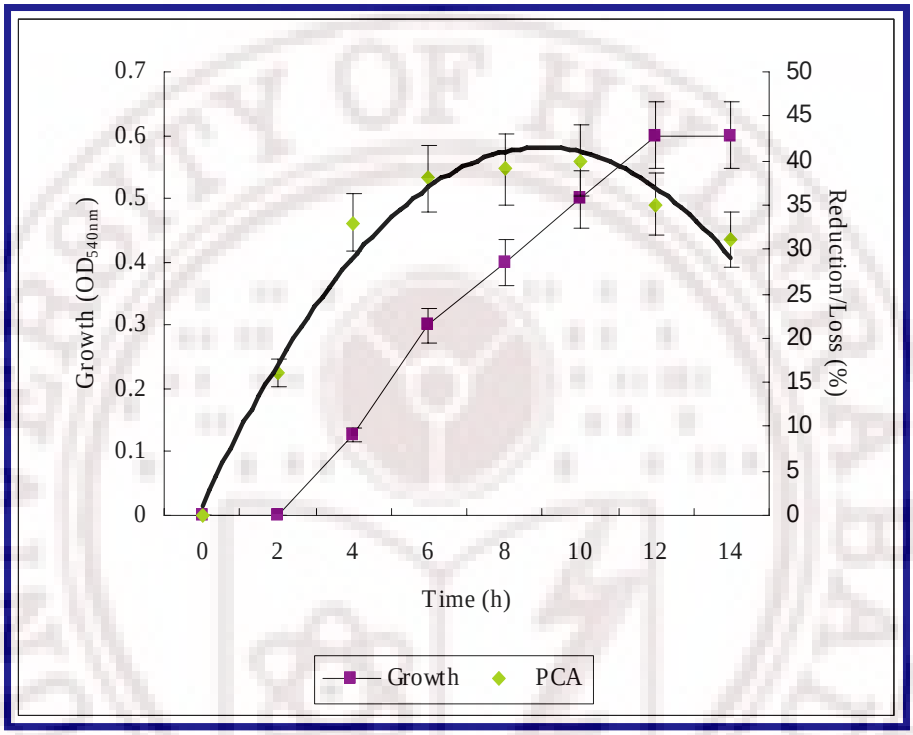


Fig 12: Chemoorganoheterotrophic growth of *Stenotrophomonas* sp. HCU1 on pyrazine-2-carboxylate as sole nitrogen source

(PCA = pyrazine-2-carboxylate, Ring reduction is % increase in absorbance of the pyrazine-2-carboxylate compared to its absorbance at initial hour.)

Experimental conditions are same as in fig 11 except for 0.5 % v/v (0.4 $O.D_{540nm}$) of pyrazine-2-carboxylate (as a nitrogen source) induced log phase culture of strain HCU1 was used.

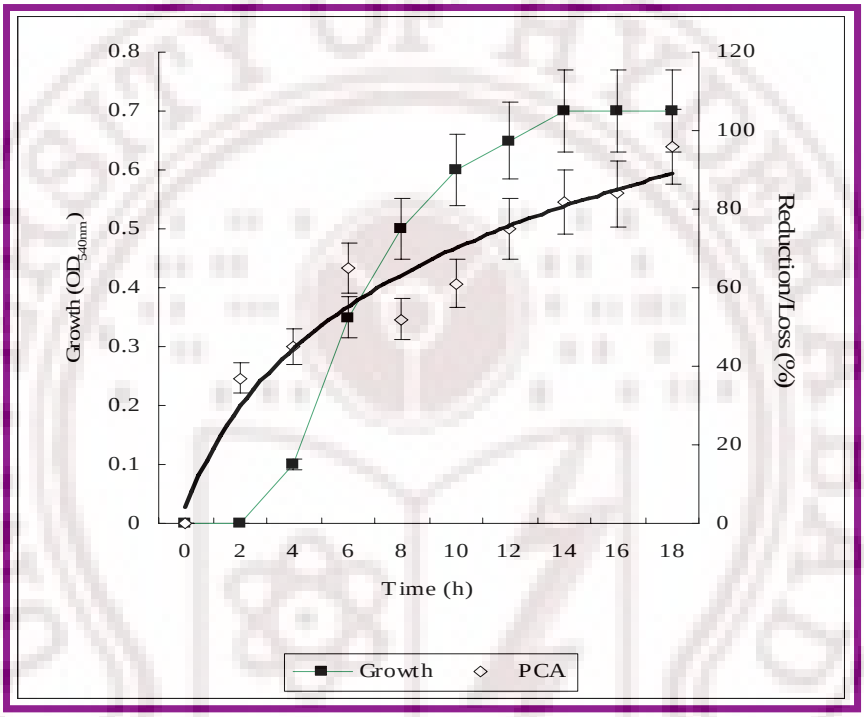


Fig 13: Chemoorganoheterotrophic growth of *Stenotrophomonas* sp. HCU1 and reduction of pyrazine-2-carboxylate

Experimental conditions are same as in fig 11 except for 0.5 % v/v (0.4 $O.D_{540nm}$) of pyrazine-2-carboxylate (as a supplement) induced log phase culture of strain HCU1 used as inoculum.

3.3.2 **Reduction of pyrazine-2-carboxylate by *Stenotrophomonas* sp. HCU1 at different concentrations of pyrazine-2-carboxylate**

Influence of pyrazine-2-carboxylate concentration on its reduction by the *Stenotrophomonas* sp. HCU1 was studied from 0.5 mM to 5 mM. There was no difference in the biomass of the strain HCU1 at any of the concentrations used. Maximum reduction of pyrazine-2-carboxylate (86 %) at around 1.5 mM (Fig 14) was observed and then decreased with increased concentrations of pyrazine-2-carboxylate. Though reduction up to 5 mM was observed it was less than 20 %. There was no difference in the biomass at any of the concentrations used.

3.3.3 **Influence of carbon sources on ring reduction of pyrazine-2-carboxylate**

Influence of different organic substrates as carbon sources on the ring reduction of pyrazine-2-carboxylate was studied by using the growing cells of *Stenotrophomonas* sp. HCU1. The carbon sources used include (Table 13) malate, acetate, citrate, fumarate, oxaloacetate, 2-oxoglutarate, pyruvate, succinate, D-glucose and L-glutamate. Maximum reduction of pyrazine-2-carboxylate of 83 % was demonstrated with malate as carbon source.

In order to study the influence of malate concentration on reduction of pyrazine-2-carboxylate, strain HCU1 was grown on different concentrations of malate (Fig 15) with ammonium chloride as nitrogen source. The concentrations used were in the range of 0 to 3 % w/v (0-220 mM). Ring reduction of pyrazine-2-carboxylate increased with the increasing concentrations of malate and reached a maximum at 0.3 % w/v (22 mM) of malate concentration and then declined.

3.3.4 **Influence of ammonium chloride concentration on ring reduction of pyrazine-2-carboxylate**

Influence of ammonium chloride concentration on reduction of pyrazine-2-carboxylate by strain HCU1 was studied from 0-1.2 % w/v (0-70 mM). Eighty to hundred percent ring reduction of pyrazine-2-carboxylate was observed at 0.12 % w/v (7 mM) of ammonium chloride (Fig 16) and then remained constant beyond 0.12 % w/v.

3.3.5 **Reduction of pyrazine-2-carboxylate on batch transfers**

Pyrazine-2-carboxylate induced culture of *Stenotrophomonas* sp. HCU1 was batch transferred to pyrazine-2-carboxylate supplemented medium as a carbon, nitrogen and as an additional supplement (data not shown). There was a decrease in the ring reduction of pyrazine-2-carboxylate from 100 to 20 % by *Stenotrophomonas* sp. HCU1 on third transfer when used as an additional supplement, while growth of strain HCU1 remained the same till third transfer. Similarly, ring reduction of pyrazine-2-carboxylate by strain HCU1 was also decreased from 80 and 40 % to 10 % and 25 % used as carbon and nitrogen source, respectively. Increase in the growth yield on third transfer was observed with pyrazine-2-carboxylate as carbon and nitrogen source.

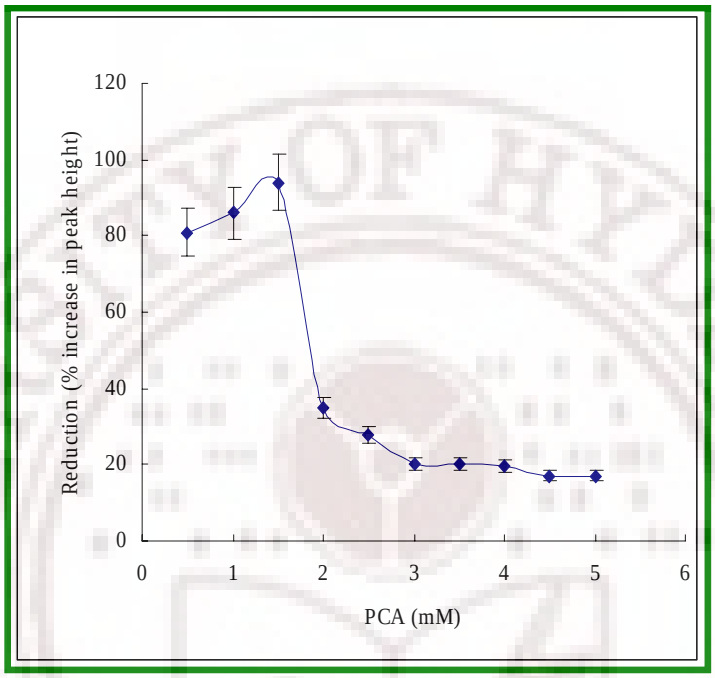


Fig 14: Ring reduction of pyrazine-2-carboxylate by *Stenotrophomonas* sp. HCU1 at different concentrations of pyrazine-2-carboxylate

Experimental conditions are same as in Fig 13 except for different concentrations of pyrazine-2-carboxylate used. Data pertains to the analysis of pyrazine-2-carboxylate levels in the culture supernatants after 14 h.

Carbon source (0.3 % w/v)	Growth (OD_{540nm})	Reduction of pyrazine-2-carboxylate (%)
Control	0.05	36
Citrate	0.40	18
Fumarate	0.40	20
Glucose	0.42	0
Glutamate	0.46	0
Malate	0.48	83
2-oxoglutarate	0.40	24
Oxaloacetate	0.45	8
Pyruvate	0.50	15
Succinate	0.55	26

Table 13: Influence of organic substrates on the reduction of pyrazine-2-carboxylate by *Stenotrophomonas* sp. HCU1

(Control = without pyrazine-2-carboxylate)

Experimental conditions are same as in fig 14 except for the different organic substrates used as carbon sources. Data pertains to the analysis of pyrazine-2-carboxylate levels in the culture supernatants after 14 h.

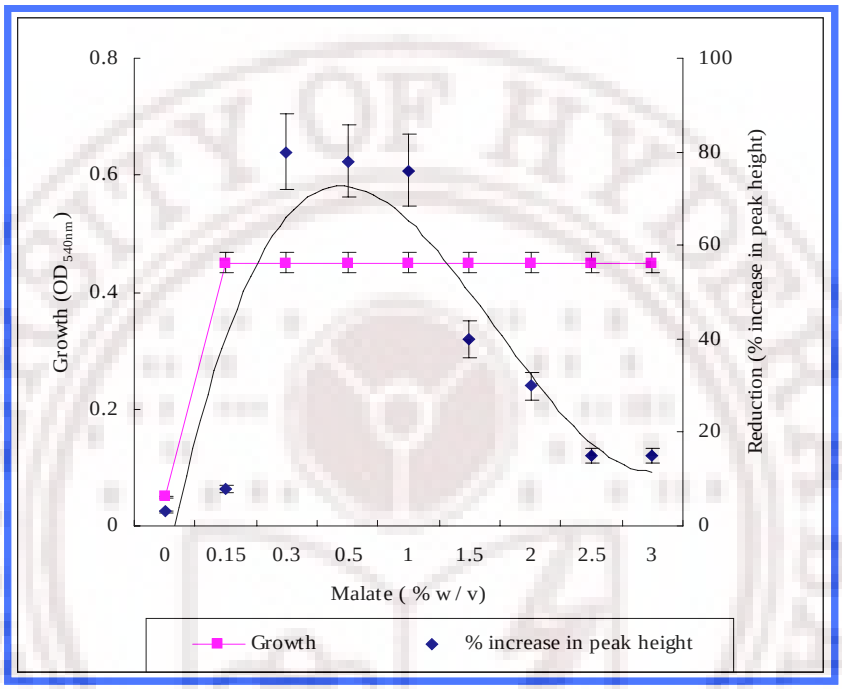


Fig 15: Influence of malate concentration on the reduction of pyrazine-2-carboxylate by the growing cells of *Stenotrophomonas* sp. HCU1

Experimental conditions are same as in fig 14 except for different concentrations of malate used. Culture was harvested after 14 h of aerobic incubation and pyrazine-2-carboxylate levels in culture supernatant were analyzed in HPLC.

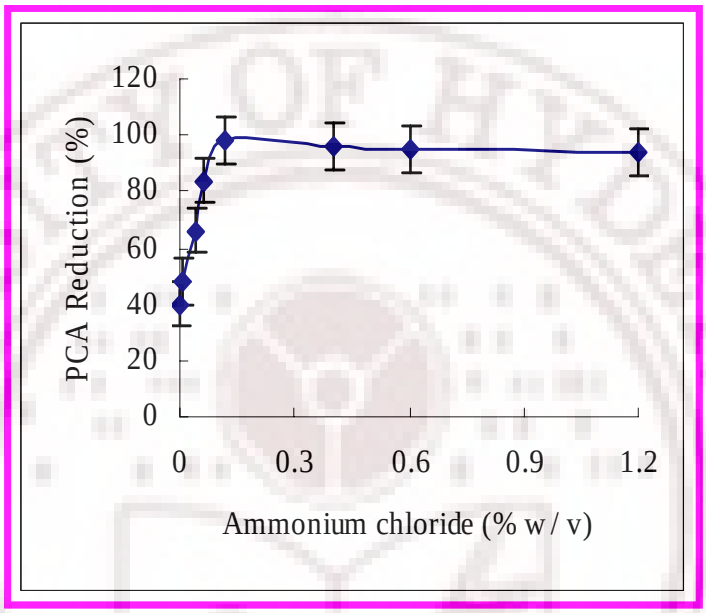


Fig 16: Influence of ammonium chloride concentration on reduction of pyrazine-2-carboxylate by *Stenotrophomonas* sp. HCU1

Experimental conditions are same as in fig 14 except for the different concentrations of ammonium chloride used. Culture was harvested after 14 h of aerobic incubation and pyrazine-2-carboxylate levels in the culture supernatants were analyzed using HPLC.

3.3.6 **Reduction of pyrazine-2-carboxylate with resting cells of strain HCU1**

Reduction of pyrazine-2-carboxylate was also demonstrated with the resting cells of *Stenotrophomonas* sp. strain HCU1. Ring reduction (Fig 17) increased with time and reached a maximum up to 30 % at around 30 h of incubation. However, biomass remained the same. Though ring reduction was demonstrated with resting cells of strain HCU1, it was comparatively lesser when compared to the reduction observed with growing cells.

3.3.7 **Reduction of other N-heterocyclic compounds by *Stenotrophomonas* sp. HCU1**

The capability to reduce various N-heterocyclic compounds was studied using the growing cells of strain HCU1. Among the N-heterocyclic compounds tested, (Table 14) a maximum of 78 % reduction was observed with pyrazine-2-carboxylate. In addition to pyrazine-2-carboxylate, the strain HCU1 also reduced compounds like guanine, uracil and nicotinic acid and imidazole.

3.3.8 **Toxicity of pyrazine-2-carboxylate on the growth of *Stenotrophomonas* sp. HCU1**

Growth of strain HCU1 was demonstrated on Biebl and Pfennig's broth supplemented with pyrazine-2-carboxylate up to a concentration of 10 mM. Hence the growth of the strain HCU1 was studied beyond 10 mM concentration of pyrazine-2-carboxylate. Growth of the strain and ring reduction of pyrazine-2-carboxylate was demonstrated up to a concentration of 600 mM. The 50 % inhibitory concentration (IC_{50}) (Fig 18) and minimum inhibitory concentration (MIC) of pyrazine-2-carboxylate is 600 and 800 mM respectively for *Stenotrophomonas* sp. HCU1. .

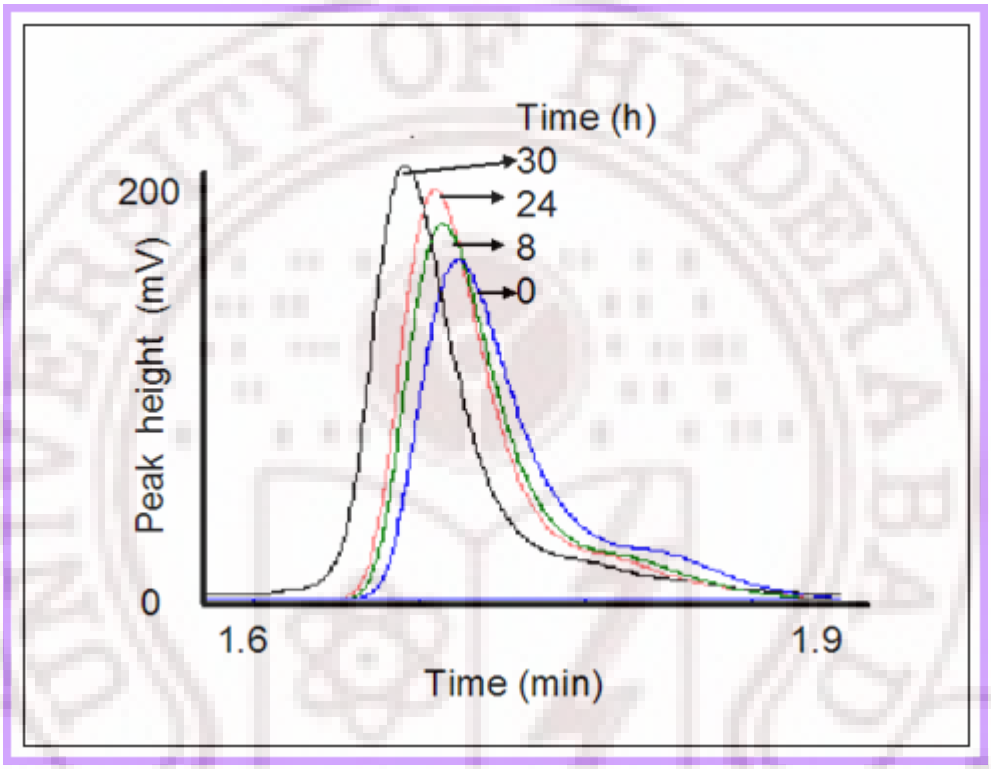


Fig 17: HPLC chromatogram showing time course of reduction of pyrazine-2-carboxylate by the resting cells of *Stenotrophomonas* sp. HCU1

Resting cells of strain HCU1 were pelleted out at different time intervals and pyrazine-2-carboxylate levels in the supernatant were analyzed in HPLC.

N-heterocyclic compound (1.5mM)	Growth (OD_{540nm})	Reduction of N-heterocyclic compound (%)
Control	0.45	-
4-Cyanopyridine	0.18	0
Dimethylaminopyridine	0.46	0
Guanine	0.46	20
Imidazole	0.45	15
Nicotinic acid	0.45	20
Pyrazine	0.48	20
Pyrazine-2-carboxylate	0.48	78
Pyrazinamide	0.46	0
Quinoline	0.25	0
Uracil	0.46	18

Table 14: Growth of *Stenotrophomonas* sp. HCU1 on N-heterocyclic compounds (supplement) and their reduction

Experimental conditions are same as in fig 14 except for the different N-heterocyclic compounds used as supplement. Strain HCU1 grown on different N-heterocyclic compounds was harvested after 14 h and N-heterocyclic compound levels in the culture supernatants were analyzed using HPLC.

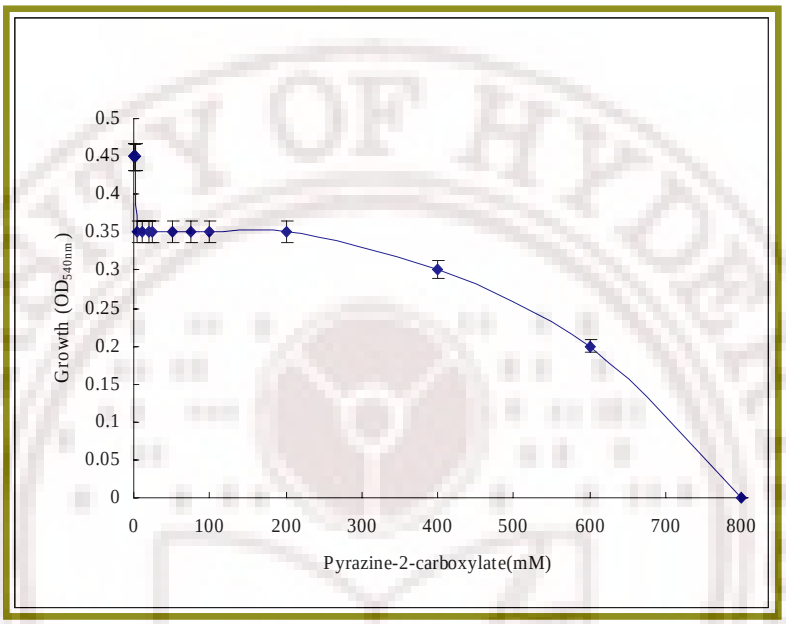


Fig 18: Minimum inhibitory concentration of pyrazine-2-carboxylate on growth of *Stenotrophomonas* sp. HCU1

Experimental conditions are same as in fig 14 except for the different concentrations of pyrazine-2-carboxylate used. Data pertains to analysis of the culture supernatant after 18 h of incubation. IC_{50} and MIC of pyrazine-2-carboxylate is 600 and 800 mM respectively.

3.4 **Isolation and characterization of metabolites of pyrazine-2-carboxylate metabolism**

3.4.1 **Metabolite profiling of the culture supernatant of strain HCU1 grown on pyrazine-2-carboxylate as supplement**

In order to know the metabolites produced in the presence of pyrazine-2-carboxylate by *Stenotrophomonas* sp. HCU1, the culture supernatant of *Stenotrophomonas* sp. grown with and without pyrazine-2-carboxylate was concentrated and the crude concentrate was extracted into methanol. The methanol extract was analyzed using LC-MS. The metabolite of mass (*m/z*) 129 (in the negative mode) was detected in the presence of pyrazine-2-carboxylate, while it was absent in the culture supernatant of *Stenotrophomonas* sp. HCU1 grown without pyrazine-2-carboxylate (Fig 19).

3.4 2 **Isolation and purification of metabolites**

The concentrated crude extract on dissolving in methanol resulted in the precipitation of a white solid which is termed as fraction A and supernatant is termed as fraction B. Metabolites were isolated from both fraction A and B using semi preparative HPLC (Flow chart 1&2).

3.4.2.1 **Characterization of metabolite isolated from fraction A:** The white precipitate (fraction A) from the methanolic supernatant was dried and resuspended in methanol and was purified using semipreparative HPLC using methanol, water, acetonitrile (1:1:0.25) as the solvent system. Different fractions corresponding to the metabolites were collected and major fraction (A1) giving positive reaction to ferrous sulphate reagent was further analyzed. The metabolite had a t_R of 1.55 minutes. The purity of the compound was checked at different wavelengths from 200 to 400 nm. The metabolite was characterized using UV, IR, and ^{1}H, ^{13}C NMR and mass spectroscopic analyses and based on these

results the structure of the compound was elucidated and identified. Infra red analysis of the compound (Fig 20A) indicated the partial ring saturation with peaks (cm^{-1}) at 3445 corresponding to N-H stretching, 1319 corresponds to secondary amine stretching, 1595 corresponding to N-H bending, 1408 corresponding to CH_2 bending and 675 corresponding to = C-H bending vibrations. Ring saturation or presence of cyclic imine was confirmed by the positive reaction to ninhydrin test (plate 1). Proton NMR analysis (Fig 21A) of the compound reveals it as partial ring saturated compound and is a mixture of two isomers differing in the position of double bond. Further purification of the isomer mixture was not possible owing to the low yields of the product. ^{13}C NMR (Fig 21B) of the compound also confirms the partially saturated ring structure of the compound. GC-MS analysis (Fig 20B) of the compound showed mass of 128 m/z. The molecular formula of the compound is $C_5H_9N_2O_2$ and the IUPAC name of the compound is 1, 2, 5, 6-tetrahydropyrazine-2-carboxylic acid (Fig 20B). This compound is a white amorphous solid having no absorption in the visible region.

3.4.2.2 **Characterization of metabolite isolated from fraction B:** The metabolites obtained from the methanolic supernatant (fraction B) were purified using semipreparative HPLC and peaks corresponding to the metabolite fractions were collected. The peak fractions were checked for purity in analytical HPLC at two different wavelengths. The major metabolite resolving at 1.5 minutes (B1) was characterized. Infra red analysis (Fig 22A) of the compound showed peaks (cm^{-1}) 3416, 1711 and 1095-1398 corresponding to carboxylic (COOH) group stretching vibrations. 1022-1398(cm^{-1}) stretching corresponds to C-N group stretching. Peak at 1614 and 3416 cm^{-1} corresponds to N-H group bending and stretching. Peak at 1398 cm^{-1} correspond to CH_3 group

bending and 1406 corresponding to secondary amine bending. Saturation of the aromatic ring of pyrazine-2-carboxylate or presence of secondary imine was confirmed by the positive reaction to ninhydrin. ^{1}H and ^{13}C NMR (Fig 23A&B) analysis of the metabolite B1 indicated pyrazine ring cleavage having a mass of 134 (Fig 22B). Based on the NMR and mass spectroscopic analyses (Fig 22C, 23A&B), the metabolite was identified as ring cleavage product of pyrazine-2-carboxylate named, 2-amino-2-hydroxy-3-(methylamino) propanoic acid and the compound is a orangish sticky compound.

3.4.2.3 **Isolation of two other metabolites of pyrazine-2-carboxylate catabolism:** During purification of metabolite B1 using semi preparative HPLC, two more metabolites were isolated from fraction B in water, acetonitrile solvent system in gradient mode. The masses of the metabolites in the HPLC peak fractions showed 127 and 129 in the negative mode (Fig 24A&B). The metabolites were not further purified and characterized owing to their very low yields. However, based on the mass analysis, the metabolites B2 and B3 were predicted to be 1, 4-dihydropyrazine-2-carboxylic acid and piperazine-2-carboxylic acid, respectively. The metabolites isolated at different time intervals showed positive reaction to cyclic imino acids (plate 1).

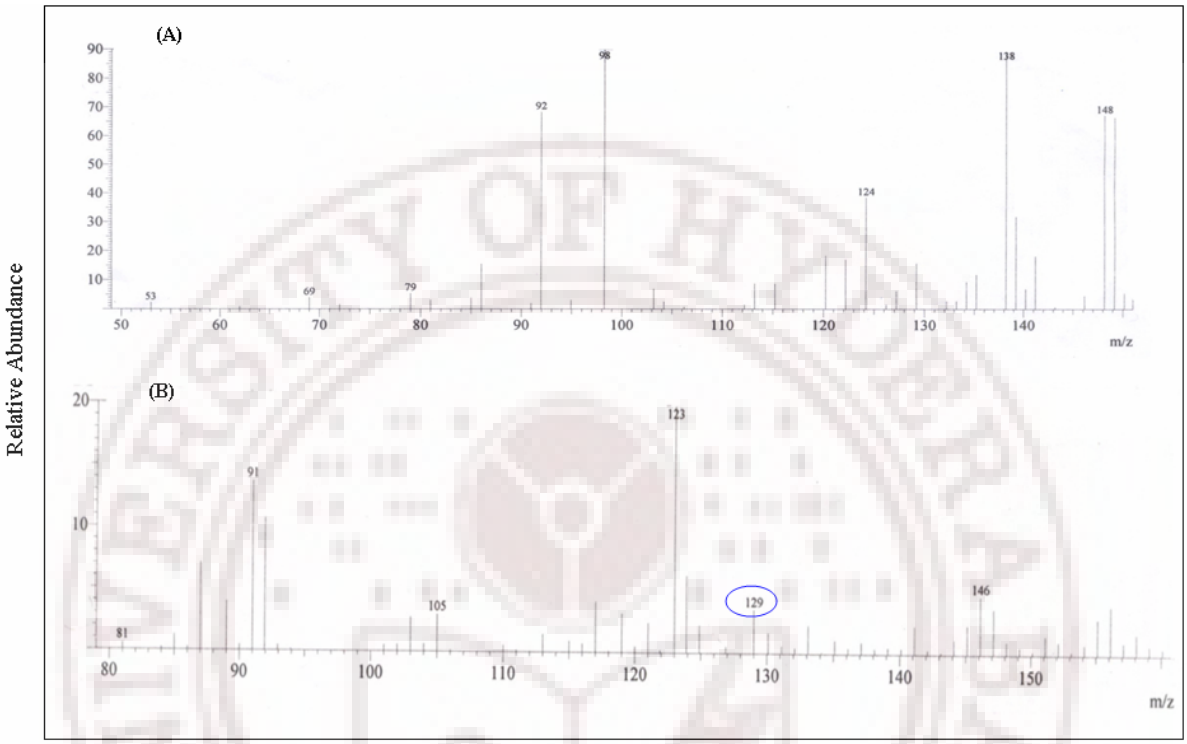
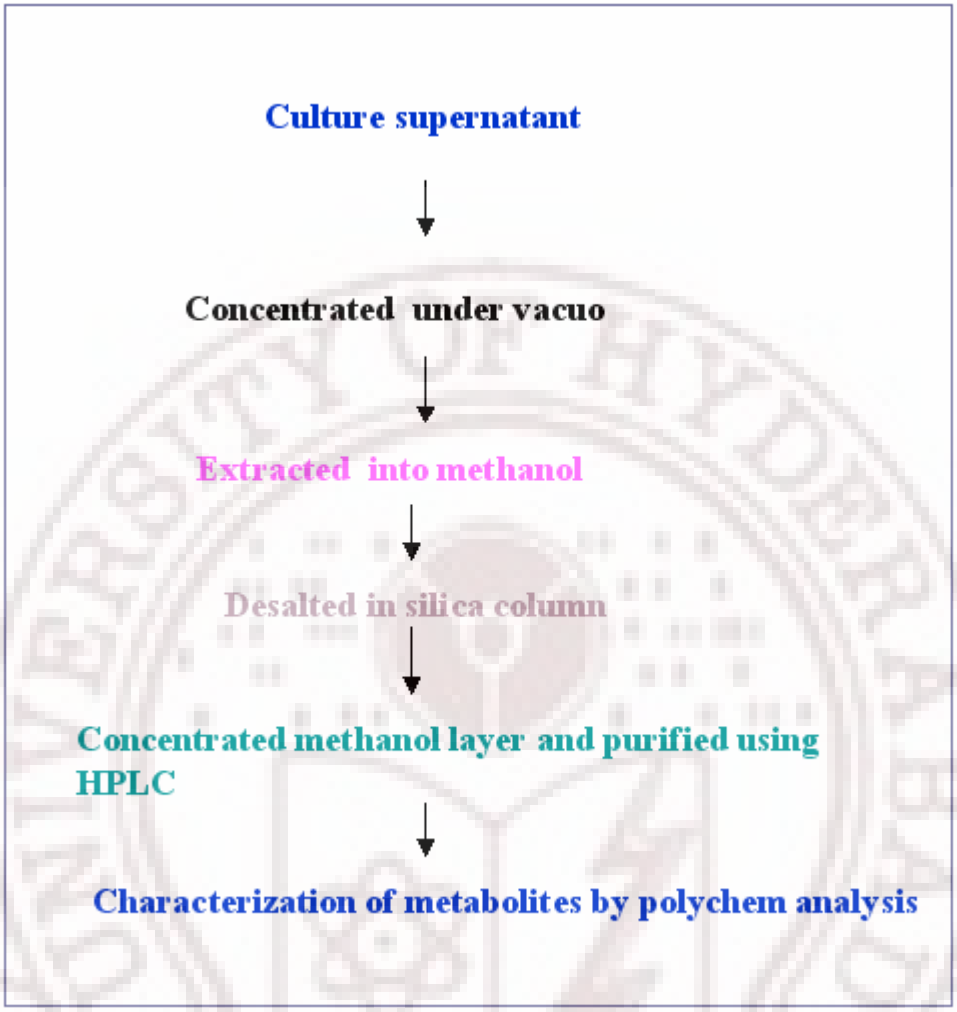


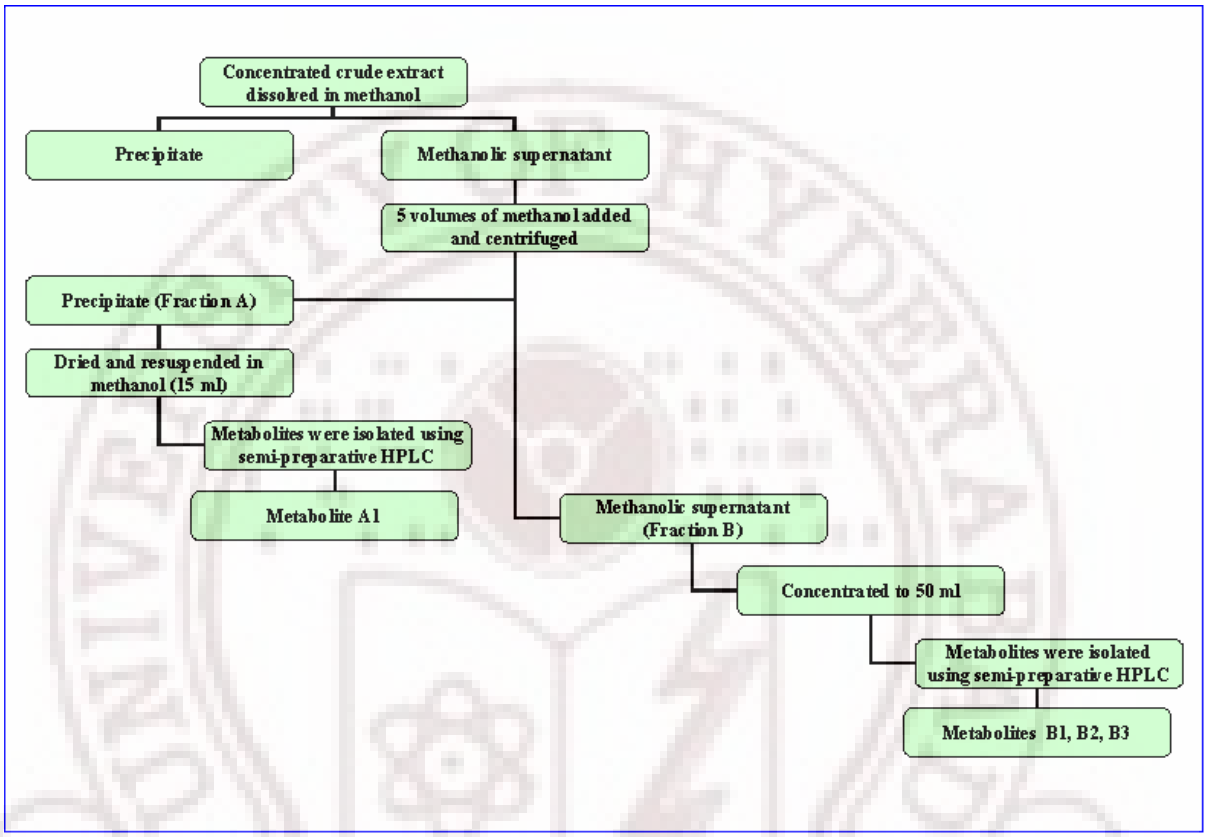
Fig 19: LC-MS metabolite profiling of the culture supernatant of strain HCU1 grown with and without pyrazine-2-carboxylate

(A) Represents the ion masses of the metabolites of culture supernatant of strain HCU1 grown without pyrazine-2-carboxylate obtained from the peak integration of LC chromatogram (data not shown)

(B) Represents the ion masses of the metabolites of the culture supernatant of the strain HCU1 grown with pyrazine-2-carboxylate. The encircled one is the mass of the metabolite, which was absent in the control supernatant.



Flow chart 1: Isolation of metabolites of pyrazine-2-carboxylate catabolism



Flow chart 2: Isolation and purification of metabolites from the culture supernatant of strain HCU1

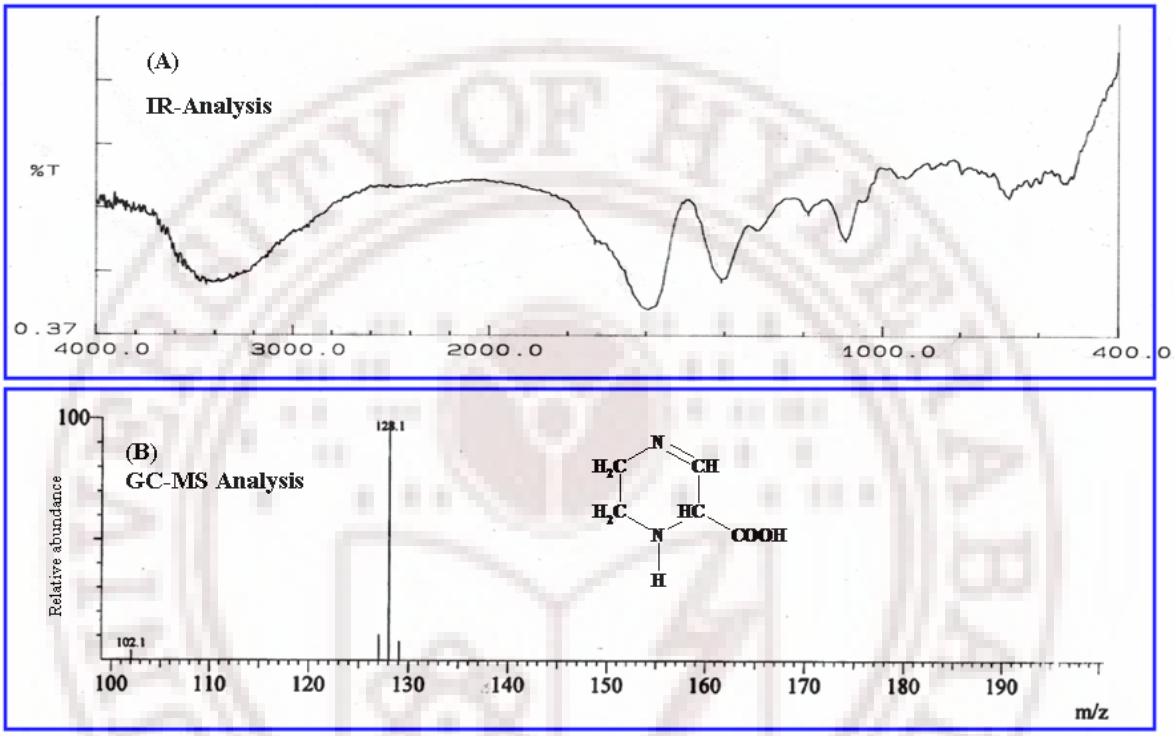


Fig 20: (A) IR and (B) Mass spectroscopic analyses and structure of the metabolite A1

Analyses was done as described in materials and methods

(A) Infra Red spectral analysis (KBr pellet; cm^{-1}): 3445-N-H stretching, 1595-N-H bending, 1408-CH_2 bending, 1319-C-N (2° amine) stretching, 675- =C-H bending vibrations.

(B) Mass spectrum of metabolite A1 showing m/z of 128 and its predicted structure.

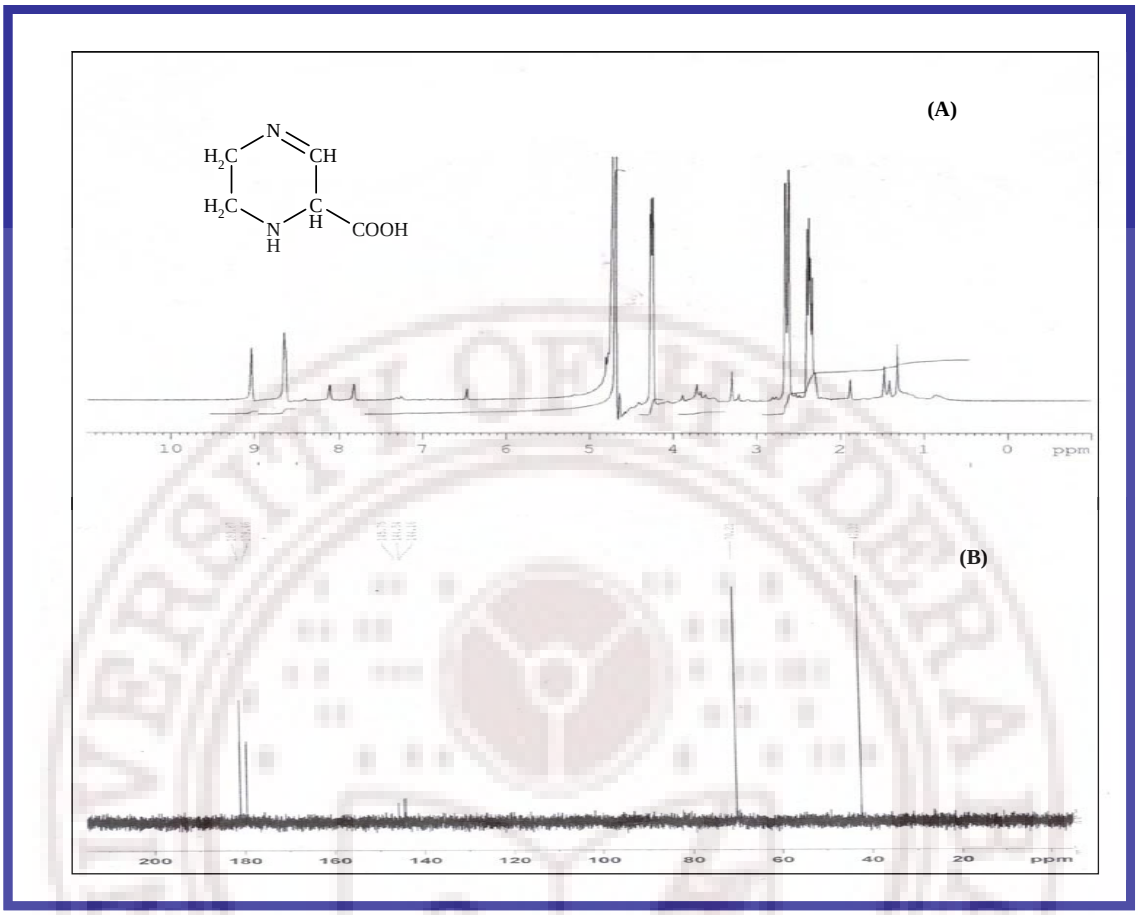


Fig 21: Structural elucidation of the metabolite A1 based on NMR analyses

(A)- ^{1}H NMR (D_2O, 400 MHz) : δ 2.6 (q, 2H), 3.5 (t, 2H) , 9.03 (s, 1H) , 1.6 (q, 1H) 4.8(t, 1H), 4.3(d)

(B)- ^{13}C NMR (D_2O, 400 MHz): δ 180.87 (-COOH), 179.48 (-CH_2-NH-COOH), 144.54 (-C=N), 70.22 (-CH_2-NH), 42.32 (CH_2-CH_2)

The metabolite is a white amorphous solid with absorbance in the UV region.

The IUPAC name of the compound is 1, 2, 5, 6-tetrahydropyrazine-2-carboxylic acid.

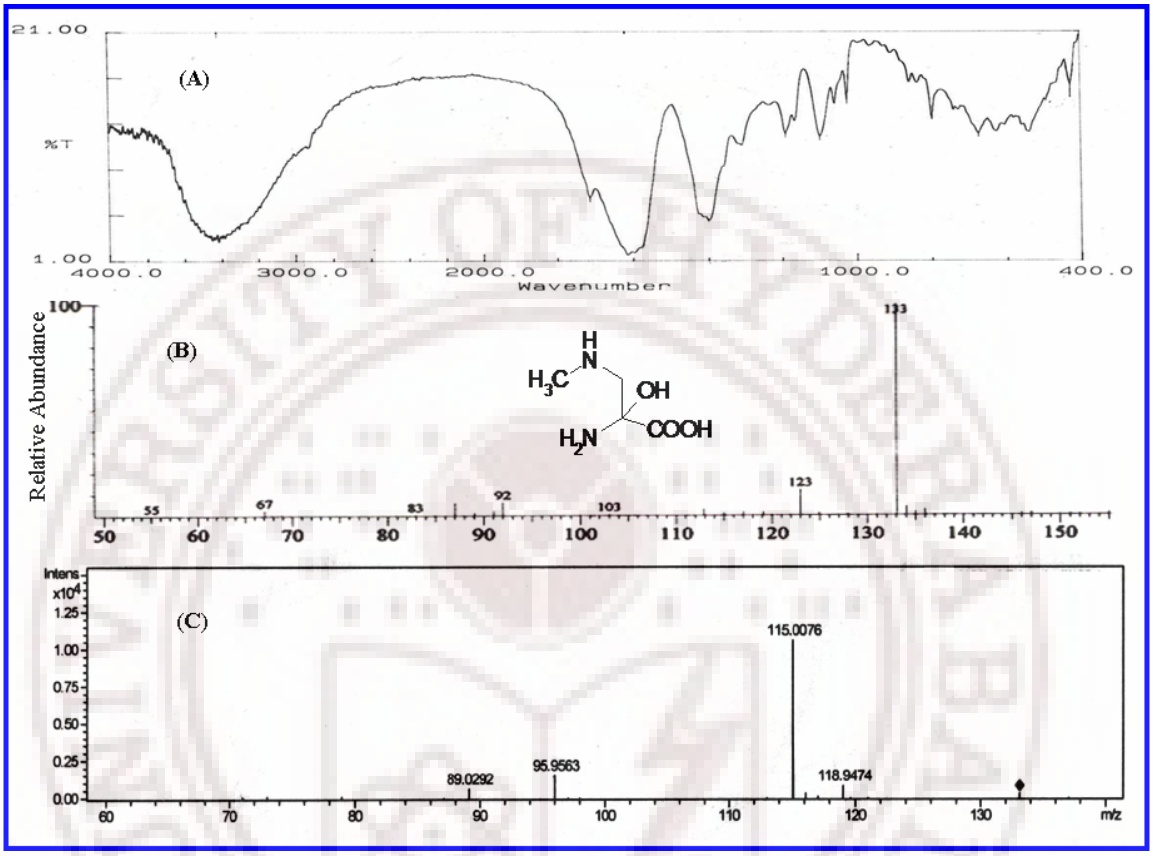


Fig 22: IR and mass spectroscopic analyses and structure of the metabolite B1

Analysis was done as described in materials and methods

(A) Infra red spectral analysis (KBr pellet; cm^{-1}): 3416, 1711 and 1095 correspond to -OH and -COOH group stretching, 3416 –N-H stretching, 1614.56-N-H bending, 1398.52-CH_3 bending, 1022-C-N group.

(B) LC-MS (C) MS-MS spectrum of the compound in acetonitrile, water (1:1) gradient.

The structure of the metabolite was predicted based on NMR, IR and LC-MS analyses.

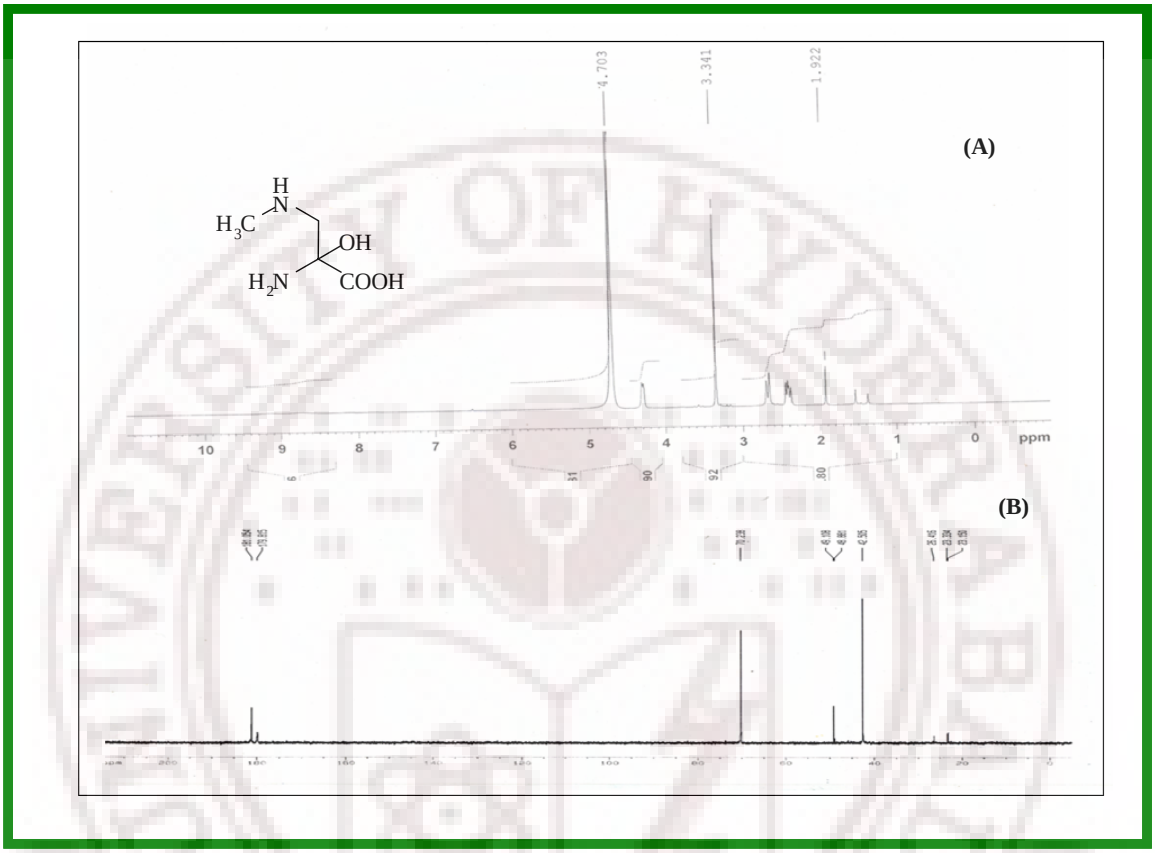


Fig 23: NMR analyses of the metabolite B1

(A)-^{1}H NMR (D_2O, 400MHz): δ 8.7 (s, 1H) , 9.2 (s, 1H), 4.7 (s, 1H), 4.3 (d, 2H), 2.4 (q, 1H), 2.7 (d).

(B)- ^{13}C NMR (D_2O, 400MHz) : δ 181.05 (-COOH), 42.50 (-CH_3-NH-CH_2), 49.1 (CH_3-NH-CH_2-C-COOH), 70.23 (NH_2-C(OH)-COOH).

The IUPAC name of the compound is 2-amino-2-hydroxy-3-(methylamino) propanoic acid.

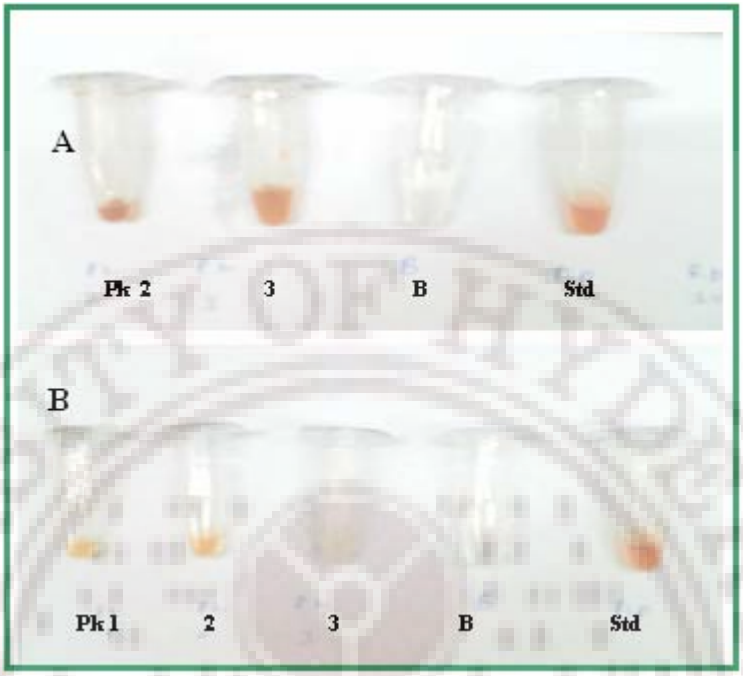


Plate 1: HPLC purified metabolite fractions showing positive reaction to cyclic imino acids (Piez *et al.*, 1956)

Log phase culture of strain HCU1 was inoculated on to Biebl and Pfennig's broth supplemented with 1.5 mM pyrazine-2-carboxylate and incubated at 30 ± 2°C under agitation at 150 rpm. Culture was harvested at regular intervals of time and metabolites were isolated from the culture supernatant.

(A) Corresponds to the metabolites isolated from the culture supernatant at 8 h using semi preparative HPLC showing positive reaction to cyclic imino acids. Peak 2 and 3 are the metabolite fractions showing positive reaction, B = blank (without the compound) and Std = cyclic imino acid (piperazine-2-carboxylate).

(B) Metabolites isolated at 16 h from the culture supernatant of strain HCU1. Peak fractions 1 and 2 showed positive and 3 showed negative for cyclic imino acids.

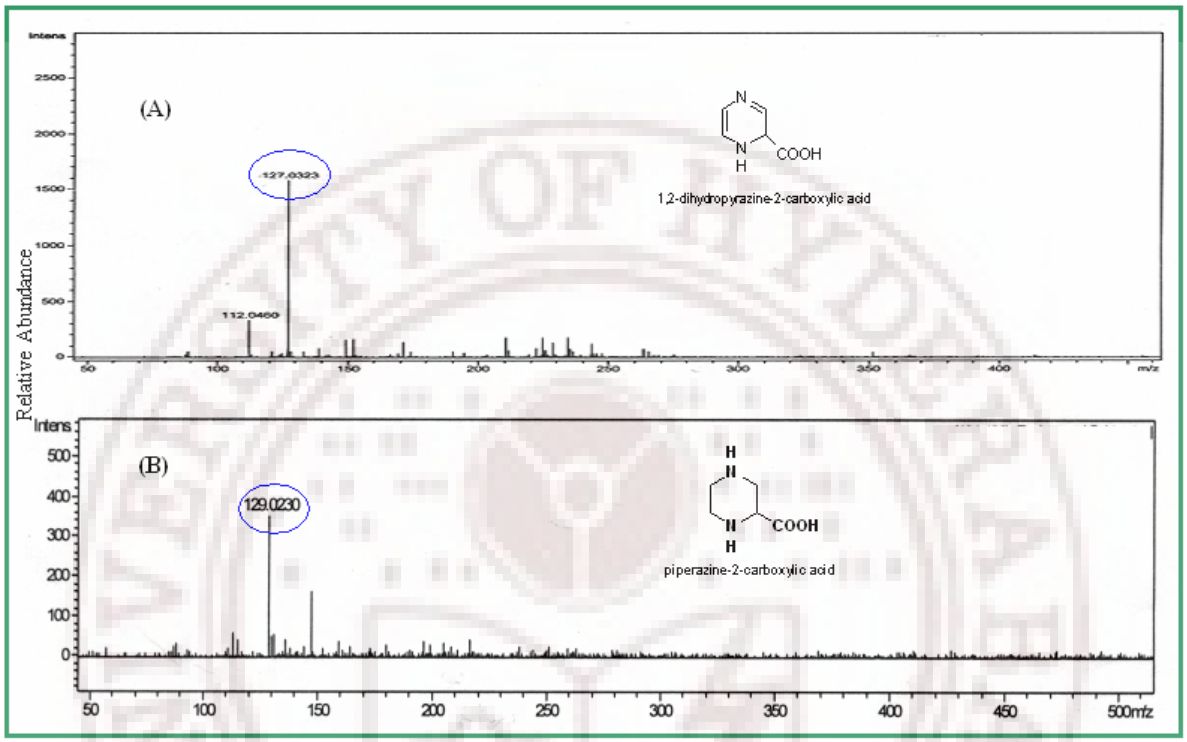


Fig 24: Mass spectroscopic analyses and structure of the metabolites isolated from fraction B

(A) Mass spectrum of the metabolite B2 isolated from fraction B with a t_R of 2.4 minutes using acetonitrile, water as the solvent in gradient mode. The metabolite has m/z of 127 (mH^{-1}) and the compound predicted is 1, 2-dihydropyrazine-2-carboxylate.

(B) Mass spectrum of the metabolite B3 with a t_R of 3.2 minutes with the above said solvent system in gradient mode.

3.5 **Mining for the enzyme involved in pyrazine-2-carboxylate reduction**

3.5.1 **Work with cell free extracts**

3.5.1.1 **Reduction of pyrazine-2-carboxylate with crude cell free extracts:** Ring reduction of pyrazine-2-carboxylate was observed with whole cells of *Stenotrophomonas* sp. HCU1. Hence, assay for pyrazine-2-carboxylate reduction was studied with cell free extracts of pyrazine-2-carboxylate induced and uninduced cultures of *Stenotrophomonas* sp. HCU1. Pyrazine-2-carboxylate ring reduction activity was observed with both induced and uninduced cell free extracts.

3.5.1.2 **Influence of pyrazine-2-carboxylate concentration on the ring reduction activity by cell free extracts:** Pyrazine-2-carboxylate ring reduction activity of the cell free extracts was studied with different concentrations of pyrazine-2-carboxylate ranging from 0-500 µmoles (data not shown). Reduction of pyrazine-2-carboxylate increased with increasing concentration and optimum pyrazine-2-carboxylate concentration for maximum reduction was 200 µmoles.

3.5.1.3 **Coenzyme requirement for pyrazine-2-carboxylate reduction:** Ring reduction activity of the cell free extracts was studied in the presence of different coenzymes like NAD, NADH, NADPH and $FADH_2$. Among them, maximum reduction of pyrazine-2-carboxylate was observed with NADPH followed by NADH (Fig 25). However, reduction was inhibited in the presence of ATP with NADH and NADPH. Optimum concentration of NADPH (Fig 26) for the reduction of pyrazine-2-carboxylate was 200 µmoles.

3.5.1.4 **Kinetics of pyrazine-2-carboxylate reduction:** Time course of reduction of pyrazine-2-carboxylate was studied with pyrazine-2-carboxylate induced cell free

extracts of *Stenotrophomonas* sp. HCU1. Reduction of pyrazine-2-carboxylate (Fig 27) increased between 10-20 minutes of assay and then decreased after 30 minutes. At 60 minutes of assay, there is an appearance of peak (at t_R =1.79 min) corresponding to the transformed product (Fig 28). Based on the GC-MS analysis (Fig 29) of the assayed sample, ring reduced and ring cleavage products of pyrazine-2-carboxylate corresponding to 128 and 134 masses were predicted.

3.6 **Isolation and purification of the enzyme involved in pyrazine-2-carboxylate reduction**

3.6.1 **Isolation of enzyme** Cell free extracts of pyrazine-2-carboxylate induced culture was subjected to 0-90 % ammonium sulphate saturation. 0-30 % ammonium saturated protein fraction showed maximum reduction of pyrazine-2-carboxylate. Hence 30 % ammonium sulphate saturated fraction was used for isolation of protein using ion exchange chromatography with a step gradient of 0-1 M sodium chloride (Flow chart 3) (Fig 30B). The active fractions 64-69 were pooled and further reloaded onto DEAE-cellulose column and eluted with 0.3-0.5 M NaCl. The active fractions 9-11 (Fig 30A) eluted with 0.4 M NaCl showing highest absorbance of protein were analyzed on Native PAGE. Purity of the protein was checked on 10 and 6 % Native PAGE (Fig 31A) and detected using silver staining. Two subunits of masses ~43 and 23 kDa were observed on SDS-PAGE analysis (Fig 31B).

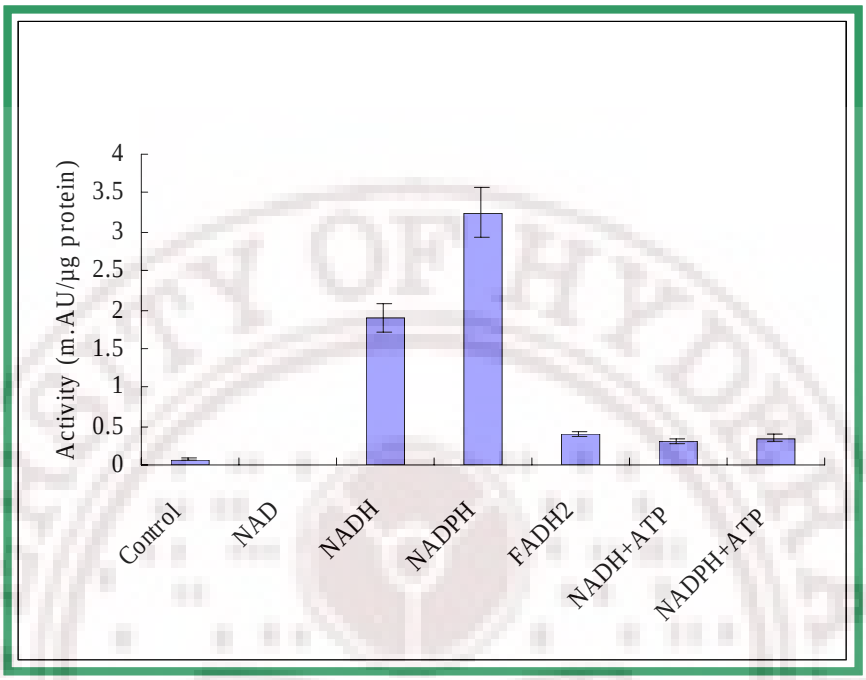


Fig 25: Ring reduction of pyrazine-2-carboxylate by the cell free extracts of strain HCU1 in the presence of different coenzymes

(Control = without coenzyme addition; NAD = Nicotinamide adenine dinucleotide; NADH = Reduced nicotinamide adenine dinucleotide; NADPH = Reduced nicotinamide adenine dinucleotide phosphate; $FADH_2$ = Reduced flavin adenine dinucleotide; ATP = Adenosine triphosphate).

Assay mixture (1 ml) contained 200 µmoles of pyrazine-2-carboxylate; 100 µ moles of coenzyme, 5 mM $MgCl_2$ and Tris buffer pH-7.4. Assay was started by adding 40 µl (500 $\mu g.ml^{-1)}$ of the cell free extract and was stopped by adding 5 µl of 1N HCl and pyrazine-2-carboxylate levels of the assay mixture were analyzed in HPLC.

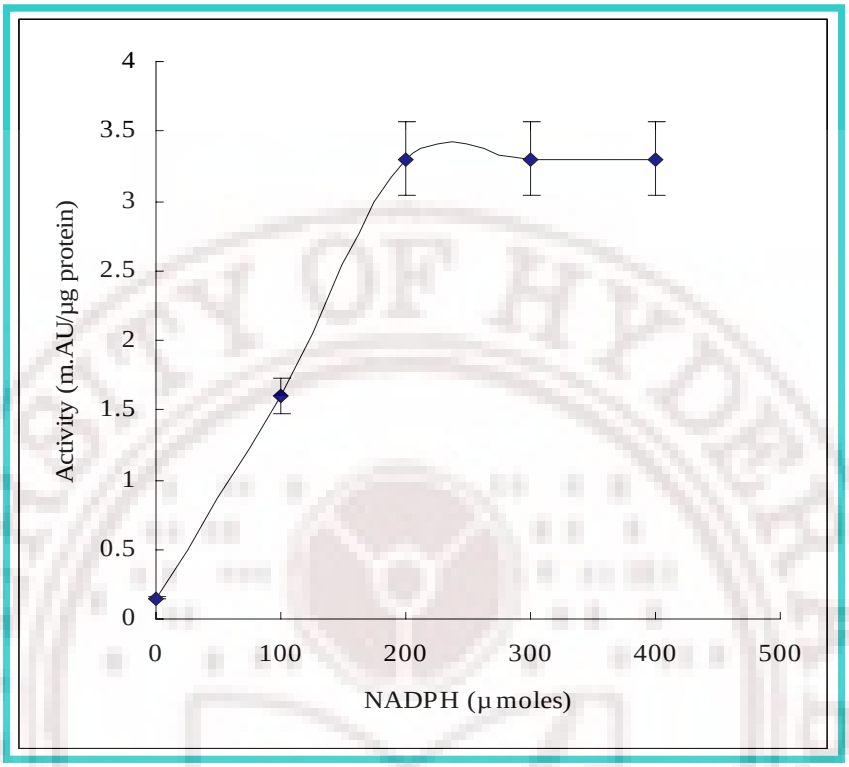


Fig 26: Influence of NADPH concentration on the reduction of pyrazine-2-carboxylate by the cell free extracts of *Stenotrophomonas* sp. HCU1

Experimental conditions are same as in fig 25 except for the different concentrations of NADPH used**.** The assay was stopped after 20-25 minutes by adding 5 µl of 1N HCl and pyrazine-2-carboxylate levels of the supernatant were analyzed using HPLC.

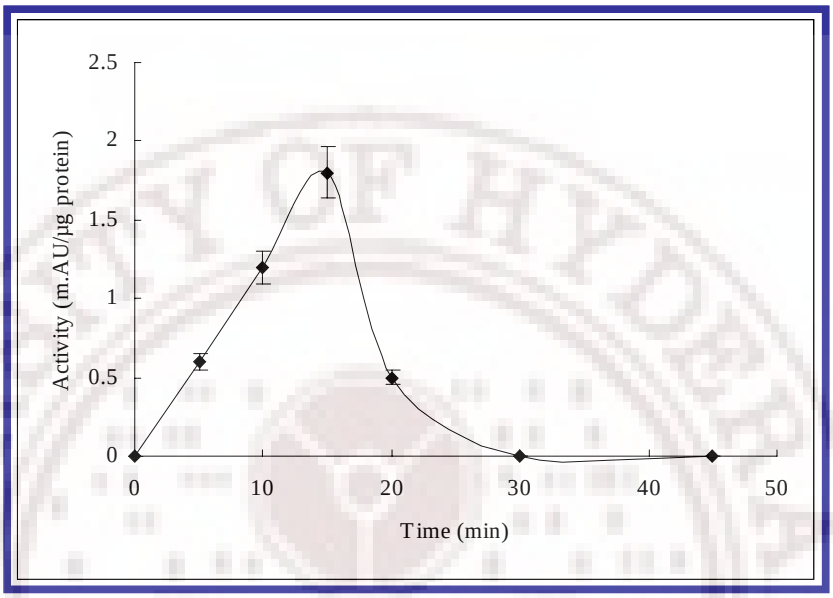


Fig 27: Kinetics of pyrazine-2-carboxylate reduction by the cell free extracts of *Stenotrophomonas* sp. HCU1

Experimental conditions are same as in fig 25. Aliquots were drawn from the assay mixture at different time intervals and assay was stopped by adding 5 µl of 1N HCl and incubated at 0 °C. Pyrazine-2-carboxylate levels of the sample were analyzed using HPLC.

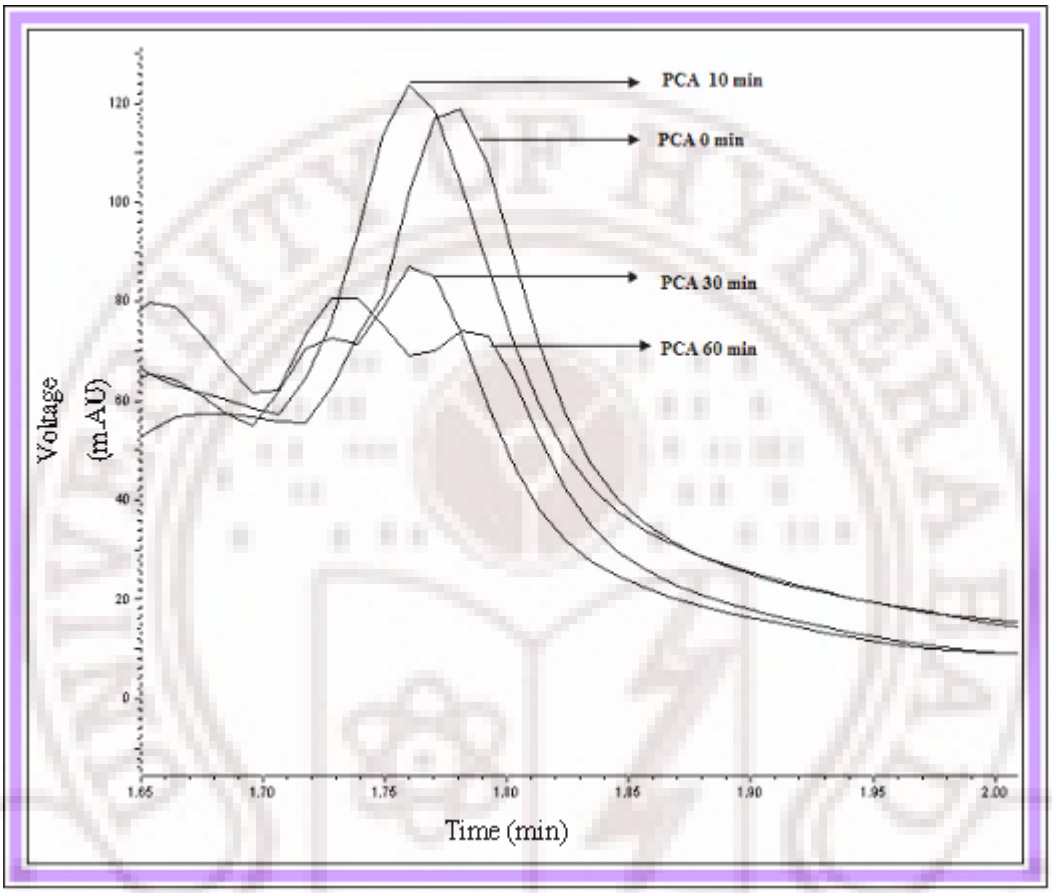


Fig 28: HPLC chromatogram showing time course of reduction of pyrazine-2-carboxylate by the cell free extract of strain HCU1

Data pertains to the analysis of the assay sample at different time intervals in HPLC.

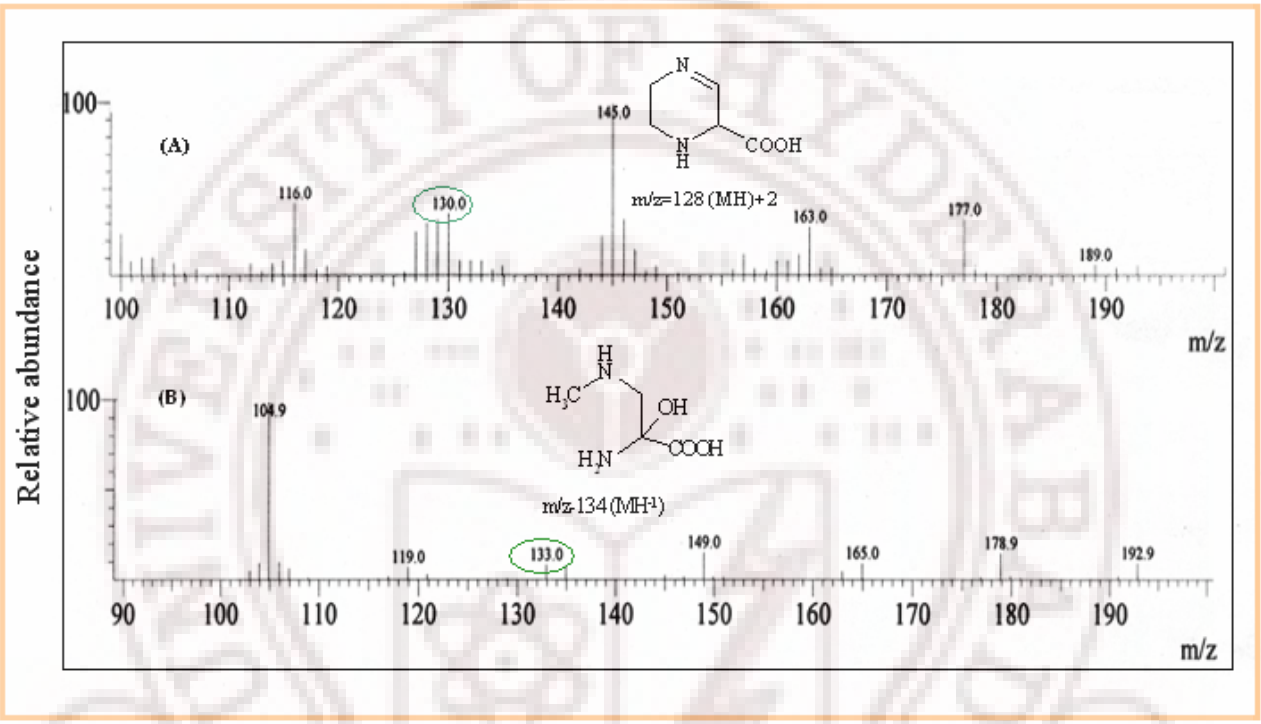
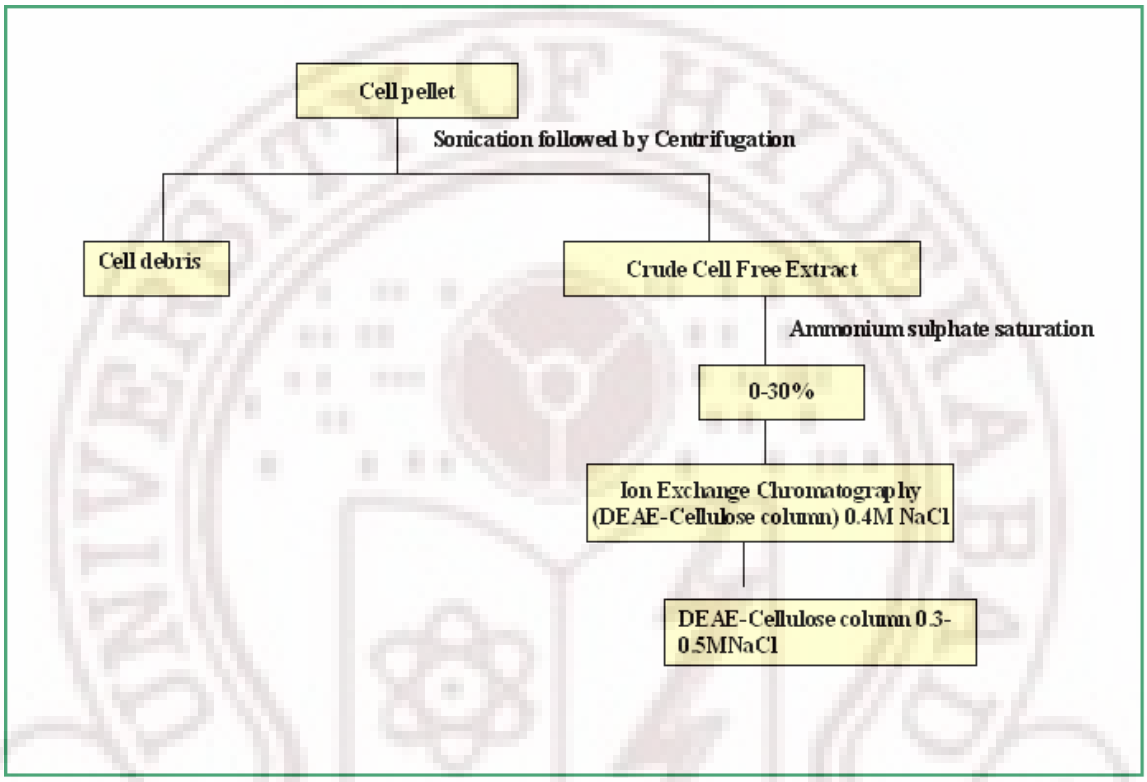
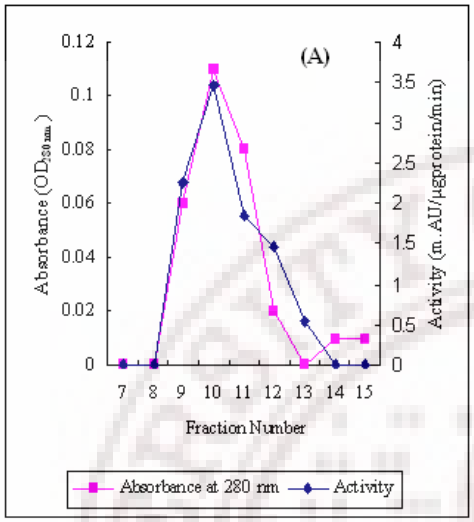


Fig 29: GC-MS metabolite profiling of the crude cell free extracts of strain HCU1 showing reductase activity

Assay sample was extracted into methanol and methanol extract was analyzed on GC-MS Shimadzu 2010.



Flow chart 3: Steps involved in the isolation and purification of the protein catalyzing pyrazine-2-carboxylate ring reduction



Purification steps (B)	Total Protein (volume)	Concentration(mg/µg)	Specific activity (m.AU/µg/min)
Crude cell free extract	30	20	0.156
30% Ammonium sulphate saturation	10	7	0.5
Ion exchange chromatography(0.4M NaCl) in DEAE-Cellulose)	10	600	4.46
DEAE column purification	2	200	14.3

Fig 30: Elution profile of the reductase protein from DEAE-Cellulose column and steps involved in purification and recovery

(A) Pyrazine-2-carboxylate ring reduction activity was measured for all fractions eluted with a linear gradient of 0.3-0.5 M NaCl. The protein fraction eluted from 0.4 M NaCl was analyzed for pyrazine-2-carboxylate ring reduction activity and the same was plotted on X-axis. Protein absorption and activity (in terms of ring reduction) were plotted on primary and secondary Y-axis respectively.

(B)Summary of the steps involved in protein purification.

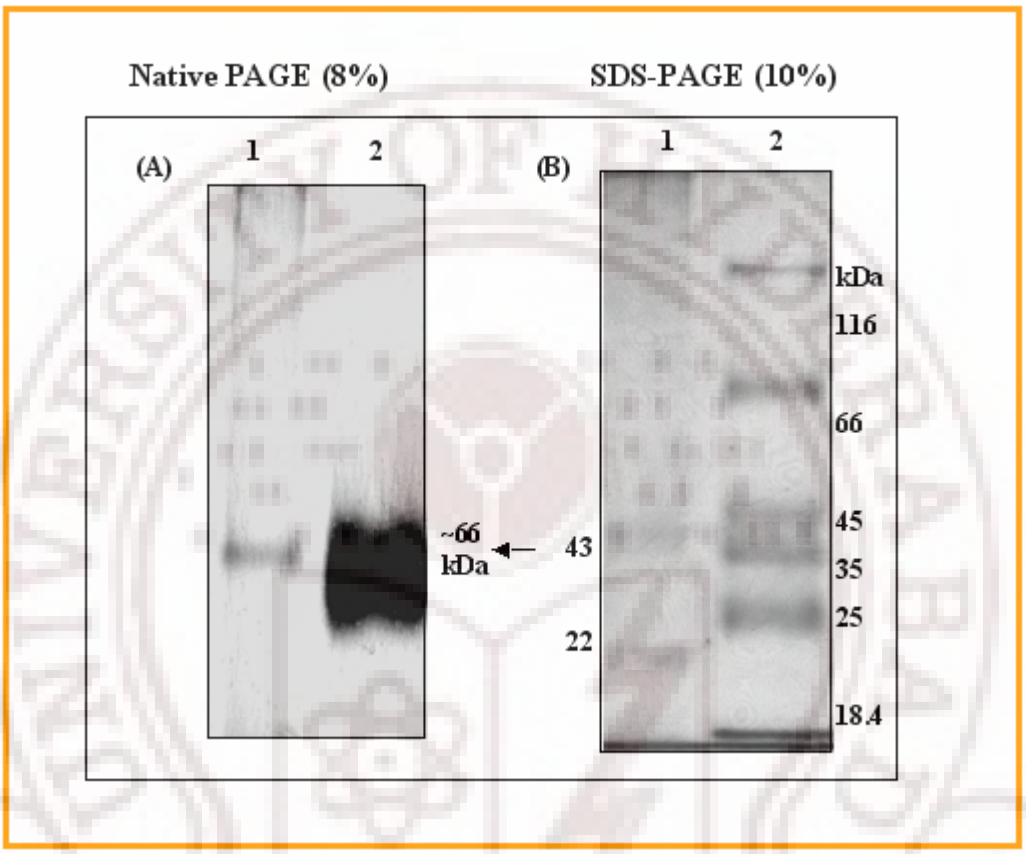


Fig 31: Native and SDS-PAGE analysis of the purified pyrazine-2-carboxylate reductase

A) Lane1 = Purified pyrazine-2-carboxylate reductase

Lane 2 = Bovine serum albumin

B) Lane 1 (SDSPAGE) = Subunits of pyrazine-2-carboxylate reductase

Lane 2 = Protein markers

3.6.2 **Molecular weight determination using Fast Protein Liquid Chromatography (FPLC):** Native molecular weight of purified protein fraction was determined using gel exclusion chromatography (Sephacryl, G-100). Void volume of the column was determined using Blue Dextran and calibrated using standard protein markers; catalase (240 kDa), glucose oxidase (90 kDa), bovine serum albumin (67 kDa) and peroxidase (40 kDa) in FPLC chromatography (Fig 32). Based on the elution volume of protein with standard protein markers, molecular weight of the protein was found to be ~65 kDa.

3.6.3 **Pyrazine-2-carboxylate ring reduction activity by purified protein**

The purified protein fraction catalyzed the ring reduction of pyrazine-2-carboxylate and the same was detected in HPLC (Fig 33A). The assay mixture was extracted into methanol and analyzed on LC-MS. A metabolite of molecular mass (m/z) 129 was detected in LC-MS (Fig 33B). The compound was identified as 1, 2, 5, 6-tetrahydropyrazine-2-carboxylate. Since the protein catalyzed the ring reduction of pyrazine-2-carboxylate, it was named as pyrazine-2-carboxylate reductase. Characters of pyrazine-2-carboxylate reductase are shown in Table 15.

3.7 **Characterization of protein**

3.7.1 **Enzyme kinetics** Pyrazine-2-carboxylate reductase activity was studied with time. Increase in reduction of pyrazine-2-carboxylate was observed with time and the activity was optimum at fifteen minutes and then remained constant beyond fifteen minutes (Fig 34A).

3.7.2 **Kinetic constant (K_m and V_{max})** Pyrazine-2-carboxylate reductase activity of the enzyme was checked with varying concentrations (Fig 34B) of pyrazine-2-carboxylate. The enzyme showed maximum activity at 1 mmole of pyrazine-2-carboxylate. The K_m of

the enzyme fraction is 0.47 mmoles and V_{max} is 18.2 (m. AU/µg.min^{-1}) for pyrazine-2-carboxylate reduction activity (Fig 34C).

3.7.3 **Effect of pH** Pyrazine-2-carboxylate reductase activity was studied at different pH ranging from 5-9. The activity was optimum at pH 7.4 (Fig 34E).

3.7.4 **Effect of temperature** The pyrazine-2-carboxylate reductase activity at different temperatures was assayed from 10-50 ºC. The enzyme has an optimum activity at a temperature 20-30 ºC (Fig 34D).

3.7.5 **Substrate specificity of pyrazine-2-carboxylate reductase** Various analogues of pyrazine-2-carboxylate like nicotinic acid, pyrazine, pyrazinamide, imidazole, uracil, guanine, picolinic acid, dimethylaminopyridine and piperazine were used for studying the substrate specificity of reductase enzyme. The enzyme was specific towards pyrazine-2-carboxylate, pyrazine, nicotinic acid, guanine, uracil and imidazole (Table 16). However, the activity was not observed with dimethylaminopyridine, dipicolinic acid and piperazine. Pyrazine-2-carboxylate reductase activity was inhibited with nicotinic acid and uracil.

3.8 **MALDI-TOF (Peptide Mass Finger printing) analyses**

The ~65 kDa pyrazine-2-carboxylate reductase was subjected to MALDI-TOF analysis as described in materials and methods. The protein was identified using public domain MASCOT search by incorporating the standard parameters with three different data bases (Swissport, NCBI and MSDB). Total protein finger printing pattern of the reductase showed fragments of 0.8, 1.042, 1.363, 1.564 and 2.053 kDa (Fig 35A). Mascot search (http://www.matrixscience.com) of the peptide finger printing analysis indicated 59.80 and 43.80 score with the 4-hydroxyphenylpyruvate dioxygenase of *Bacillus* sp. sg-1 and

aspartate semialdehyde dehydrogenase of *Thermoanaerobacter tencongensis* MB-4, respectively. MS-MS fragmentation (Table 17) 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 transferase of *Xylella fastidiosa* Temicula 1 with 6.79 score and 1.042 kDa peptide mass fragmentation of 0.882 kDa (Fig 35B) matched with soluble pyridine nucleotide transhydrogenase of *Mycobacterium abscessus* with a score of 9.80.

From the experimental evidences it is inferred that the peptide finger printing analysis of pyrazine-2-carboxylate reductase did not show a significant score with any of the known proteins.

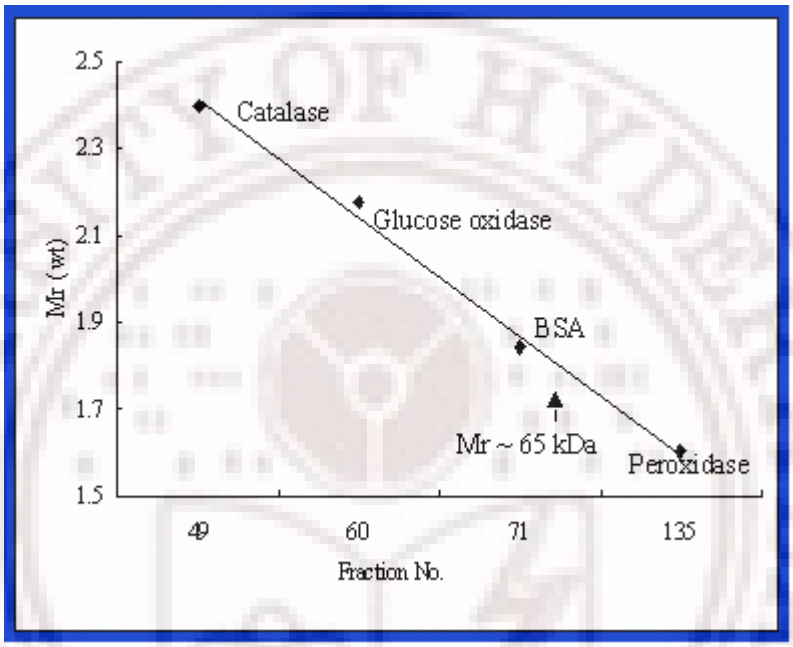


Fig 32: Elution profile of the standard proteins and pyrazine-2-carboxylate reductase using FPLC chromatography

Native molecular mass of the reductase enzyme was calculated based on the relative elution volume with standard protein markers.

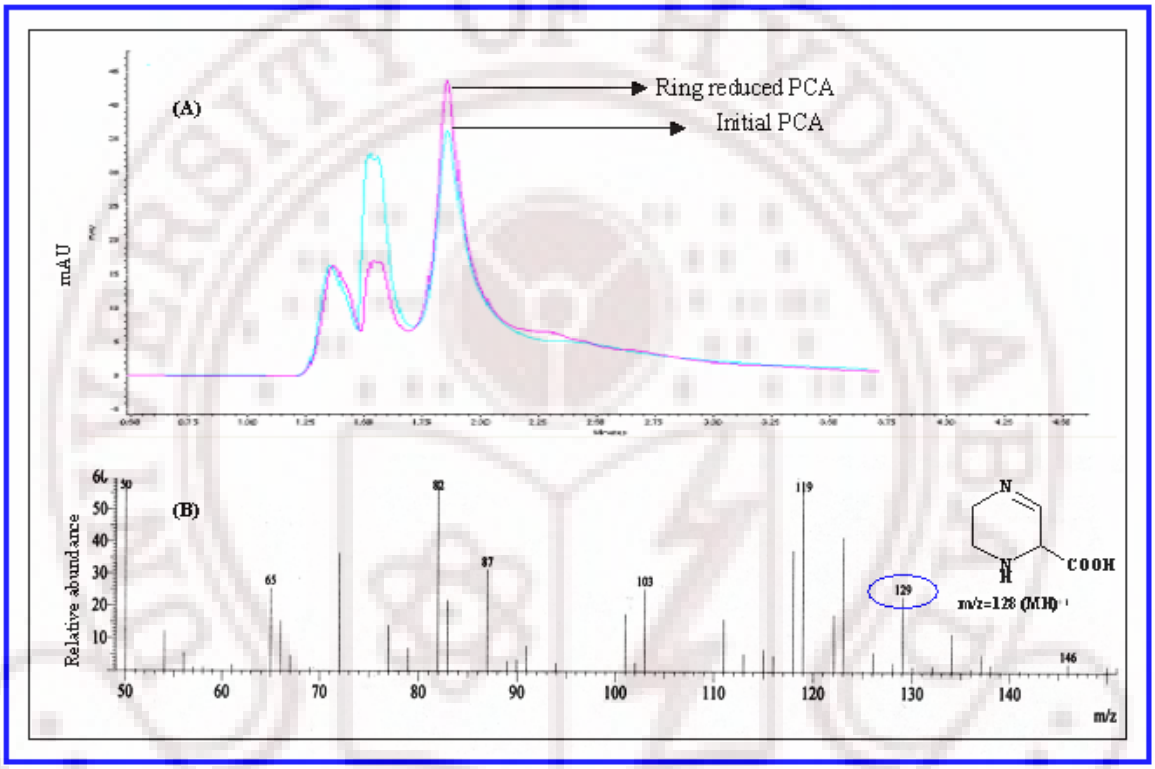


Fig 33: HPLC chromatogram showing the reduction of pyrazine-2-carboxylate by purified protein and LC-MS analysis showing the ring saturated product

(A)Enzyme was assayed for pyrazine-2-carboxylate ring reduction and pyrazine-2-carboxylate levels of the assay mixture were analyzed in HPLC.

(B)The assay mixture was extracted in methanol and product of the enzyme activity was detected using LC-MS.

Properties	Character
K_m	0.475 mmoles
Vmax	18.2 (m. AU/µg.min^{-1})
pH	7.4
Temperature	30 °C
Cofactors and coenzymes required Metal cofactor	NADPH Mg^{+2}
Molecular weight	~ 65kDa

Table 15: Characters of pyrazine-2-carboxylate reductase

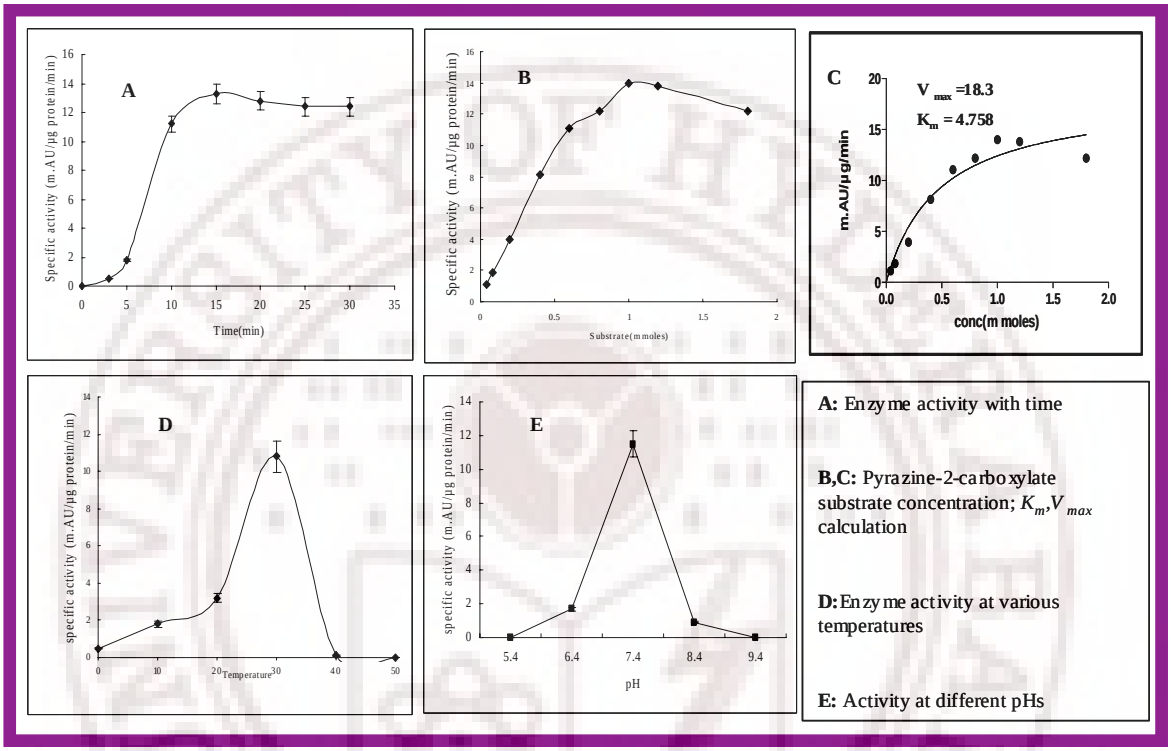


Fig 34: Characterization of pyrazine-2-carboxylate reductase

Substrate	Activity (%)
Pyrazine-2-carboxylate	100
Pyrazine	25
Pyrazinamide	0
Piperazine	0
Dimethylaminopyridine	0
Nicotinic Acid	20
Dipicolinic Acid	0
Uracil	15
Guanine	12
Imidazole	10
Nicotinic Acid + PCA	-
Uracil + PCA	-

Table 16: Substrate specificity of pyrazine-2-carboxylate reductase

(PCA= Pyrazine-2-carboxylate; - = Inhibition of activity)

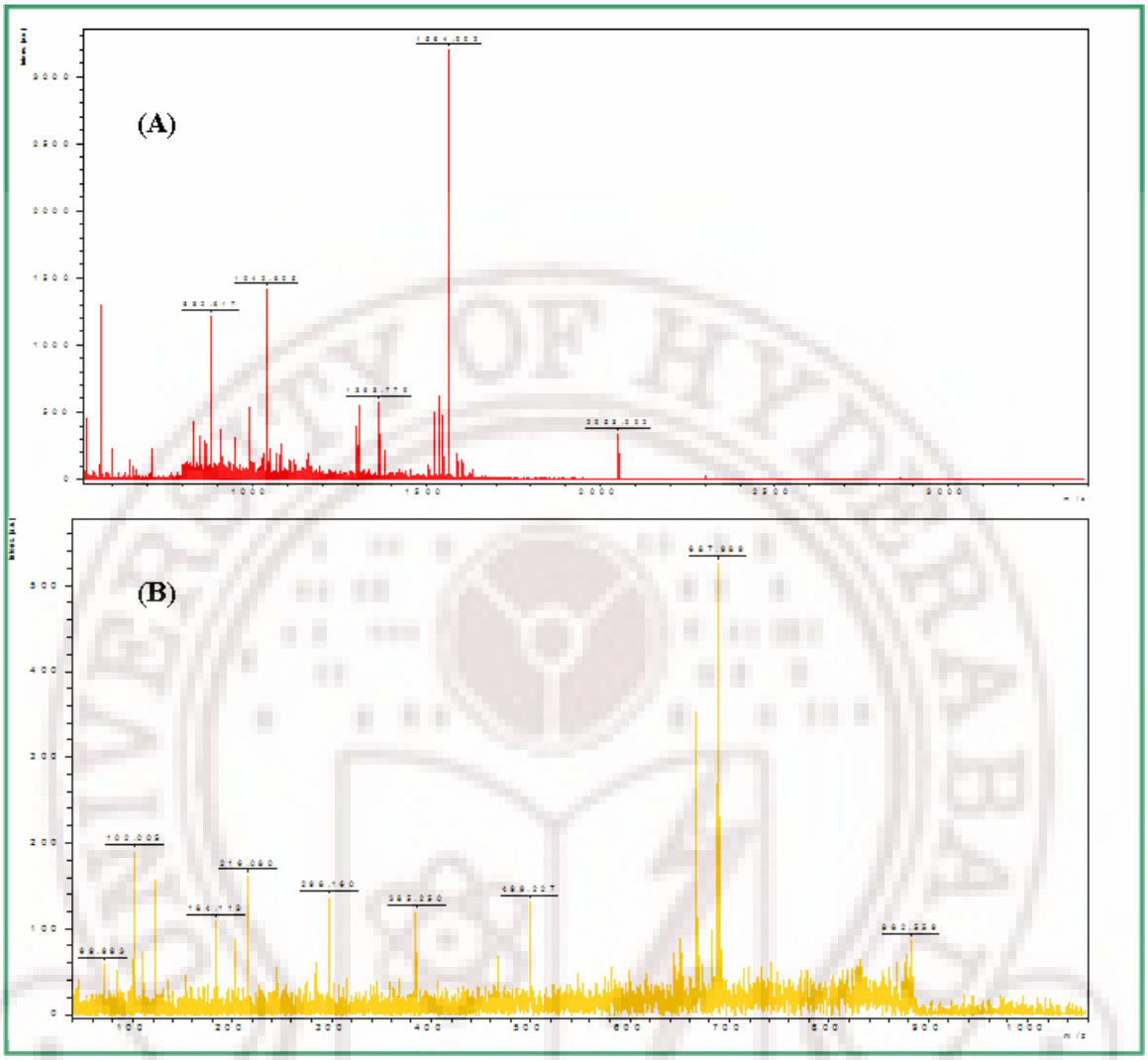


Fig 35: (A) MALDI-TOF finger printing analysis of pyrazine-2-carboxylate reductase

(B) MALDI- MS/MS peptide analysis of the 0.8 kDa peptide

Peptide mass MS/MS	Probable protein	Sequence coverage (%)	Database Accession No.	Amino acid sequences	Organism
2053	-	-	-	-	-
1564	2-methylthioadenylate synthetase	16.68	gi27365365	QSRLAYDLIEEVK	*Vibrio vulnificus*CMCP6
1363.7	Dehydrogenase(putative), aromatic amino acid amino transferase	6.79 6.79	gi149915814 gi28197970	GAAPRRPILAEGR ALGAPEFDFIQR	*Roseobacter* sp. AZWK-3B *Xylella fastidiosa* temicula1
1042.6	Oxidoreductase,2OG-Fe(II)oxygenase family protein	24.54	gi167838986	KTALDELPR	*Burkholderia thailandensis* MSMB43
882.6	Soluble pyridine nucleotide trans hydrogenase	9.80	gi169630115	TEHVIRK	*Mycobacterium abscessus*

Table 17: Amino acid sequences found during MALDI-TOF MS/MS analyses of peptides of mass 2.052, 1.564, 1.363, 1.042 and 0.8 kDa along with the probable protein and sequence coverage

Discussion

4. Discussion

Proteinaceous materials and N-heterocyclics appear to be the major nitrogen containing compounds in humic substances, of which pyrazines are the components of the heterocyclic organic matter (Schulten and Schnitzner, 1998). Pyrazine family among the N-heterocyclic molecules can be considered as new generation lead molecules owing to their usage as antibiotics (Moser *et al.,* 2008), herbicides (Stefan *et al.,* 1999), fungicides (Temple and Caroll, 2000) and pharma drugs (Lee *et al.,* 2001; Prochaska *et al.,* 1991; Noel *et al.,* 2006). Although there are reports on the bacterial catabolism of N-heterocyclic compounds (Vogels and van'der Drift, 1976; Berry *et al.,* 1987 Kaiser *et al.,* 1996; Fetzner, 1999), including pyrazines (Ref pages 23-30), biochemical pathways of pyrazine degradation were not studied in detail. Antituberculotic drug, pyrazinamide was transformed by bacterial encoded pyrazinamidase which was not metabolized by *Mycobacterium* (Zhang and Mitchison, 2003). Thus it was of interest to isolate a bacterium and to study the metabolism of pyrazine-2-carboxylate.

Isolation of bacterial strains capable of metabolizing pyrazine-2-carboxylate from soil samples was carried out using enrichment culture technique. The rationale behind isolating bacteria from different soil samples was because of the presumed presence of pyrazines in organic rich garden soil (Schulten and Schnitzner, 1998) and oil contaminated soil rich in polycyclic aromatic hydrocarbons (Henner *et al.,* 1997). Fifty bacterial strains and two enrichment cultures were studied in the screening process. Highest utilization of pyrazine-2-carboxylate was demonstrated with isolate C6 (37 %) among the seven pyrazine-2-carboxylate utilizing bacterial isolates (Table 8). Such a similar screening was done earlier for bacterial strains capable of utilizing alkylated

pyrazines (Rappert *et al.,* 2007) and 6-methylnicotinate (Tinschert *et al.,* 2000) from the waste gas treatment plant of fish meal processing company and sewage sludge, respectively.

Repeated utilization of pyrazine-2-carboxylate by bacterial isolate C6 on batch transfers could not be demonstrated (Fig 8). Such a decreased utilization and decrease in biomass was observed in 2,4,6-trintrotoluene utilizing strains TNT8, TNT32, *Pseudomonas* sp. clone (Vorbeck *et al.,* 1998), *Nocardiodes simplex* CB 22-2 (Beherend and Wagner, 1999) and pyridine degrading *Azoarcus evansii* (Rhee *et al.,* 1997). This could be plausibly explained by formation of dead end metabolites (Vorbeck *et al.,* 1998), toxicity of the metabolites (Beherend and Wagner, 1999; Rhee *et al.,* 1997) and decreased biomass could be due to carry over of nitrogen during first transfer resulting in the higher growth yield and subsequent decrease in transfers (Vorbeck *et al.,* 1998). The possible explanation for decreased utilization of pyrazine-2-carboxylate by bacterial isolates on transfers may be due to the loss of the genetic potential or curing of plasmids. Attempts were made to revive and enhance the pyrazine-2-carboxylate utilization potential of the isolate C6. These include, culturing in nutrient broth and pyrazine-2-carboxylate supplemented Biebl and Pfennig's broth by varying the carbon sources like fumarate, glucose, pyruvate, succinate, oxoglutarate and oxaloacetate (Data not shown). Despite the attempts made, pyrazine-2-carboxylate utilization potential could not be enhanced and revived either with isolate C6 or with other bacterial isolates (in this study).

Protonation of pyrazines resulted in the increased absorbance at the absorbance maxima (Chia and Trimble, 1971). The absorbance of the diprotonated pyrazine was almost doubled compared to the non-protonated pyrazine. During the process of

screening, increased absorbance at 268 nm corresponding to pyrazine-2-carboxylate was observed in the culture supernatants of seven bacterial isolates and two enrichment cultures (Table 9). Initially it was presumed that increase in absorbance could be due to the synthesis of pyrazines in the presence of supplemented pyrazine-2-carboxylate. However, when the supernatant samples tested with 1 % ferrous sulphate reagent showed orange red colour, characteristic of pyrazines (Oliveto, 1962) and when quantitatively analyzed, did not show an increase in the pyrazine content. This new property by the bacterial cultures could be due to the ring saturation or ring reduction of pyrazines as reported by Chia and Trimble (1971). Ring reduction was observed during the pyridine degradation by *Bacillus* strain 4, *Nocardia* sp. Z1 (Watson and Cain, 1975), degradation of ethyl and methyl pyridine by *Gordonia nitida* (Lee *et al.,* 2001) and nicotinic acid by *Clostridium* sp. (Holcenberg and Tsai, 1969) involving the formation of 1,4-dihydropyridine (Watson and Cain, 1975), 1,4,5,6-tetrahydronicotinic acid (Holcenberg and Tsai, 1969) and 1,4-dihydro-3-ethylpyridine (Lee *et al.,* 2001), respectively. Ring saturated products of picric acid were reported in *Rhodococcus erythropolis* (Reiger *et al.,* 1999) and *Nocardiodes* sp. CB22-2 (Beherend and Wagner, 1999). Such ring saturated products were observed during 2,4,6-trinitrotoluene degradation by *Mycobacterium* strain (Vorbeck *et al.,* 1994), strains TNT8, TNT32 (Vorbeck *et al.,* 1998), *Enterobacter cloaceae* (French *et al.,* 1998) and *Pseudomonas fluorescens* I-C (Pak *et al.,* 2000). Aromatic ring hydrogenated products of 2,4,6-trinitrophenol and 2,4-dinitrophenol by *Nocardiodes simplex* were also reported (Ebert *et al.,* 1999). Addition of hydride ion to the aromatic compounds resulted in the formation of hydride and dihydride

meisenheimer complexes which were actively metabolized (French *et al.*, 1998) and in some cases were dead end metabolites (Vorbeck *et al.,* 1998).

From the experimental results on the screening for pyrazine-2-carboxylate utilizing bacteria, it was inferred that utilization of pyrazine-2-carboxylate was of restricted metabolic potential and further among the bacterial isolates utilizing pyrazine-2-carboxylate, the utilization capability could not be demonstrated on batch transfers. Ring reduction of pyrazine-2-carboxylate by bacterial isolates was a significant observation. Highest ring reduction (40 %) of pyrazine-2-carboxylate observed with bacterial isolate C45 was the strain of choice for studying the catabolism of pyrazine-2-carboxylate.

As the taxonomic identity of a pyrazine-2-carboxylate metabolizing isolate C45 was not studied, it was characterized based on polyphasic taxonomic approach. Isolate C45 named as strain HCU1. 16S rRNA gene sequencing, BLAST search and 16S rRNA dendrogram analyses indicated 98.7 and 99.2 % sequence similarities with the 16S rRNA gene sequences of type strains, *Stenotrophomonas maltophilia* (ATCC 13637) and *Pseudomonas geniculata*, respectively (ATCC 19374) (Fig 10). The genus *Pseudomonas* was subdivided into five groups based on rRNA-DNA hybridization studies by Palleroni (Anzai *et al.,* 2000) into rRNA group's I-V. *Stenotrophomonas maltophilia* earlier called as *Pseudomonas maltophilia* belonged to the RNA group V and was defined as the type species of *Stenotrophomonas palleroni*-belonging to the gamma beta subclass of proteobacteria. Based on 16S rRNA gene sequence analyses members of *Pseudomonas beteli*, *Pseudomonas geniculata* and *Pseudomonas hibiscicola* are more closely clustered to the genus *Stenotrophomonas* with 99.2-99.5 % homology rather than to genus

Stenotrophomonas (Anzai *et al.,* 2000). Presently 8 species are included in the genus *Stenotrophomonas* (http://www.bacterio.cict.fr/). The 16S rRNA gene sequence similarity of 99.2 and 98.7% with *Pseudomonas geniculata* and *Stenotrophomonas maltophilia*, respectively indicate the need for genomic hybridization which helps for proper taxonomic affiliation of strain HCU1. Since there is an ambiguity of affiliation of *Pseudomonas geniculata* (Anzai *et al.,* 2000), the later genera was considered for strain HCU1.

Strain HCU1 is an obligate aerobe with chemoorganoheterotrophic growth mode as reported for *Stenotrophomonas maltophilia* (Table 10) (Palleroni, 2005). Growth was demonstrated on nitrate by strain HCU1 (Table 11B). *Stenotrophomonas maltophilia* was reported to reduce nitrate but could not utilize nitrate as growth substrate (Palleroni, 2005) whereas strain HCU1 could utilize nitrate for growth. Lack of methionine requirement (as a growth factor) and gelatinase activity were the biochemical variations of the strain HCU1 with the type strain *Stenotrophomonas maltophilia* (Table 12) (Palleroni, 2005). Metabolome foot printing analysis of the strain HCU1 was almost similar with the *Stenotrophomonas maltophilia* K279A, except for the amine formation as observed by the peak at wave number 2400 cm^{-1} (Fig 9A,B). *Stenotrophomonas maltophilia* is often a dominant member of rhizosphere microbial community of plants (Berg *et al.,* 1996). The strain has been used as biocontrol agent of fungal plant pathogens in agriculture and in bioremediation (Berg *et al.,* 1996, Elad *et al.,* 1987). *Stenotrophomonas maltophilia* is an important nosocomial pathogen associated with the significant fatality in immunosuppressed patients (Garrison *et al.,* 1996). The name

maltophilia is because of the production of maltophilin, an antifungal compound active against human and plant pathogenic fungi (Palleroni, 2005).

Pyrazine-2-carboxylate utilization by strain HCU1 adds to the list of microbial utilization of different pyrazines (Mattey and Harle, 1976; Rappert *et al.,* 2006; 2007; Nishimura *et al.,* 1991). Microbial utilization of hydroxylated and alkylated pyrazines as sole source of carbon, energy and supplement by growing and resting cells of *Pseudomonas* sp. PZ3 (Mattey and Harle, 1976), *Mycobacterium* sp. DM-11(Rappert *et al.,* 2006), *Rhodococcus erythropolis* DP-45 (Rappert *et al.,* 2007) and *Trichoderma koningii* (Nishimura *et al.,* 1991) were reported. In the present study, 1.5 mM pyrazine-2-carboxylate was utilized by strain HCU1 as sole source of carbon (Fig 11) and nitrogen (Fig 12). However, the utilization of pyrazine-2-carboxylate by strain HCU1 differs from other pyrazine utilizing bacteria with respect to ring reduction/saturation as observed by increase in absorbance at 268 nm in the culture supernatant (Fig 17). In addition to the heterocyclic ring saturation by bacterial species (Holcenberg and Tsai, 1969, Rhee *et al.,* 1997, Lee *et al.,* 2001), complete ring reduction of naphthalene, a PAH to octahydronapthoic acid, by a sulphate reducing enrichment culture as sole carbon and energy (Meckenstock *et al.,* 2000) and ring reduction of benzoate to cyclohexane carboxylic acid by *Syntrophus acidotrophicus* (Mouttaki *et al.,* 2008) were also reported. Increase in the absorbance of pyrazine-2-carboxylate when used as a supplement (Fig 13) was highest, indicating the ring saturation of the metabolite by growing cells of *Stenotrophomonas* sp. HCU1. Detailed study was taken up to characterize the ring saturated metabolite of pyrazine-2-carboxylate.

Carbon sources influenced the ring reduction of pyrazine-2-carboxylate and malate (Table 13) was found to be the appropriate carbon source at 22 mM (Fig 15) with ammonium chloride as nitrogen source at 7 mM (Fig 16). The obvious reasons for high reduction of pyrazine-2-carboxylate in the presence of malate could be due to *dpkA* gene encoded putative malate/lactate dehydrogenase (based on N-terminal sequences) from *Pseudomonas putida,* catalyzing the ring reduction of piperdine-2-carboxylate to pipecolate (Muramatsu *et al.,* 2004). Though resting cells of strain HCU1 has the capability to reduce pyrazine-2-carboxylate (Fig 17), it was less when compared to growing cells (Data not shown). Reduction of other N-heterocyclic compounds (Table 14) by strain HCU1 clearly indicates that reductive metabolism was not restricted to pyrazines by *Stenotrophomonas* sp. HCU1 but to other N-heterocyclic compounds.

IC_{50} and minimum inhibitory concentration values (400 and 600 mM) of pyrazine-2-carboxylate on the growth of the strain HCU1 (Fig 18) indicated that pyrazine-2-carboxylate has no lethal effect on the *Stenotrophomonas* sp. HCU1 at low concentrations. The toxicity of pyrazine-2-carboxylate on the growth of bacteria was not known. However, the mechanism of toxicity of pyrazinamide (anti-tuberculotic drug) on *Mycobacterium tuberculosis* was due to formation of pyrazinoic acid by bacterial encoded pyrazinamidase (Zhang *et al.,* 1999). Pyrazinoic acid thus formed was excreted by weak efflux pump. Protonated pyrazinoic acid was reabsorbed in to the bacilli because of the inefficiency of the efflux pump causing cellular damage (Zhang and Mitchison, 2003).

Bacterial transformations of pyrazines were reported in few bacterial species which could not utilize the transformed products as growth substrates. These include

oxidative decarboxylation at position C3 catalyzed by 6-methylnicotinate-2-oxidoreductase in *Ralstonia/Burkholderia* (Tinschert *et al.,* 2000) and hydroxylation at C5 by quinolinate dehydrogenase in *Ralstonia eutrophus* (earlier called *Alcaligene*s *eutrophus*), respectively (Uchida *et al.,* 2003). Oxidation of pyrazine carboxylic acids by *Pseudomonas putida*, *Pseudomonas acidovorans* and *Alcaligenes faecalis* DSM 6929 were known (Kiener *et al.,* 1994). Nitrilase and nicotinic acid dehydrogenase catalyzed transformation of 2-cyanopyrazine to pyrazine-2-carboxylate in *Agrobacterium* sp. (Wieser *et al.,* 1997). Such bacterial strains with nonspecific oxidoreductases were exploited for the large scale synthesis/transformation of pyrazine carboxylic acids to their hydroxylated or carboxylated educts, which in turn are the precursors for the synthesis of many pharma drugs (Petersen and Kiener, 1999; Schmid *et al.,* 2001).

Bacterial catabolism of pyrazines follows a common metabolic pathway of aromatic compounds as reported in pyrazine degrading bacteria. Initially pyrazines are hydroxylated and then undergo oxygenase catalyzed ring cleavage with the release of ammonium. N-formylglycinamide, (Mattey and Harle, 1976), leucine and 2-hydroxyimino-3-methyl-1-pentanol which were the products of aspergillic acid degradation were known (Nishimura *et al.,* 1991). LC-MS metabolite profiling analysis of the control and pyrazine-2-carboxylate induced culture supernatants of strain HCU1 (Fig 19A, B) indicated a reductive metabolism of pyrazine-2-carboxylate by strain HCU1. Masses (*m/z*) 129 and 123 (in negative mode) corresponding to the ring reduced metabolite and pyrazine-2-carboxylate were observed in the induced supernatant. Mass (*m/z*) 124 in the control supernatant does not correspond to pyrazine-2-carboxylate (Fig 19A). 1,2,5,6-tetrahydropyrazine-2-carboxylic acid (A1) was found to be the ring reduced

metabolite 128 (m/z) based on IR (Fig 20A) ^{1}H and ^{13}C NMR (Fig 21A, B) and mass analyses (Fig 20B). ^{1}H NMR analysis of the compound indicated it as a mixture of two isomers of molecule differing in the position of double bond. Further purification of the isomers could not be done owing to the low yields of the product.

UV active orangish oily solid was identified as 2-amino-2-hydroxy-3-(methylamino) propanoic acid (B1) based on IR (Fig 22A) ^{1}H and ^{13}C NMR (Fig 23A, B) and mass (Fig 22B, C) analyses as ring cleavage product of pyrazine-2-carboxylate of mass (*m/z*) 134. N-formylglycinamide (Mattey and Harle, 1976) and leucine and 2-hydroxyimino-3-methyl-1-pentanol (Nishimura *et al.*, 1991) were the known ring cleavage products of pyrazines. Such a complete ring saturated metabolite was also reported earlier in the case of trinitrotoluene (Vorbeck *et al.*, 998; Ebert *et al.*, 1999).

Two other metabolites, 1,2-dihydropyrazine-2-carboxylic acid and piperazine-2-carboxylic acid were predicted as partial and complete ring saturated metabolites of masses 126 and 130 (*m/z*) respectively based on their molecular mass analysis (Fig 24 A,B; Plate 1). Further characterization of these metabolites could not be done owing to their low yields of the metabolite on purification. These metabolites gave colour reaction for cylic imino acids (Piez *et al.*, 1956).

Based on the characterized metabolites, a putative pathway of pyrazine-2-carboxylate catabolism by *Stenotrophomonas* sp. HCU1 is proposed (Fig 36). According to the proposed pathway, pyrazine-2-carboxylate was first reduced by two hydride addition to 1,2-dihydropyrazine-2-carboxylate (*m/z* 126) followed by four and six hydride addition resulting in 1,2,5,6-tetrahydropyrazine-2-carboxylate (*m/z* 128) and piperazine-2-carboxylate (*m/z* 130), respectively. Ring saturation may be followed by

ring hydroxylation and subsequent ring cleavage to 2-amino-2-hydroxy-3-(methylamino) propanoic acid. Catabolic pathway of pyrazine-2-carboxylate in strain HCU1 differs from other reported pathways of hydroxylated pyrazine in *Pseudomonas* sp. (Mattey and Harle, 1976), alkylated pyrazine in *Mycobacterium sp.* (Rappert *et al.,* 2006) and *Rhodococcus erythropolis* (Rappert *et al.,* 2007) in having initial reductive steps followed by ring hydroxylation and cleavage. However, in the above reported pathways ring hydroxylation precedes ring cleavage (Watson and Cain, 1975). Except in the case of *Trichoderma koningii,* (Nishimura *et al.,* 1991) direct ring cleavage of aspergillic acid without ring hydroxylation was observed. Metabolic/catabolic pathways involving ring saturation/reductive steps is not uncommon and was reported for pyridine and nicotinic acid degradation in both aerobic and anaerobic organisms (Watson and Cain, 1975; Holcenberg and Tsai, 1969; Rhee *et al.,* 1997; Lee *et al.,* 2001). In contrast to the reported pathways, pyrazine-2-carboxylate ring reduction is followed by ring cleavage in *Stenotrophomonas* sp. HCU1. Similar ring reduction pathways of aromatic compounds were reported earlier, in the case of picric acid, trinitrotoluene, 2,4,6-trinitrophenol (Ebert *et al.,* 1999), 2,4,6-trinitrotoluene (Vorbeck *et al.,* 1998).

Molecular masses in GC-MS spectrum (Fig 28, 29) of enzymatic analysis matched with the predicted metabolite masses of the ring saturated and ring cleavage products of the proposed pathway. Further the isolation of ~65kDa protein (Fig 30, 31, 32) which catalyzed the ring reduction of pyrazine-2-carboxylate to 1,2,5,6-tetrahydropyrazine-2-carboxylate with a molecular mass (*m/z*) of 128 confirms the ring reduction step of the proposed pathway (Fig 33). Ring reduction of other N-heterocyclic compounds (Table 16) shows the non-specificity/broad substrate specificity of the

enzyme for N-heterocyclic compounds and some of the properties of the enzyme were studied (Fig 34, Table 15-16).

The pyrazine-2-carboxylate reductase differs from other aromatic ring reductase of *Nocardiodes simplex* FJ2-1 (Ebert *et al.*, 1999) *Xen B* reductase of *Pseudomonas fluorescens* (Pak *et al.*, 2000) and 2-aminobenzoylCoA monoxygenase/reductase of denitrifying *Pseudomonas* sp. (Altenschmidt *et al.*, 1992) in the absence of F_{420} or FAD requirement. F_{420} dependent NADPH reductase in *Nocardiodes simplex* catalyzing the ring reduction of 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 reductase of *Stenotrophomonas* 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. However, there are no reports on the reductive catabolism of pyrazine group of compounds. MALDI analysis of the enzyme has not scored more than 59 on BLAST search (www.matrixscience.com) and no significant similarity to any known protein was found on MS-MS fragmentation of the peptide fragments (Fig 35, Table 17). Based on the evidences of flavin/F_{420} independent nature, substrate specificity and peptide mass finger printing analysis, the enzyme was named as pyrazine-2-carboxylate reductase/1,2,5,6-tetrahydropyrazine-2-carboxylate synthetase. It is therefore presumed that pyrazine-2-carboxylate reductase enzyme is a novel enzyme belonging to the class of oxidoreductases with flavin independent nature.

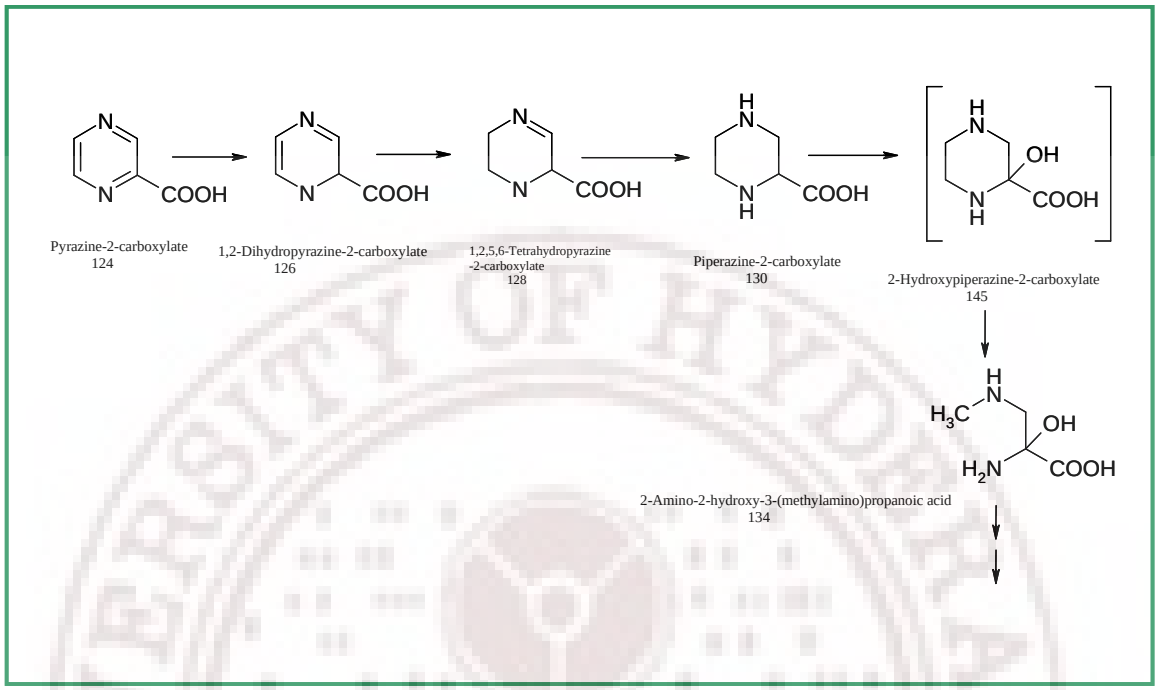


Fig 36: Proposed putative pathway of pyrazine-2-carboxylate catabolism by *Stenotrophomonas* sp. HCU1

Conclusions

5.Conclusions:

- Isolated a bacterium capable of degrading pyrazine-2-carboxylate and identified it as a strain belonging to the genus *Stenotrophomonas*
- The unique character of the strain in metabolism of pyrazine-2-carboxylate was its capability to degrade the substrate by ring reduction followed by pyrazine ring cleavage.
- Malate augmented the ring reduction process and its role needs to be studied in detail.
- The isolated and identified metabolites confirm the reductive pyrazine-2-carboxylate degradation pathway of pyrazine-2-carboxylate in *Stenotrophomonas* sp. HCU1.
- ~65 kDa protein was identified as a novel enzyme based on MALDI-TOF, MS/MS analysis for which it was named as pyrazine-2-carboxylate reductase based on the activity of the enzyme.

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