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Antimicrobial activity and metabolic effects of alkylpyrazines on facultative pathogens

MASTER'S THESIS

to achieve the university degree of

Diplom-Ingenieurin

Master's degree programme: Biotechnology

submitted to

Graz University of Technology

Supervisor

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Institute of Environmental Biotechnology

Graz University of Technology

Graz, January 2016

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Acknowledgements

I would like to express my deep and sincere gratitude to my supervisor Univ.-Prof. Dipl.-Biol. Dr. rer. nat. Gabriele Berg for giving me the possibility to do my master thesis at the Institute of Environmental Biotechnology. Her guidance and intellectual support during this time have been of great value for me.

I am deeply grateful to my co-supervisor Mag. rer. nat. Dr. rer. nat. Stefan Liebminger, who let me take part in his highly interesting project. His advices and constructive discussions with him provided me help in scientific questions. I am very thankful for his trustfulness and engagement during the whole time.

I also want to thank the whole working group for inspiring discussions and for the great time we spent together at the institute. Especially I would like to thank Zohartze Monteagudo for her helping hands and enthusiastic support.

Last but not least, I am heartily thankful to my family, my boyfriend and friends for their invaluable support, encouragement and motivating words throughout my studies.

Abstract

Alkylated pyrazines are volatile organic compounds, which are almost ubiquitously distributed in nature. They are found as secondary metabolites of microorganisms and animals and they highly contribute to the flavour of vegetables and heated food. Despite the high abundance of alkylpyrazines, their microbiological activities are not well studied. The aim of this thesis was to test the antimicrobial potential of those compounds on facultative pathogens. Thereby, the focus was set on the understanding of the structure-activity relationship and the metabolic effects initiated by alkylpyrazines. Seven derivatives were tested, whereby 5-isobutyl-2,3-dimethyl-pyrazine (IBDM) was applied as a structural homology model. Moreover, the bacteria *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* as well as the fungi *Saccharomyces cerevisiae, Candida albicans* and *Penicillium sp.* were used. By the tools of time-kill kinetic studies and antimicrobial efficacy tests including the determination of minimal inhibitory concentrations, the antimicrobial activity was studied. Fluorescence assays such as the *Bac*Light Bacterial Viability Kit and the *Bac*Light Membrane Potential Kit as well as enzyme assays and an *E. coli* Knockout Collection were used to identify potential cellular targets of alkylpyrazines. Within this thesis it was proven that alkylpyrazines exhibit strong bactericidal as well as fungicidal activities. It was demonstrated that IBDM is able to reduce high cell concentrations of pathogens of nosocomial infections in an exposure time of 5 min. This fast-acting effect demonstrates that alkylpyrazines are potential candidates for disinfectants. Furthermore, it was shown that the antimicrobial efficiency of the derivates correlates with their hydrophobicity, where IBDM, which was the most effective pyrazine in this study, exhibits the highest log P value. It was observed that alkylpyrazines interact with proteins and that the presence of proteins can reduce the antimicrobial efficiency. Moreover, it was interpreted that IBDM has enzyme inhibiting activities. Fluorescence assays revealed that alkylpyrazines cause membrane damages by disrupting the inner plasma membrane and depolarizing the membrane potential. The findings in this thesis lead to the assumption that alkylated pyrazines are targeting the cell membrane. The hydrophobic compounds are presumably able to penetrate into the bilayer of cell walls, where they might interact with membrane proteins. This interference may cause a deformation of the membrane integrity and an increase of the permeability, leading to a higher passive flux of protons and thus to the disruption of the membrane potential.

Kurzfassung

Alkylierte Pyrazine sind flüchtige organische Verbindungen, die nahezu ubiquitär in der Natur verbreitet sind. Sie stellen sekundäre Metabolite von Mikroorganismen und Tieren dar und tragen stark zum Geschmack von Gemüse und erhitzten Lebensmitteln bei. Trotz des ubiquitären Vorkommens von Alkylpyrazinen sind ihre mikrobiologischen Aktivitäten nicht gut untersucht. Das Ziel dieser Masterarbeit war es, das antimikrobielle Potential dieser Verbindungen an fakultativen pathogenen Mikroorganismen zu testen. Der Fokus wurde dabei auf das Verständnis von Struktur-Aktivität Beziehungen sowie auf die Detektion von metabolischen Effekten gesetzt, welche durch Alkylpyrazine verursacht werden. Sieben Derivate wurden getestet, wobei 5-Isobutyl-2,3-Dimethylpyrazin (IBDM) als Struktur-Homolog eingesetzt wurde. Die Mikroorganismen *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae, Candida albicans* und *Penicillium sp.* wurden verwendet. Mithilfe von antimikrobiellen Wirksamkeitstests inklusive der Bestimmung von minimalen Hemmkonzentrationen wurde die antimikrobielle Aktivität untersucht. Fluoreszenz-Tests wie das "*Bac*Light Bacterial Viability Kit" und das "*Bac*Light Bacterial Membrane Potential Kit" sowie Enzym Assays und eine *E. coli* Knockout Kollektion wurden zur Aufdeckung der potentiellen zellulären Ziele von Alkylpyrazinen eingesetzt.

Im Zuge dieser Masterarbeit konnten starke bakterizide und fungizide Aktivitäten ausgehend von Alkylpyrazinen nachgewiesen werden. Es wurde gezeigt, dass IBDM nach einer Einwirkung von 5 min hohe Zellkonzentrationen an fakultativen Pathogenen von nosokomialen Infektionen abtöten kann. Die schnelle Wirksamkeit von Alkylpyrazinen macht sie zu potenziellen Kandidaten als Desinfektionsmittel. Des Weiteren konnte belegt werden, dass die antimikrobielle Effizienz der getesteten Derivate mit ihrer Hydrophobizität steigt. Beispielsweise weist IBDM, welches in dieser Studie das effektivste Pyrazin war, den höchsten log P Wert auf. Zudem wurde beobachtet, dass Alkylpyrazine mit Proteinen interagieren und dass die Anwesenheit von Proteinen die antimikrobielle Effizienz reduzieren kann. Darüber hinaus wurde eine Enzym-inhibierende Wirkung von IBDM festgestellt. Mithilfe von Fluoreszenz-Tests wurden Membranschäden nachgewiesen, welche sich in der Permeabilisierung der inneren Plasmamembran und der Depolarisierung des Membranpotentials äußern. Die Ergebnisse dieser Arbeit führen zu der Annahme, dass das primäre Angriffsziel der alkylierten Pyrazine die Zellmembran

ist. Die hydrophoben Verbindungen dringen in die Doppelmembranschicht der Zellhülle ein, wo sie mit Membranproteinen interagieren. Diese Interferenz verursacht eine Deformation der Membranintegrität und eine Erhöhung der Permeabilität, was einen höheren passiven Protonenfluss und damit den Verlust des Membranpotentials zur Folge hat.

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1 Introduction

1.1 Chemical properties of pyrazines

Pyrazine molecules are monocyclic heteroaromatic compounds with two para-oriented nitrogen atoms in the ring (1,4-diazines) [1]. They can be substituted at one or more of the four ring carbon atoms [2]. The structural formula is illustrated in Figure 1. Pyrazine derivatives can be structured in four subcategories [1]:

1) Unsubstituted pyrazines
2) Pyrazine derivatives with a hydrocarbon substituent (alkyl, alicyclic or alkylaryl)
3) Pyrazine derivatives with an oxygenated functional group and aliphatic side chain (alkoxy or acetyl)
4) Pyrazine derivatives with a thiol or sulphide functional group in the aliphatic side chain

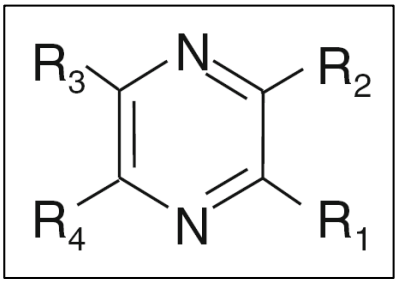


Figure 1: Structural formula of a pyrazine molecule [2].

Alkylated pyrazines are the most abundant representatives of pyrazine compounds [3]. Due to the relatively low molecular weight of pyrazines and their ability to evaporate at normal atmospheric temperatures and pressure, they are classed as volatile organic compounds (VOCs) [4]. Numerous pyrazine compounds have an intensive odour and very low aroma threshold values, which means that their flavour is recognized at very low concentrations [1, 2]. Due to these properties and the broad aroma spectrum of pyrazines, they are used as flavour ingredients in various food products [2, 5]. Moreover, these compounds are found naturally in vegetables and heated food [6, 7]. Pyrazines are also widespread among animals such as insects and terrestrial vertebrates, where they are used as semiochemicals for intra- or interspecific communication [8]. The high volatility and the low olfactory thresholds are ideal conditions for a quick and highly transient information transfer [9].

A pyrazine is a weak diacid base (pK_1 = 0.57; pK_2 = -5.51) with unique physico-chemical properties due to its low lying unoccupied π-molecular orbital [5, 10]. Shimazaki *et al*. [11] evaluated the odour activity of pyrazine compounds based on their conformational and electronic properties. In the study it was suggested that

the presence and the orientation of the lone-pair orbital on the nitrogen atom and the substituents of the ring are elementary, contributory factors of the odour quality and intensity. Lone-pair orbitals extend to the outside of the pyrazine ring and are proposed to be responsible for the interaction with the functional groups of the olfactory receptor sites (e.g. G-protein-coupled receptors). The type and the position of the functional substituents on the pyrazine ring are essential for the odour thresholds and aroma spectrum [11]. Due to the molecular structure of pyrazines they can be selectively detected by their target receptor [4].

1.2 Natural occurrence and sources of pyrazines

1.2.1 Pyrazines in food

For humans the main source of pyrazine uptake is food, where pyrazines are found in concentrations between 0.001 and 40 ppm [7]. Substituted pyrazines, especially 3-alkyl-2-methoxypyrazines, are found naturally in various raw vegetables.

3-isobutyl-2-methoxypyrazine was detected in fresh beans, Indian cress, green and red pepper and in potatoes. 3-sec-butyl-2-methoxypyrazine belongs to the key aroma substances in beetroots, silverbeets, carrots and in parsnips. Moreover, 3-isopropyl-2-methoxypyrazine contributes to the aroma of green peas, pea shells, broccoli, broad been seeds, cucumber, asparagus, lettuce, cabbage, thistle and tomatoes [6, 12]. These alkyl-2-methoxypyrazines are responsible for the typical earthy, green and bell pepper-like flavour of vegetables [13]. 3-isobutyl-2-methoxy-pyrazine serves also as impact flavour compound of several wines, giving a typical grassy aroma, associated with grapes and wine [14 - 17].

Furthermore, pyrazines play an important role in the aromas of heated food, where they are formed through the Maillard reaction. A non-enzymatic, heat-induced condensation between amino acids and α-dicarbonyl compounds (sugars) leads to the synthesis of the aroma compounds (Strecker degradation). Thereby, the nitrogen atoms of the pyrazine come from the amino acid and sugar is the source of the carbon atoms. High levels of pyrazines are formed at temperatures between 120 and 150°C and the substitution pattern of the pyrazines is influenced by the

amino acid type [2]. 2-acetylpyrazine, for instance, is the key substance for a popcorn-like flavour [18] and 2-ethyl-3,5-dimethylpyrazine is a major contributor to the aroma of a baguette crust. Over 70 alkylpyrazines have been identified in heat-processed foods [19], where they are mainly responsible for a roasty and nutty aroma [13]. In Table 1 selected food products, such as roasted beef, nuts, coffee and cocoa and the corresponding pyrazine derivatives are listed. Due to the wide distribution of pyrazine compounds in foods, they could be identified in the exhaust gas streams of food industries such as coffee- and cacao-productions [20, 21].

In addition, pyrazines are also found in fermented food, where they are synthesized during the microbial fermentation process. Also in microorganisms the pyrazines are formed by the reaction between amino acids and sugars [2]. Examples are explained in more detail in chapter 1.2.3.

Table 1: Selected pyrazines reported in food products. Almost all compounds are alkylated.

Food	Pyrazine derivatives	References
Roasted beef	2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine	[22]
Roasted peanuts, peanut butter preparations	2,6-dimethylpyrazine, ethylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2-ethyl-5-methyl- and 2-ethyl-6-methylpyrazine, 3-ethyl-2,5-dimethylpyrazine	[23], [24]
Peanut, hazelnut and pumpkin seed oil	In all oils: 2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine Great impact in pumpkin seed oil: 2-ethyl-3,5-dimethylpyrazine, 2-isobutyl-3-methylpyrazine and 2-methyl-6-furfurylthiopyrazine	[25], [26]
Roasted sesame seeds	2-acetylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine	[27]
Maple syrup	Most abundant: 2,6-dimethylpyrazine, 2,5-dimethyl- and 2-methylpyrazines 2-ethyl-3-methyl-, 2-ethyl-5-methyl-, 2-ethyl-3,5-dimethyl-, 3-ethyl-2,5-dimethyl- and 2,3,5,6-tetramethylpyrazine	[28]
Potato chips	2-ethyl-3,5-dimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, 2-ethyl-3-ethyl-5-methylpyrazine	[21]
Roasted powder and brew of coffee	3-isopropyl-2-methoxypyrazine, 2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, 3-isobutyl-2-methoxypyrazine	[29]
Cocoa powder	2-ethyl-3,6-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, 2-isobutyl-3-methoxypyrazine	[30]
Bread	2-methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2-ethylpyrazine, 2-ethyl-3-methylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 2-methyl-6-propylpyrazine, 3-methyl-3-ethylpyrazine, 2-acetylpyrazine	[31]
Popcorn	2-acetylpyrazine, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2-ethyl-5-methylpyrazine, 2,5-dimethyl-3-ethylpyrazine	[18]

1.2.2 Pyrazines in animals and plants

Pyrazines have been reported in several animals and plants with a high diversity of functions. Thereby they play an important role in chemical communication by serving as alarm or trail pheromones, as deterrents or attractants without having a harmful or beneficial effect [8]. For social insects the maintenance of colony integrity and cohesiveness depends on volatile secretory products like pyrazines [9]. In 1989, pyrazines were already identified in 180 species of ants, 29 wasps, 7 flies and 2 bees [32]. Moreover, 2-methoxy-3-alkylpyrazines have been detected in 30 species of aposematic beetles, butterflies and plant bugs in 1990 [33].

Alkylpyrazines were discovered firstly in the mandibular gland secretions of ponerine ants of the genus *Odontomachus*, in which they are implicated in alarm reactions of the workers. The derivatives 2,6-dimethyl-3-pentyl-, -butyl-, -propyl-, and -ethyl-pyrazines were identified in the species *O. brunneus* and 2,5-dimethyl-3-isopentylpyrazine was found in the secretions of *O. hastatu*s and *O. clarus* [34]. The alkylated pyrazine derivatives 3-(2-methylpropyl)-2,5-dimethylpyrazine, 3-(2-methyl-butyl)-2,5-dimethylpyrazine and 3-(3-methylbutyl)-2,5-dimethylpyrazine were found as alarm pheromones in the secretions of the Australian formicine ant of the genus *Calomyrmex* [35].

The volatile 3-ethyl-2,5-dimethylpyrazine was recorded to serve as trail pheromone for eight species of *Myrmica* ants and four species of *Pogonomyrmex* ants, being secreted by poison glands. Functions such as food gathering and recruitment were associated with this pyrazine derivate [36, 37].

Alkyldimethylpyrazines are also part of the defensive spray of the phasmid insect *Phyllium westwoodii*. A mixture of 3-isobutyl-2,5-dimethyl-pyrazine, 2,5-dimethyl-3-(2-methylbutyl)pyrazine, 2,5-dimethyl-3-(3-methylbutyl)-pyrazine and glucose is sprayed by this leaf insect, when it is disturbed by a natural predator [38]. Moreover, it was found that the seven-spot ladybird emits pyrazine volatiles, when it is attacked [39]. Also the tiger moth and the male melon fly use pyrazines as warning signals for predators. However, both insects release those molecules also as attractants for sexual partners [8]. The biological function as sex pheromone component was recorded for the wasp *Zaspilothynnus trilobatus* too. Three alkylpyrazines and one hydroxymethylpyrazine were identified as the key

compounds of the attractant emitted by this wasp species. Interestingly, these pyrazine derivatives are also used as semiochemicals by the orchid *Drakaea glyptodon*. Thereby, the wasps are sexually attracted to serve as pollinators for the plant [40].

Volatile emission by plants is widely distributed. On the one hand they can implicate sexual deception, and on the other hand they can serve as filter for unwanted visitors such as herbivores [40]. In 1986, a pyrazine-binding protein was found in the nasal mucosa of cows and rabbits, which supports the hypothesis that pyrazines function as alerting signals for herbivores [41]. In addition, pyrazines may serve as warning system for fire, due to the fact that the smoke of a burning wood is highly pyrazine-laden [8].

Pyrazines have also been recorded in some terrestrial vertebrates. The urine of wolfs, for instance, has a fear-inducing impact on cattle and rats. In a recent study it was proved that the urine of the grey wolf (*Canis lupus*) contains a mixture of the compounds 2,6-dimethylpyrazine, trimethylpyrazine and 3-ethyl-2,5-dimethyl-pyrazine. These volatiles function in predator-prey chemosignalling, inducing avoidance and freezing behaviour in laboratory mice. The signalling pathway of the volatiles was investigated by observing the immunoreactivity for Fos, a marker of neuronal excitation, in the accessory olfactory bulb. It was reported that the wolf urine and the pyrazine mixture itself triggered the expression of Fos-immunoreactive cells. Thereby, it was concluded that the pyrazine derivatives in the urine provoke aversive and fear-related responses via stimulation of the murine vomeronasal system [42]. This fear-inducing effect was also observed for ungulates (Hokkaido deers) when exposed to the pronounced pyrazine cocktail of wolf urine [43]. On this basis, a further function of pyrazines can be established, they are serving as kairomones. Kairomones are chemicals for interspecific communication, which are released from an emitter and have a beneficial effect for the receiver organism [44].

1.2.3 Microbial formation of pyrazines

Further natural sources of pyrazines are microorganisms, where they are a major class of volatiles emitted by bacteria and fungi [3, 45]. Already in 1962 the first evidence was published that bacteria are able to produce pyrazines. Thereby, tetramethylprazine (TMP) was found as an aroma component of natto, fermented soybeans, where it was synthesized by *Bacillus subtilis* [46]. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* was also reported to be a TMP producer [47]. Pyrazine derivatives such as methylpyrazine, 2,5-dimethylpyrazine, trimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were identified as metabolites of *Bacillus cereus* [48]. Moreover, *Paenibacillus polymyxa,* a plant growth-promoting soil bacteria living in rhizosphere*,* releases a mixture of 19 alkylpyrazines in the medium, when it is cultivated in TSB medium. Thereby, 2,5-diisopropylpyrazine is the most abundant compound in this mixture [49, 50]. The species *Pseudomonas perolens* and *taetrolens* have been reported to be responsible for a potato-like odour in several meats, including beef, pork and lamb, due to their production of 2-methoxy-3-isopropylpyrazine [51]. Furthermore, the *Corynebacterium glutamicum* was found to biosynthesize a variety of pyrazines. The volatile profile of this microorganism includes large quantities of trimethylpyrazine and tetramethylpyrazine [52]. For the myxobacterium *Chondromyces crocatus* a mixture of 27 pyrazines was identified, containing 2,5-dialkylpyrazines and 3-methoxy-2,5-dialkylpyrazines in high amounts. In addition, pyrazine metabolites have also been recorded in the volatile profile of marine bacteria including *Sulfitobacter pontiacus* from the North Sea and species of the *Roseobacter* clade [45].

The biosynthesis of pyrazines is also widely distributed among fungi, especially hydroxylated pyrazines [3]. The most commonly known example is the metabolite aspergillic acid (2-hydroxy-3-isobutyl-6-sec-butylpyrazine-1-oxide) of *Aspergillus flavus,* which is used as an antibiotic [53]. Moreover, *A. sclerotiorum* was found to produce the pyrazine-ring containing antibiotics neohydroxyaspergillic acid and neoaspergillic acid [54]. The yeast *Candida pulcherrima* synthesizes pulcherriminic acid (2-hydroxy-3,6-diisobutylpyrazine-1,4-oxide), a derivate of the red pigment pulcherrimin, which exhibits antifungal properties against *Fusarium*, *Mucor* and *Penicillium* [55, 56].

Until now the functions of the pyrazine compounds in the microbial world are not well studied. In general it is assumed that bacterial volatiles can serve (1) as inter- and intraspecific communication signals, (2) as quorum sensing molecules (cell-to-cell communication), (3) as an outlet for carbon, or (4) as growth promoters or inhibiters [57].

In some cases, bacterial pyrazine metabolites are responsible for the occurrence of undesired flavours in food [58]. Aroma defects in coffee, for instance, can be caused by musty smelling alkylmethoxypyrazines, which are released by bacteria [30]. The same bacterial pyrazine compounds can also lead to a mouldy off-flavour in eggs, dairy products and fish [59].

In addition to the microbial formation of pyrazines, microorganisms are also able to use pyrazine compounds as a carbon and nitrogen source. Already in 1976 it was reported that a *Pseudomonas* species degrades 2-hydroxypyrazines as energy source [60]. Furthermore, Rappert *et al.* described that *Mycobacterium fortuitum* and *Mycobacterium* sp. DM-11 can use 2,3-diethyl-5-methylpyrazine as a sole source of carbon and energy. The same was reported for *Rhodococcus erythropolis* being capable to grow on 2,5-dimethylpyrazine. Moreover, *Rhodococcus opacus* is able to utilize tetramethylpyrazine as a sole source of carbon and nitrogen [2].

1.3 Application fields of pyrazines

1.3.1 Flavour industry

Due to the broad aroma spectrum of pyrazine compounds and their low odour threshold values, they are commonly used as flavouring additives in various foods [2, 5]. It has been estimated that the daily uptake of pyrazine food additives is about 3 µg/kg bodyweight.

Approximately two thirds of the total annual production volume of pyrazines as flavouring agents can be assigned to the derivatives 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine and acetylpyrazine [1]. Bacteria such as *Bacillus subtilis*, *Lactococcus lactis* and *Corynebacterium glutamicum* are used for the industrial production of tetramethylpyrazine, which exhibits a musty, fermented or

coffee odor [2, 61]. Trimethylpyrazine creates a baked potato or roasted nut aroma in foods and acetylpyrazine is used due to its popcorn- and bread crust-like flavour profile [62]. Furthermore, alkoxypyrazines, which can have a nutty but also green bell pepper or tomato aroma, are added as flavour ingredients to food.

Pyrazine compounds can enhance the flavour of various food products by giving a new taste and scent or by adding a characteristic flavour, which was lost during the cooking or packaging process [63]. The major food products, which are added with pyrazines, are baked goods, beverages, breakfast cereals, chewing gum, candies, soups, instant coffee/tea, jams, cake, chocolate, cocoa, coffee, corn chips, gingerbread, pizza, popcorn and tacos as well as egg, milk, fish and meat products [64].

1.3.1.1 Safety evaluation of pyrazine derivatives used as flavouring substances

The Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) evaluated the safety of pyrazines used as flavour ingredients in 2002. Thereby, the rat acute oral LD_{50} values were determined and ranged from 500 to 2500 mg/kg, which indicates a low level of toxicity. In the short-term toxicity studies no evidence of histopathologic changes, and hence no adverse effects were found. Moreover, the carcinogenicity and genotoxicity studies showed no negative influence of pyrazine derivatives. Due the obtained data in this report and the fact that the intake of pyrazine compounds as natural components of food is higher than the intake as flavour additives, it was concluded that pyrazine derivatives are safe for the human consumption [1].

1.3.2 Pharmaceutical and agricultural industry

Pyrazine derivatives exhibit a wide range of biological activities including anti-tubercular, antiviral, antimicrobial, anticancer, antiepileptic and antiinflammatory activities. As a consequence, numerous compounds having a pyrazine nucleus are used as pharmaceuticals [65]. Moreover, pyrazines exhibiting herbicidal, insecticide or nematicide are applied as agricultural chemicals [3].

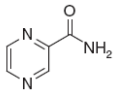
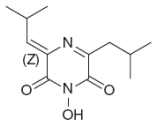
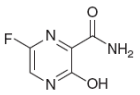
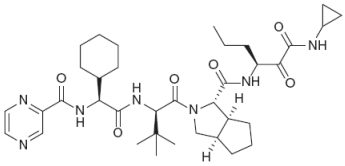
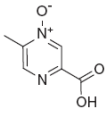
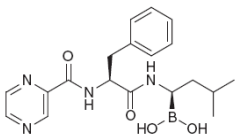
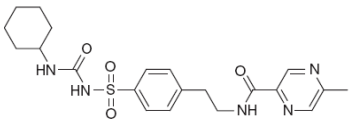
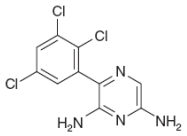
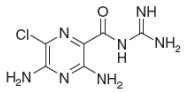
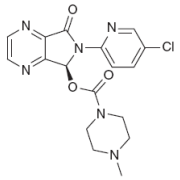
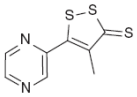
In Table 2 marketed drugs and groups of medicinally important pyrazine derivatives are listed.

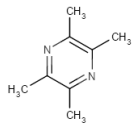
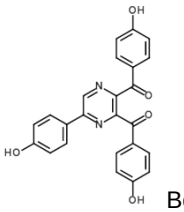
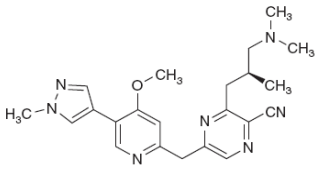
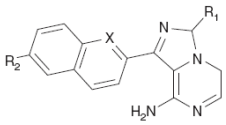
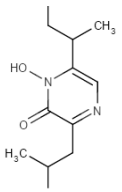
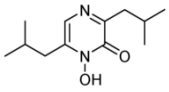
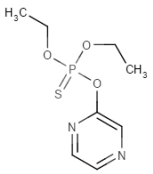
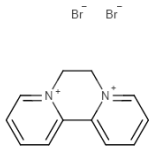
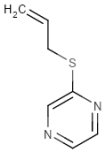
The probably most commonly known pyrazine pharmaceutical is pyrazinamide (PZA), an important sterilising tuberculosis drug. The application of this compound helps to shorten the chemotherapy for tuberculosis and can be perfectly combined with the antibiotic rifampicin. PZA needs an acid pH and is only active against old and slow growing cultures of *Mycobacterium tuberculosis* (semi-dormant). It was reported that PZA enters the bacterium via passive diffusion and is then converted by nicotinamidase into its active form – pyrazinoic acid (POA). POA is excreted by passive diffusion and a deficient efflux system. Due to an acidic environment a part of the POA is protonated (HPOA), which again enters the bacilli. Thereby, the accumulation of protons eventually causes cytoplasmic acidification leading to the disruption of the proton motive force and to an inhibition of the membrane transport [66].

Moreover, it was proposed that POA inhibits the fatty acid synthases I and II, which are involved in the formation of mycolic acids, important structural elements of the mycobacterial cell wall [10]. A recent study suggested that the target of PZA is the ribosomal protein S1 (RpsA), causing an inhibition of the *trans*-translation in *M. tuberculosis* [67]. Zhang *et al*. reported in 2013 that panD encoding aspartate decarboxylase is the biological target of PZA. PanD is essential for the synthesis of pantothenate and co-enzyme A. The hypothesis was proposed due to the fact that mutations in panD led to resistance of *M. tuberculosis* [68]. To this day, the mode of action of PZA is not completely understood and will be further investigated in more details.

As can be seen in the table, there are just a few pyrazine derivatives which are known for antibacterial or antimicrobial activity. In the table the hydroxylated pyrazines aspergillic acid, neoaspergillic acid and emimcyin are exhibiting these properties. In this regard, alkylpyrazines, which will be examined in this study, are not known for showing antimicrobial activity. Ligustrazine (tetramethylpyrazine) is one of the few alkylated pyrazines, which are used as drugs. The compound is known for its anti-inflammatory activity and hypotensive action, but not for exhibiting antimicrobial activity [69, 70].

Table 2: Relevant pyrazine derivatives and their biological activity and structure.

Pyrazine derivate Trivial and IUPAC[1] name	Biological activity	Structure[2]	Reference
Pyrazinamide; pyrazine-2-carboxamide	Antitubercular activity		[66]
Flutimide; (3Z)-1-Hydroxy-5-isobutyl-3-(2-methylpropylidene)-2,6(1H,3H)-pyrazinedione	Antiviral activity		[71]
Flavipiravir; 6-fluoro-3-hydroxypyrazine-2-carboxamide			[72]
Telaprevir (protease inhibitor for treatment of hepatitis C)			[73]
Pyrazinediazohydroxide	Antineoplastic activity		[74]
Acipimox; 6-methyl-1-oxo-1λ⁵-pyrazine-3-carboxylic acid	Hypolipidemic activity		[75]
Bortezomib; [(1R)-3-methyl-1-[(2S)-3-phenyl-2-(pyrazin-2-ylformamido) propanamido] butyl]boronic acid	Treatment of multiple myeloma (proteasome inhibitor)		[76]
Glipizide; N-[2-(4-{[(cyclohexyl-carbamoyl) amino]sulfonyl}phenyl)ethyl]-5-methylpyrazine-2-carboxamide	Antidiabetic activity		[77]
Elpetrigine; 3-(2,3,5-Trichlorophenyl)-2,6-pyrazinediamine	Antiepileptic activity (sodium channel and calcium channel blocker)		[78]
Amiloride; 3,5-diamino-6-chloro-N-(diamino-methylidene)pyrazine-2-carboxamide	Diuretic activity, treatment of cystic fibrosis (epithelial sodium channel blocker)		[79]
Eszopiclone; (5S)-6-(5-Chloro-2-pyridinyl)-7-oxo-6,7-dihydro-5H-pyrrolo [3,4-b] pyrazin-5-yl 4-methyl-1-piperazine carboxylate	Hypnotic activity		[80]
Oltipraz; 4-methyl-5-(pyrazin-2-yl)-3H-1,2-dithiole-3-thione	Antischistosomal and chemoprotective activity		[81]

Ligustrazine; 2,3,5,6-Tetramethylpyrazine	Anti-inflammatory and antihypertensive activity		[69, 70]
Botryllazine A and B; [5-(4-Hydroxyphenyl)-2,3-pyrazine-diyl]bis[(4-hydroxy-phenyl) methanone], (4-Hydroxyphenyl)[6-(4-hydroxy-phenyl)-2-pyrazinyl] methanone	Cytotoxic activities against human tumor cell lines	 Botryllazine A	[82]
Cyanopyrazine derivatives (as Chk1 inhibitors)	Anticancer activity (inhibitors of protein kinases)		[76]
Imidazo[1,5-a]pyrazine derivatives	Antiprotozoal activity	 R_1 = *i*-Pr, *tert*-Bu R_2 = H, CH_3O, CH_3CH_2O X = CH, N	[83]
Aspergillic acid; 6-(butan-2-yl)-1-hydroxy-3-(2-methyl propyl)-1,2-dihydropyrazin-2-one	Antibacterial activity		[53]
Neoaspergillic acid; 1-hydroxy-3,6-diisobutyl-2(1H)-pyrazinone	Antibacterial activity, inhibitory activities against human cancer cell lines		[84]
Thionazine; ethyl pyrazin-2-yl ethoxy (sulfanylidene)phosphonite	Insecticidal and nematicidal activity		[65]
Diquat-dibromide; 6,7-Dihydrodipyrido[1,2-a:2',1'-c] pyrazinediium dibromide	Herbicidal activity		[5]
2-(Allylthio)pyrazine	Chemoprotective activity (hepatoprotective effects)		[85]
Dihydropyrazines	Mutagenic activity, DNA-cleaving activity		[86]

[1] References for IUPAC nomenclature: [87] and [88].

[2] References for structural formulas: [76], [87] and [88].

1.4 Objectives of this study

In previous experiments performed at the Institute of Environmental Biotechnology, it was shown that natural occurring alkylpyrazines show strong inhibitory and antimicrobial effects on infectious pathogens. Due to the fact that there is almost no information about an antimicrobial activity of alkylated pyrazines, the mode of action causing these effects is not known. These circumstances make these compounds valuable for carrying out closer explorations. The objective of this thesis was to investigate the biological activities of selected alkylpyrazines in more details by treating various facultative pathogenic microorganisms. Thereby, the focus was set on the understanding of the metabolic effects initiated by the pyrazine derivates. By the tools of microbiological and biochemical methods, including antimicrobial susceptibility tests and fluorescence assays, the potential cellular target of alkylpyrazines was examined. The results of this work should provide a fundament for an evaluation of the biochemical potential of these molecules.

2 Materials and Methods

2.1 Alkylated pyrazine derivatives

In this study seven different alkylated pyrazine derivatives were used. Their exact chemical names, the corresponding abbreviations, which are used in this thesis, as well as the producing companies are listed in Table 3. Due to the availability of technical resources, the derivate 5-isobutyl-2,3-dimethylpyrazine has been used in many experiments as a model for the activity of alkylated pyrazines.

Table 3: Pyrazine derivates used in this study.

Chemical name	Abbreviation	Company
5-isobutyl-2,3-dimethylpyrazine	IBDM	Sigma-Aldrich
2-isobutyl-3-methylpyrazine	IBM	Sigma-Aldrich
2-isobutylpyrazine	IB	Alfa Aesar
2-isopropyl-5-methylpyrazine	IPM	Sigma-Aldrich
2-isopropylpyrazine	IP	Alfa Aesar
2,5-dimethylpyrazine	2,5-DMP	Sigma-Aldrich
2,3-dimethylpyrazine	2,3-DMP	Sigma-Aldrich

2.2 Antibacterial activity

2.2.1 Bacterial strains

The following bacterial strains were used in this study: *Escherichia coli* K-12 (*E. coli*), *Staphylococcus aureus* 25923 (*S. aureus*) and *Pseudomonas aeruginosa* QC14-3-8 (*P. aeruginosa*). The strains were obtained from the strain collection of the Institute of Environmental Biotechnology (Graz University of Technology).

2.2.2 Time-kill kinetics – IBDM

The goal of this experiment was to examine the antibacterial activity of the pyrazine derivate IBDM. An overnight culture of *E. coli* was prepared in a flask filled with 20 ml LB medium. The flask was incubated overnight in the shaker at 30°C and 110 rpm. On the next day, the sample preparation was carried out. The

optical density of the culture was measured photometrically (Eppendorf BioPhotometer). Using the OD_{600} value, the bacterial suspension was adjusted with PBS-Buffer to a defined cell concentration of 10^7 CFU/ml in a total volume of 1 ml. An OD_{600} value of 0.02 was equivalent to log 7 *E. coli* cells. Amounts of 0.5, 1, 2 and 3 µl of IBDM were added to the samples. Samples without the addition of the pyrazine derivate were negative controls. All samples were prepared in 1.5 ml tubes (Eppendorf) in triplicates. They were incubated at 30°C for 6 h with agitation (110 rpm). The tubes were positioned transversely during treatment to improve the mixing. One fraction of controls was spread on LB plates to determine the cell concentration (CFU/ml) at time point 0 (t_0). After 2, 4 and 6 h of incubation, the samples were plated on agar plates to determine the number of viable cells and thereby the antibacterial activity of the derivate. All samples were plated out in duplicates. The experiment was repeated twice.

2.2.3 Antibacterial efficacy of IPM, IBM, IP and IB

The objective was to test if the pyrazine derivatives IBM, IPM, IB and IP exhibit antibacterial activity. Firstly, an overnight culture of *E. coli* was prepared in 20 ml LB medium. The culture was incubated overnight in the shaker at 30°C and 110 rpm. On the next day, the antibacterial assay was performed. In the first step the optical density of the culture was measured photometrically. The bacterial suspension was adjusted with PBS-Buffer to defined cell concentrations of 10^5 – 10^7 CFU/ml in a total volume of 1 ml. To reach a cell number of 10^5 CFU/ml the *E. coli* culture broth was adjusted to an OD_{600} of 0.0002. The samples were treated with 3 µl (0.3%) of the compounds IBM, IPM, IB and IP. Samples without the addition of pyrazine were negative controls. All samples were prepared in 1.5 ml tubes in triplicates. Incubation was carried out at 30°C and 110 rpm for 4 h. The tubes were positioned transversely during treatment to improve the mixing process. One fraction of controls was spread on LB agar plates to determine the cell concentration at t_0. After 4 h of incubation, aliquots of each sample were spread on agar plates for the enumeration of viable cells. All samples were plated out in duplicates.

2.2.4 Antibacterial efficacy of 2,3- and 2,5-DMP

The purpose of this experiment was to examine if the pyrazine derivatives 2,5- and 2,3-dimethylpyrazine show antibacterial activity. An overnight culture of *E. coli* was prepared in a flask filled with 20 ml LB medium. The flask was incubated overnight in the shaker at 30°C at 110 rpm. On the next day, the antibacterial assay was performed. Therefore, the optical density of the culture was measured with a photometer. Using the OD_{600} value, the bacterial suspension was adjusted with PBS-Buffer to a defined cell concentration of 10^3 CFU/ml in a total volume of 1 ml. The samples were treated with 3, 6, and 12 µl of 2,3-DMP and 2,5-DMP. Samples without the addition of a pyrazine derivate were negative controls. All samples were prepared in 1.5 ml tubes in triplicates. They were incubated at 30°C and 110 rpm for 6 h. One fraction of controls was spread on LB agar plates to determine the cell concentration at t_0. After 2, 4 and 6 h of incubation, aliquots of each sample were spread on LB plates to determine the number of viable cells and thereby the antibacterial activity of the derivatives. All samples were plated out in duplicates.

2.2.5 Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [89]. The MIC is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism under defined conditions [90]. The MBC is defined as the lowest concentration of the agent required for a 99.9% reduction of viable bacteria after a distinct exposure time and subculture to antibiotic-free medium [91]. Both values are used to determine susceptibilities of bacteria to antimicrobial agents and to evaluate the activity of the substances [90].

The bacterial strains *E. coli* and *S. aureus* were used for the test. The antibacterial susceptibility testing was performed for the pyrazine derivatives IBDM, IPM, IP, IB, and IBM. The Mueller Hinton Broth (MHB) was used, which is the standard medium for the MIC determination [89].

The microorganisms were grown on LB agar plates at 30°C overnight. On the next day, five distinct colonies were transferred to a flask filled with 20 ml of LB medium. The flask was incubated at 37°C and 110 rpm for approximately 5 h. Then the samples were prepared in 1.5 ml tubes. Each tube contained MHB-medium and a pyrazine derivate (concentration between 1 and 20 mg/ml). The EUCAST method was slightly modified. Normally, a two-fold dilution series of the antimicrobial agent should have been prepared (e.g. from 10 mg/ml to 5 mg/ml to 2.5 mg/ml etc.) [89]. In this study the concentration was lowered in steps of 1 mg/ml to be able to compare precisely the antimicrobial efficacy of the pyrazine derivatives. The optical density of the bacterial culture was measured spectro-photometrically. The suspension was diluted with MHB-medium. For the test, each sample was inoculated with $5*10^5$ CFU/ml, which corresponds to an OD_{600} of 0.001 for *E. coli* and to an OD_{600} of 0.0035 for *S. aureus*. Sterility controls as well as negative controls were prepared, containing MHB-medium and the bacterial inoculum. The final volume of the samples was 1 ml. All samples were prepared in triplicates. The samples were well mixed by vortexing, horizontally positioned in a rack and incubated at 37°C for 18 h with agitation (110 rpm). To verify the cell number of the inoculum, additional controls were prepared and dilutions of them were spread on LB agar plates. The plates were incubated overnight at 30°C.

The MIC was read after the incubation as the lowest concentration of the pyrazine agent preventing visible growth. To ensure the results aliquots were plated in duplicates on LB agar plates. For the determination of the MBCs, aliquots of the samples treated with a higher concentration than the MIC, were spread in duplicates on LB agar plates. For the negative controls serial dilutions were prepared in PBS-Buffer and plated. The plates were incubated for 24 h at 30°C. The lowest concentration of the pyrazine derivate, which showed no growth on the plates, was considered as the MBC. The experiment was repeated three times. The single steps of the procedure are demonstrated in Figure 2.

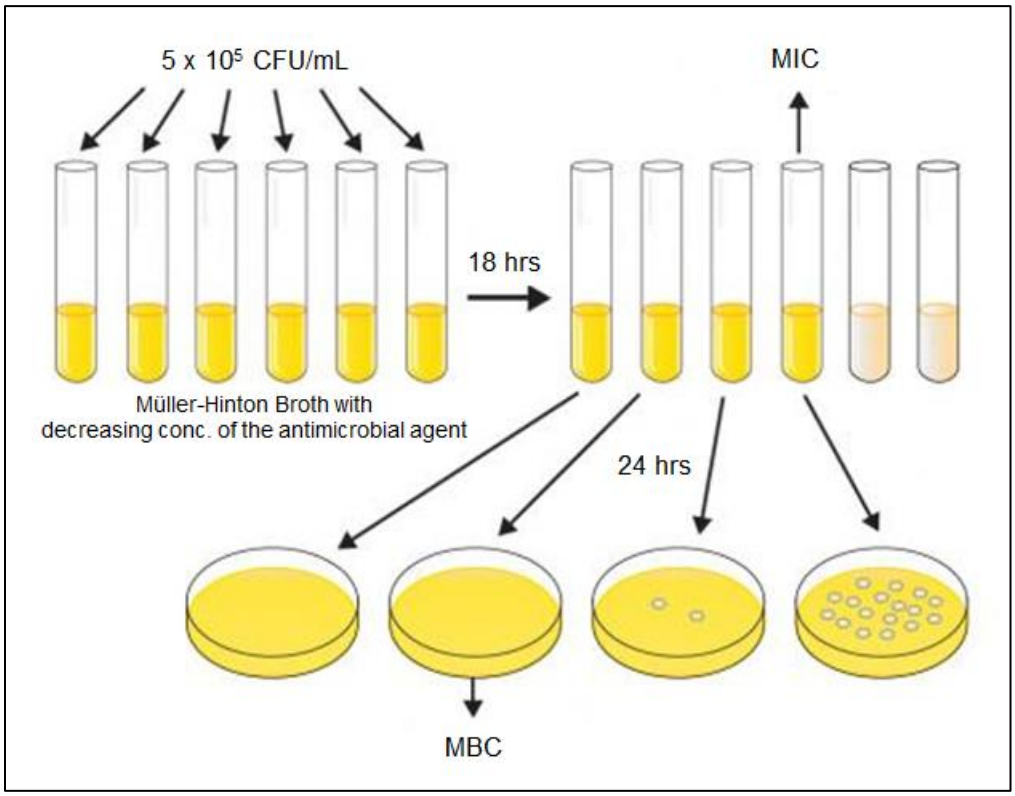


Figure 2: Antimicrobial susceptibility testing by determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) [92; modified].

2.2.6 5 min bactericidal testing with IBDM

The objective was to verify the antibacterial efficacy of the pyrazine derivate IBDM, which can be achieved in a contact time of 5 minutes. Therefore, overnight cultures of *E. coli*, *P. aeruginosa* and *S. aureus* were prepared. Flasks filled with 20 ml LB medium were inoculated with the bacteria and incubated overnight in the shaker at 30°C and 110 rpm.

On the next day, the antibacterial assay was performed. Firstly, the optical density of the cultures was measured with a photometer. The bacterial suspensions were adjusted with PBS-Buffer to defined cell concentrations of $10^4 – 10^6$ CFU/ml in a total volume of 1 ml. To reach a cell number of 10^6 CFU/ml the *E. coli* culture was adjusted to an OD_{600} of 0.002, the suspension of *S. aureus* to an OD_{600} of 0.06 and the *P. aeruginosa* culture to an OD_{600} of 0.01. For the treatment of *S. aureus* NaCl (0.85%) was used instead of PBS-Buffer. The cell suspensions were exposed to different amounts of IBDM (0.3, 0.6, 0.9, 1.2, 1.8, 2.4 and 3.0%). Samples without the addition of IBDM were negative controls. All samples were

prepared in 1.5 ml tubes in triplicates. The samples were well mixed by vortexing and incubated at room temperature for 5 min with agitation (90 rpm). After incubation, aliquots of each sample were spread onto LB agar plates to determine the number of viable cells. For the controls, dilution series with PBS-Buffer or NaCl were plated. All samples were plated out in duplicates. The experiment was repeated two times.

2.3 Antifungal activity

2.3.1 Fungal strains

The following fungal strains were used in this study: *Candida albicans* H5 (*C. albicans*), *Saccharomyces cerevisiae* B-72030 (*S. cerevisiae*) and a *Penicillium species* (*Penicillium sp.*). The strains were obtained from the strain collection of the Institute of Environmental Biotechnology (Graz University of Technology).

2.3.2 Determination of MIC and MFC

The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) with slight modifications [93]. The MIC is defined as the lowest concentration of an antifungal agent that inhibits fungal growth under defined conditions [93]. The MFC is defined as the lowest concentration of the agent required for a 99% killing of fungal cells after a distinct exposure time and subculture to antibiotic-free medium. Both values are used to determine the susceptibility or resistance of fungal strains to antifungal agents and to evaluate the efficiency of the substances [94].

The fungus *C. albicans* was used for the test. The *in vitro* antifungal susceptibility testing was performed for the pyrazine derivatives IBDM, IPM, IP, IB and IBM. A synthetic medium, RPMI-1640 with glutamine and supplemented with glucose (2%), was used, which is the standard medium for the MIC determination [93]. The

EUCAST method was slightly modified. Normally, the samples would be prepared in 96-well microdilution plates [93]. Due to the fact that the pyrazine derivatives are volatile compounds, a more closed system was needed. Therefore, 1.5 ml tubes were used for the sample preparation. Moreover, a two-fold dilution series of the antifungal agent is usually prepared (e.g. 10 mg/ml to 5 mg/ml to 2.5 mg/ml etc.) [93]. In this study the concentration was lowered in steps of 1 mg/ml to be able to compare precisely the antimicrobial efficacy of the pyrazine derivatives. A further modification of the EUCAST methodology was the use of an overnight culture of *C. albicans*.

Each Eppendorf tube contained 500 µl of the 2x RPMI medium and the double amount of the final pyrazine concentration (1 and 20 mg/ml). The optical density of the fungal culture was measured with a photometer. The suspension was diluted with sterile distilled water to an OD_{600} of 0.09, which corresponds approximately to $5*10^5$ CFU/ml. 500 µl of the inoculum suspension were added to each sample, whereby the final inoculum concentration was diluted to $2.5*10^5$ CFU/ml per sample. Sterility controls contained drug-free RPMI medium and sterile distilled water. For the negative controls the drug-free medium was mixed with the inoculum suspension. All samples were prepared in duplicates at least. The samples were well mixed by vortexing and incubated at 37°C for 24 h without agitation. To verify the cell number of the inoculum, additional controls were prepared and dilutions of them were spread on PDA plates. The plates were incubated at 37°C for 24 h. The MIC was read after the incubation spectro-photometrically (at 530 nm). The MIC is defined as the lowest concentration of the pyrazine agent preventing visible growth, which means that turbidity due to fungal growth should not be detected. To ensure the results, aliquots were spread in duplicates on PDA plates. For the determination of MFCs, aliquots of the samples treated with a higher concentration than the MIC, were spread in duplicates on PDA plates. For the negative controls serial dilutions were prepared in PBS-Buffer and plated. The plates were incubated for 24 h at 37°C and examined after the incubation. To ensure the results, the plates were incubated further 24 h at 37°C. The lowest concentration of the pyrazine derivate, which showed no growth on the plates, was considered as the MFC. The experiment was repeated two times.

2.3.3 5 min fungicidal testing with IBDM

The goal was to verify the antifungal efficacy of the pyrazine derivate IBDM, which could be achieved in a contact time of 5 minutes. Therefore, overnight cultures of *S. cerevisiae* and *C. albicans* were prepared. A flask filled with 20 ml yeast-peptone-dextrose medium (YPD) were inoculated with *S. cerevisiae*. For *C. albicans* potato dextrose medium (PDA) was used. The flasks were incubated overnight in the shaker at 30°C and 110 rpm. On the next day, the antifungal assay was performed. Firstly, the optical density of the cultures was measured with a photometer. The yeast suspensions were adjusted with PBS-Buffer to defined cell concentrations of 10^4 – 10^6 CFU/ml in a total volume of 1 ml. To reach a cell number of 10^6 CFU/ml the *S. cerevisiae* culture was adjusted to an OD_{600} of 0.5 and the broth of *C. albicans* to an OD_{600} of 0.9. The cell suspensions were treated with different amounts of IBDM (0.3, 0.6, 0.9, 1.2, 1.8, 2.4 and 3.0%). Samples without the addition of IBDM were negative controls. All samples were prepared in 1.5 ml tubes in triplicates. The samples were well mixed by vortexing and incubated at room temperature for 5 min with agitation (90 rpm). After incubation, aliquots of each sample were spread onto agar plates to determine the number of viable cells. For *S. cerevisiae* YPD agar plates were used and the samples with *C. albicans* were spread on PDA plates. For the controls, dilution series with PBS-Buffer were plated. All samples were plated out in duplicates. The experiment was repeated two times.

2.3.4 Antifungal assay – *Penicillium sp.* spores

The goal of this experiment was to verify the antifungal efficacy of the pyrazine derivate IBDM against spores of a *Penicillium species*. An agar plate with sporulating *Penicillium sp.* was used and a tube with 1 ml PBS-Buffer was prepared. A sterile toothpick was dipped into the plate, so the fungal spores got attached to it, and then the toothpick was dipped into the tube. This procedure was done several times using always a new toothpick. After vortexing the suspension, 10 µl were added to a Thoma-chamber to calculate the amount of spores in the sample. Three 4 x 4 squares were counted out. At average 10^4 spores were in 1 µl of the suspension. In the next step, the spores were treated with 3 and 6 µl of

IBDM. Detailed instructions can be found in Table 4. PCR-tubes were used as reaction vessels. Samples without the addition of the pyrazine compound were negative controls (B1 – B4). C1 – C3 were sterility controls. The samples were incubated at room temperature for 15 min. After incubation, each sample was transferred onto the middle of a DG18 agar plate. For the distribution of the sample, the plate was tilted back and forth. The incubation of the agar plates was carried out at room temperature for 96 h. The fungal growth was observed and photographically documented after 48, 72 and 96 h.

Table 4: Treatment conditions for the antifungal assay with *Penicillium sp.* spores. Samples A1 – A8 were treated with IBDM; samples B1 – B4 were negative controls; samples C1 – C3 were sterility controls.

Sample	Fungal suspension (10^4 spores/µl) [µl]	IBDM [µl]	NaCl [µl]
A1 – A4	1	3	6
A5 – A8	1	6	3
B1 – B4	1	-	9
C1 – C3	-	-	10

2.3.5 Growth kinetics of *S. cerevisiae* in the presence of IBDM

In this experiment the influence of IBDM on the growth behaviour of the yeast *S. cerevisiae* was examined. In the first step a pre-culture was started. Therefore, an overnight culture of *S. cerevisiae* was prepared in 20 ml YPD medium. The flask was incubated in the shaker at 30°C and 110 rpm (~ 14 h). On the next day, the optical density of the pre-culture was measured at 600 nm with a photometer. The amount of yeast culture, which was required to start a new 35 ml YPD culture at an OD_{600} of 0.1 (~ 10^5 CFU/ml), was calculated. Six main cultures were prepared in 50 ml Sarstedt falcons. Flacon tubes were used instead of flasks to avoid the outgassing of the volatile pyrazine compound. OD_{600} of each main culture was determined in duplicates at t_0. Moreover, a sample of 100 µl was taken from each falcon. A dilution series was prepared with PBS-Buffer and aliquots of each sample were spread on YPD agar plates to determine the cell concentration at t_0. The samples were plated out in duplicates. In the next step, 105 µl of IBDM were added to culture 3 and 4 to reach a final concentration of 0.3% and 210 µl to culture 5 and 6 to reach a concentration of 0.6%. Culture 1 and 2 were negative

controls. Incubation of the falcons was carried out in the shaker at 30°C and 110 rpm for 12 h. Every hour OD_{600} of each culture was measured in duplicates. Additionally, a sample of 100 µl was taken from each falcon and serial dilutions were spread on YPD plates.

2.4 Mode of action

2.4.1 LIVE/DEAD *Bac*Light Bacterial Viability Kit

The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7012, Molecular Probes, Life Technologies) was used to examine if IBDM is able to reduce the membrane integrity. This two-color fluorescence assay contained the SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain propidium iodide (PI). Usually this assay is used for bacterial enumeration and viability testing. However, the test kit can also serve as rapid method for identifying membrane-active compounds [95 - 97].

The kit consists of a 3.34 mM SYTO 9 solution in DMSO and a 20 mM PI solution in DMSO. An overnight culture of *E. coli* was prepared in a flask filled with 20 ml LB medium. The culture was incubated overnight in the shaker at 30°C and 110 rpm to late log phase. On the next day, 2 x 1 ml bacterial culture were centrifuged at 10,000 x g and RT for 15 min. The supernatant was discarded und the pellet resuspended in 1 ml of 0.85% w/v NaCl. 15 ml of NaCl were mixed with 2 ml of cell suspension. The optical density of the suspension was measured photometrically. For the treatment, the OD_{600} of the cell suspension was adjusted to 0.06 with NaCl. The bacterial cells were treated with 10 mM (1.92 µl), 20 mM (3.84 µl) and 40 mM (7.68 µl) IBDM in a total volume of 1 ml. For the positive control the cells were treated with 70% v/v isopropanol and for the negative control NaCl was used. All samples were prepared in 1.5 ml tubes in triplicates. The samples were incubated at 30°C and 700 rpm for 15 min. After incubation the samples were centrifuged at 10,000 x g and RT for 15 min. The supernatant was discarded and the pellet was resuspended in 125 µl of NaCl. Next, 6 µl SYTO 9 dye (3.34 mM) and 6 µl of Propidium iodide (20 mM) were mixed. A 2x stain solution was prepared by transferring the 12 µl of stain to 2 ml sterilized dH_2O.

125 µl of stain solution were added to each sample and mixed well. The samples were incubated at room temperature in the dark for 15 min.

Each sample (250 µl) was transferred to a black 96-well microplate from Greiner bio-one (Flat-bottom, Chimney well, TC). A microtiter plate reader (Infinite® M200 from Tecan) was used for the fluorescence intensity measurement. The excitation/emission wavelengths were at 485/530 nm for the green fluorescence and at 485/630 nm for the red fluorescence (Gain 100). Data were analysed by calculating the green/red fluorescence intensity ratio [95, 96]. The experiment was carried out two times.

$$Ratio_{G/R} = \frac{\text{Fluorescence intensity}_{\text{Green}}}{\text{Fluorescence intensity}_{\text{Red}}}$$

2.4.2 *Bac*Light Bacterial Membrane Potential Kit

In this experiment it was tested if IBDM and IBM have an effect on the membrane potential of bacterial cells. The *Bac*Light™ Bacterial Membrane Potential Kit (B34950, Molecular Probes, Life Technologies) was used. This fluorescence assay included the carbocyanine dye $DiOC_2(3)$ (3,3′-diethyloxa-carbocyanine Iodide) and the proton ionophore CCCP (carbonylcyanide 3-chlorophenyl-hydrazone) [98]. The kit consisted of a 3 mM $DiOC_2(3)$ solution in DMSO, 500 µM CCCP in DMSO and a Phosphate-buffered saline solution (PBS, 10 mM sodium phosphate, 145 mM sodium chloride, pH 7.4).

An overnight culture of the bacterial strain *S. aureus* was prepared in a flask filled with 20 ml LB medium. The flask was incubated overnight in the shaker at 30°C and 110 rpm. On the next day, 4 x 1 ml of bacterial culture were centrifuged at 10,000 x g for 15 min. The supernatant was discarded und the pellet was resuspended in 0.5 ml PBS-Buffer. The samples were pooled and the optical density of the suspension was measured photometrically. For the treatment, the OD_{600} of the cell suspension was adjusted to 3 with PBS-Buffer (1.5 ml total volume). 30 µl of the stain $DiOC_2(3)$ were added to reach a final concentration of 60 µM. The sample was incubated for 30 min in the dark. After the incubation the cells were washed by centrifugation to remove the extracellular dye (10,000 x g for

15 min). The pellet was resuspended in 1.5 ml PBS-Buffer. The samples were prepared in 1.5 ml tubes in triplicates. Each sample contained 50 µl of the stained cell suspension and 210 µl PBS-Buffer. The bacterial cells were treated with 5 – 20 mM of the pyrazine derivatives IBDM and IBM, respectively. For the positive control 5.2 µl of CCCP were added to reach a concentration of 10 µM. For the negative control PBS-Buffer was added. The samples were incubated at 37°C and 700 rpm for 15 min. After incubation, each sample was diluted additionally with 30 µl of PBS-Buffer and mixed well.

250 µl of each sample were transferred to a well of a black 96-well microplate from Greiner bio-one. A microtiter plate reader was used for the fluorescence intensity measurement. The excitation/emission wavelengths were at 485/530 nm for the green fluorescence and at 485/630 nm for the red fluorescence (Gain 80). Data were analysed by calculating the red/green fluorescence intensity ratio. The experiment was carried out two times.

$$\text{Ratio}_{R/G} = \frac{\text{Fluorescence intensity}_{\text{Red}}}{\text{Fluorescence intensity}_{\text{Green}}}$$

2.4.3 β-Galactosidase assay – inner membrane permeabilization

This experiment was performed to verify if IBDM had a permeabilizing effect on the inner membrane of bacterial cells. Therefore, a β-galactosidase assay was carried out [99, 100].

An overnight culture of *E. coli* was prepared in a flask filled with 20 ml LB medium. Moreover, a solution of Isopropyl β-D-1-thiogalactopyranoside (IPTG, 40 mM) was made with sterile dH_2O. The solution was sterilized with a syringe filter (0.2 µm). 500 µl of the IPTG solution were added to the culture flask to reach a final concentration of 1 mM. Thereby, the *E. coli* cells were induced to overexpress the cytoplasmic enzyme β-galactosidase. The flask was incubated overnight in the shaker at 37°C and 110 rpm. On the next day, the culture was diluted 200-fold into fresh LB medium supplemented with 1 mM IPTG [100]. The culture was grown to a final OD_{600} of 1. The cells were washed by centrifugation (10,000 x g for 15 min) and resuspended in PBS-Buffer (half of the culture volume). The optical density

was measured. OD_{600} was adjusted with PBS-Buffer to 1.2 in a volume of 10 ml (~10^9 CFU/ml). The samples were prepared in 1.5 ml tubes in triplicates. Each sample contained 200 µl of cell suspension [99]. The bacterial cells were treated with 100 mM (5 µl), 50 mM (2.5 µl), 20 mM (1 µl) and 10 mM (0.5 µl) IBDM. PBS-Buffer was added to reach a final volume of 250 µl. For the positive control 50 µl of 70% v/v isopropanol were added. For the negative control 50 µl of PBS-Buffer were used. For the detection of the inner membrane disruption, the leakage of β-galactosidase was quantified by using the substrate o-nitrophenyl-D-galacto-pyranoside (ONPG). To start the reaction, 10 µl of ONPG (30 mM) were added to each sample [99]. The incubation of the samples was carried out at 37°C and 500 rpm for 1 h. After the treatment 10 µl of PBS-Buffer were added to each sample. The bacterial cells were removed by centrifugation (10,000 x g for 15 min). 250 µl of supernatant were transferred to a 96-well microtiter plate. ONPG is converted by β-galactosidase to o-nitrophenol, which led to a yellow colour production [100]. A microplate reader was used for absorbance measurement at 415 nm.

2.4.4 Interaction of pyrazine derivatives with proteins

The aim of this experiment is to test if alkylated pyrazine derivatives, especially IBDM, possibly interact with cellular *E. coli* proteins and BSA, respectively.

Interaction of IBDM with intracellular *E. coli* proteins

Firstly, the *E. coli* cells were disrupted via ultrasonic. Therefore, an overnight culture of *E. coli* was prepared in 20 ml LB medium. The flask was incubated overnight in the shaker at 30°C and 110 rpm. On the next day, the cell suspensions were divided into four 50 ml Greiner tubes. Previously, the tubes were weighed. The samples were centrifuged at 4,500 rpm and 4°C for 15 min. The supernatant was discarded and the tubes were weighed to calculate the amount of pellet. The tubes were frozen at -20°C for 30 min and then thawed on ice. An ultrasonic instrument (Branson Sonifier Sound Enclosure) was used. According to the manual, 2 g wet weight of *E. coli* should be dissolved in 10 ml Buffer. 1.8 g pellet were dissolved in 9 ml Tris-HCl Buffer (50 mM, pH 7.0). The

suspension was transferred into a beaker, which was positioned in a vessel filled with water and ice. The probe of the instrument was immersed into the suspension and the ultrasonic programme was started (ID #1). The suspension was divided into 1.5 ml tubes and centrifuged at 13,500 rpm and 4°C for 30 min. The supernatant, which contained the cellular proteins, was transferred into new tubes.

In the subsequent step, the interaction assay was performed with IBDM. Therefore, 50 µl of protein solution, obtained from the ultrasonic experiment, were mixed with 2 µl of IBDM. The samples were incubated for 5 min at 37°C. For the negative controls 50 µl of protein solution were mixed with 2 µl of Tris-HCl Buffer. Moreover, 50 µl of Tris-HCl Buffer were mixed with 2 µl IBDM. After the incubation, possible visible changes in the appearance of the reaction mixtures were observed.

In the next experiment the protein solution was separated by size to examine if IBDM was interacting with a certain protein and enzyme class, respectively. Therefore, Vivaspin® 500 Centrifugal Concentrators (GE Healthcare Life Sciences) were used. In the first step, 500 µl were filled in a concentrator for proteins ≥100 kDa. The sample was centrifuged with 15,000 rfc, at 4°C for 5 min. 50 µl Tris-HCl Buffer were filled into the membrane chamber and mixed with the concentrate by pipetting up and down. The supernatant was transferred to a new tube, which was kept on ice. The filtrate was added into a Vivaspin tube for proteins ≥50 kDa. The sample was centrifuged with 15,000 rfc, at 4°C for 5 min. The concentrate was treated the same way as described before. The filtrate was added to a Vivaspin tube for proteins ≥10 kDa. The sample was centrifuged with 15,000 rfc, at 4°C for 5 min. Again the concentrate was mixed with Tris-HCl Buffer and transferred to a new tube. After this procedure four protein fractions were prepared (see Table 5).

Table 5: Protein fractions separated by their size.

Fraction	Size
1	<10 kD
2	≥10 kD and <50 kD
3	≥50 kD and <100 kD
4	≥100 kD

Subsequently, the protein concentrations in these samples were determined by using a spectrophotometer (Thermo Scientific NanoDrop 2000c spectro-photometer). Every fraction was measured in triplicates. Reaction mixtures were prepared with a total protein concentration of 0.25 mg/ml in 40 µl. Tris-HCl Buffer was used and the samples were prepared in duplicates. The reaction was started by adding 1 µl of IBDM to each sample. Controls were also prepared. The samples were incubated at 37°C for 5 min. After the incubation, possible visible changes in the appearance of the reaction mixtures were observed.

Interaction of pyrazine derivatives with BSA

The objective was to verify if selected pyrazine derivatives undergo an interaction with the protein bovine serum albumin (BSA) when they get in contact with it. The derivatives IBDM, IBM, IB, IPM and IP were used in this experiment. A BSA (Sigma, 99%, for gel electrophorese) solution was prepared with sterile distilled water (10 mg/ml). Per sample 50 µl of the BSA-solution were mixed with 2 µl of a derivate. The control samples only contained the BSA-solution. All samples were prepared in 1.5 ml tubes in triplicates. They were vortexed and incubated at 37°C and 500 rpm for 5 – 25 min. Meanwhile, possible visible changes of the appearance of the reaction mixtures were followed and documented. If necessary, the amount of derivate and/or the incubation time were increased.

Influence of BSA on the antibacterial efficacy of IBDM

The goal of this experiment was to test if the presence of BSA had an influence on the efficacy of IBDM. An overnight culture of *E. coli* was prepared in a flask filled with 20 ml LB medium. The flask was incubated overnight in the shaker at 30°C and 110 rpm. Moreover, a solution of BSA was prepared with PBS-Buffer (1.5 mM). The solution was sterilized with a syringe filter (0.2 µm).

On the next day, the BSA-influence assay was performed. Therefore, the optical density of the culture was measured with a photometer. Using the OD_{600} value, the bacterial suspension was adjusted with PBS-Buffer to a defined cell concentration (10^4 CFU/ml) in a total volume of 1 ml. The *E. coli* cells were treated with IBDM, with or without the addition of BSA. The test was carried out with an IBDM concentration of 0.3% and a BSA concentration of 0.25 – 1 mM. Samples without the addition of IBDM were negative controls. Moreover, negative controls with the

addition of 0.25 – 1 mM BSA were prepared, to exclude an influence of BSA on the viability of the bacterial cells. All samples were prepared in 1.5 ml tubes in triplicates. The samples were incubated at 30°C and 110 rpm for 2 h. After incubation, the samples were spread on LB agar plates to determine the cell concentration. For the controls, serial dilutions with PBS-Buffer were prepared and plated. All samples were spread out in duplicates.

2.5 *E. coli* Keio Knockout Collection

2.5.1 Single-gene knockout mutants of *E. coli*

The Dharmacon *E. coli* Keio Knockout Collection is a library of mutants with single-gene deletions of non-essential genes. 3985 genes were knocked out, whereby two independent mutants were created for each deleted gene, generating a total set of 7980 knockout strains [101]. The mutants were constructed from the background strain *E. coli* K-12 BW25113 by Baba *et al.* [102]. Each coding region was replaced by a kanamycin resistance cassette, using the method of Datsenko and Wanner [103]. First of all, the whole knock out library was replicated by following the instructions of the manual [101]. The source microtiter plates were thawed and in the meantime, sterile 96-well microplates were filled per well with 160 µl LB broth (Lennox) supplemented with 8% glycerol and 25 µg/ml kanamycin. A microplate replicator was carefully put in the source plate and the bacterial suspensions were slightly stirred. The replicator was removed from the plate and immediately placed in the target plate. Subsequently, the replicator was incubated in an ethanol bath (98%) and flamed with a Bunsen burner. After the replicator was cooled down, it was ready for the copy of the next plate. The original plates were frozen at -80°C and the target plates were incubated at 37°C without shaking for 18 – 19 h. The copies of 90 microtiter plates were stored at -80°C [101]. In the next step, 233 different mutants were selected to carry out further experiments (see Table 6). The mutants were picked from the different plates and pooled in three 96-well microplates. The wells of the plates were filled with 160 µl of 2x LB medium (Lennox) supplemented with 8% glycerol and 25 µg/ml kanamycin. The plates were maintained at -80°C.

Table 6: List of 233 mutants from the *E. coli* Keio Knockout Collection, which were selected for further experiments. The deleted gene of each mutant is listed.

1	aceE	40	eutH	79	metQ	118	rffA	157	ybjG	196	ygdD
2	acrA	41	eutL	80	mraW	119	rihA	158	ybjJ	197	ygeG
3	acrB	42	exbD	81	mrcB	120	rimK	159	ybjO	198	ygiH
4	agaD	43	feoA	82	narQ	121	rph	160	ybjP	199	yhbW
5	allB	44	fepB	83	nlpC	122	rplA	161	ycbR	200	yhdP
6	alsB	45	fepC	84	nudB	123	rusA	162	ycdZ	201	yhdV
7	argO	46	fieF	85	nusB	124	ruvC	163	yceK	202	yheL
8	aroL	47	fkpB	86	ompF	125	sapC	164	ychE	203	yheN
9	asr	48	flgF	87	ompG	126	secG	165	ychM	204	yhiN
10	astE	49	focA	88	ompT	127	seqA	166	yciS	205	yhiS
11	bglG	50	frlA	89	ompX	128	sgcC	167	yciT	206	yhjA
12	bioC	51	fruB	90	pal	129	smpA	168	ycjG	207	yiaA
13	bioF	52	glpT	91	pdhR	130	ssuA	169	ycjP	208	yiaD
14	btuD	53	gltI	92	pdxA	131	stpA	170	ycjU	209	yibA
15	chbA	54	glxR	93	phnH	132	surA	171	ycjV	210	yibK
16	cobU	55	gmhB	94	phoU	133	tatA	172	ydaC	211	yicC
17	cpdA	56	gshA	95	plsX	134	tatB	173	ydcS	212	yidE
18	cpxA	57	hlpA	96	pphA	135	tatC	174	ydcX	213	yidQ
19	csgD	58	hlyE	97	ppiB	136	tdcB	175	ydcY	214	yieF
20	csgG	59	hscA	98	ppiD	137	tolC	176	ydcZ	215	yieG
21	cvpA	60	hscB	99	proA	138	tolQ	177	yddJ	216	yihN
22	cyoA	61	hyaC	100	proV	139	tolR	178	ydgD	217	yjaA
23	dacA	62	hyaF	101	proW	140	tonB	179	ydiF	218	yjcD
24	dacC	63	ibpB	102	pspC	141	trxA	180	yebB	219	yjeH
25	dapF	64	ihfA	103	pstA	142	trxB	181	yebZ	220	yjeO
26	dcuA	65	ilvM	104	pstC	143	umuD	182	yedE	221	yjhG
27	dcuC	66	intG	105	pstS	144	vacJ	183	yedF	222	ykgL
28	dedD	67	kdpB	106	pykF	145	visC	184	yedK	223	ylcG
29	degP	68	leuB	107	recA	146	wbbL	185	yeeV	224	ymcE
30	deoR	69	ligT	108	recB	147	wza	186	yegQ	225	ymfA
31	dnaJ	70	lon	109	rfaC	148	xapR	187	yeiL	226	yncA
32	dnaK	71	lpxL	110	rfaD	149	yahB	188	yfbT	227	yneE
33	ecnB	72	lpxM	111	rfaE	150	yahC	189	yfdG	228	ynhG
34	emrA	73	luxS	112	rfaG	151	yaiL	190	yfgO	229	yoaF
35	emrB	74	mdtB	113	rfaH	152	ybcL	191	yfhR	230	yodD
36	emtA	75	mdtJ	114	rfaL	153	ybcO	192	yfjR	231	ypjD
37	entC	76	mdtL	115	rfaP	154	ybfB	193	ygaP	232	yraI
38	envC	77	menD	116	rfaQ	155	ybgF	194	ygcN	233	yrdB
39	envZ	78	metA	117	rfaY	156	ybhR	195	ygcO		

2.5.2 Susceptibility test with selected *E. coli* mutants –potential increased sensitivity or resistance to IBDM

The objective of this experiment was to treat the wildtype *E. coli*-K12 and a selected group of mutants with the pyrazine derivate IBDM. Thereby, it was tested whether these clones were possibly more resistant or sensitive to IBDM, which could give a better understanding of the mode of action of alkylated pyrazine derivatives. Due to limited working time a group of 42 mutants was selected for the susceptibility testing. In regard to the function of the deleted gene, these mutants can be assigned to six different categories (see Table 7) [104]. Half of the selected mutants belong to the sector transport, efflux, cell wall and cell membrane synthesis. The major attention was paid to this category, due to the fact that data obtained from previous experiments lead to the hypothesis that the cellular target of the pyrazine derivatives can be narrowed to the membrane.

Table 7: Selected mutants of the *E. coli* Keio Knockout Collection. Due to the cellular functions of the deleted genes, the mutants can be assigned to six different categories [104].

Category	Description	Mutants (deleted genes)
1	DNA replication, recombination and repair	recA, recB, ruvC
2	Transport, efflux, cell wall and cell membrane synthesis	acrB, argO, cpxA, csgG, envC, envZ, mdtJ, mrcB, ompF, ompT, pal, rfaC, rfaD, rfaE, rfaG, smpA, surA, ybfB, ybjO, ybjP, yheL
3	Protein synthesis and RNA processing	rimK, rplA, ydgD, yheN, yibK
4	Central metabolic reactions	aceE, dapF, gmhB, nudB, pykF
5	Regulation	ihfA, pdhR, rfaH, seqA, xapR
6	Prophage-carried genes and cell adhesion	yfjR, ylcG

Flasks filled with 20 ml of LB medium (Lennox) containing 25 µg/ml kanamycin were inoculated with 5 µl of the selected bacterial cell suspension. The wild-type strain *E. coli* K-12 was used without the addition of kanamycin. Moreover, sterility controls were prepared. The flasks were incubated overnight in the shaker at 30°C and 110 rpm. On the next day the sample preparation was carried out. Each sample contained Mueller-Hinton Broth (MHB) with a kanamycin concentration of 25 µg/ml (with the exception of the wild-type strain) and IBDM (2, 3 or 4 mg/ml). The optical density of the bacterial culture was measured. The suspension was diluted with MHB. For the test, each sample was inoculated with $5 – 9 \times 10^5$ CFU/ml, which corresponded to an OD_{600} of 0.001 for *E. coli*. Sterility controls as well as negative controls were prepared containing MHB and the

bacterial inoculum. The final volume of the samples was 1 ml. All samples were prepared in 1.5 ml tubes in 4 replicates. The samples were well mixed by vortexing, horizontally positioned in a rack and incubated at 37°C for 24 h with agitation (110 rpm). To verify the cell number of the inoculum, additional controls were prepared and the dilutions of them were spread on LB agar plates. The plates were incubated overnight at 30°C. On the next day, aliquots of the samples were spread on LB agar plates. For samples treated with 2 mg/ml and for the negative controls serial dilutions were prepared in PBS-Buffer and plated. The plates were incubated overnight at 30°C. On the next day, the colonies on the plates were counted to determine the cell concentrations after the treatment. Thereby the results of the mutants were compared to the results of the wild-type strain. Moreover, mutants, which showed an enhanced or decreased tolerance to IBDM compared to the wild-type strain, were tested a second time.

2.6 Stability Tests – Effect of abiotic factors on alkylpyrazines

2.6.1 Influence of temperature

The objective was to examine the stability of IBDM in relation to the influence of temperature. Therefore, 2 ml PBS-Buffer were mixed with 6 µl of IBDM (0.3%) by vortexing. The samples were prepared in duplicates and stored at -20°C, 2°C, 22.5°C (RT), 37°C or 60°C for 24 h. The bacterial strain *E. coli* was used and overnight culture was prepared in a flask filled with 20 ml LB medium. The flask was incubated overnight in the shaker at 30°C and 110 rpm. On the next day, the IBDM concentration in the temperature treated samples was measured with a spectrophotometer at A_{280}. Afterwards, the bacterial cell suspension was treated with the different IBDM samples. The reaction mixtures contained 10 µl of culture broth (10^{-3} and 10^{-4} dilution, respectively) and 500 µl of the IBDM solution (0.3%). Samples without the addition of pyrazine compound were negative controls. The samples were prepared in 1.5 ml tubes in duplicates. Incubation was carried out in the shaker at 30°C and 110 rpm for 6 h. After the treatment, aliquots of each sample undiluted and diluted in PBS-Buffer were spread on LB agar plates to determine the cell number. The samples were plated out in duplicates.

2.6.2 Influence of light

The goal was to verify the stability of IBDM and IPM in relation to light exposure. Therefore, 20 µl of each derivate were added to HPLC-vials and sealed with caps. Two vials per compound were prepared. One vial of each pyrazine derivate was placed under an UV-lamp for 24 h. The remaining vials were put under an average light source (light bulb) for four days. The illumination intensity of this light source, measured by a luxmeter, was about 965 lux (4k).

The bacterial strain *E. coli* was used and overnight culture was prepared in a flask filled with 20 ml LB medium. The flask was incubated overnight at 30°C and 110 rpm. On the next day, the antibacterial assay was performed. Using the OD_{600} value, the bacterial suspension was adjusted with PBS-Buffer to defined cell concentrations (10^4 – 10^6 CFU/ml) in a total volume of 1 ml. The samples were treated with 0.3% IBDM or IPM. Therefore, the two light-exposed pyrazine samples and the control pyrazine, which was not irradiated, were used. Each sample was prepared in duplicates and well mixed by vortexing. Samples without the addition of pyrazine were negative controls. Incubation of the samples was carried out in the shaker at 30°C and 110 rpm for 2 h. After the treatment, aliquots of each sample were spread on LB agar plates for the enumeration of viable cells. For the negative controls and the samples treated with IPM serial dilutions were prepared in PBS-Buffer and spread on LB agar plates. On the next day, the colonies on the plates were counted to compare the antibacterial efficacy of the light-exposed pyrazine samples to the activity of the control pyrazines.

2.7 Growth media and solutions

Sources of supply

If not specifically mentioned, all chemicals, culture media and hardware were obtained from the following companies: Eppendorf (Hamburg, Germany), Fluka (Buchs, Switzerland), Greiner Bio-one (Kremsmünster, Austria), Carl Roth (Karlsruhe, Germany), Sarstedt (Nümbrecht, Germany), Sigma-Aldrich (St. Louis, USA), biowest (Nuaillé, France), Fermentas (St.Leon-Rot, Germany), Merck (Darmstadt, Germany) and Invitrogen (Lofer, Austria).

If not explicitly stated, all used media and solutions were autoclaved at 121°C for 15 min to ensure sterility.

For the preparation of nutritive agar plates, 15 g/L Agar-Agar were used.

Instant media such as LB medium (Luria/Miller; 10 g/L NaCl), LB medium (Lennox; 5 g/L NaCl) and Potato dextrose agar (PDA) were prepared according the manufacturer's instructions (Carl Roth, Germany).

Müller-Hinton-Broth (MHB)

Instant medium, for antibiotic susceptibility studies (MIC-determination); prepared according the manufacturer's instructions (Sigma-Aldrich, USA) – 23 g/L.

Yeast extract peptone dextrose (YPD) medium

Yeast extract ..... 10 g/L
Peptone from soya ..... 20 g/L
α-D (+) Glucose Monohydrate ..... 20 g/L

Synthetic medium RPMI-1640 with glutamine (2X)

MOPS ..... 69.06 g
RPMI-1640 ..... 20.80 g
α-D (+) Glucose Monohydrate ..... 36.00 g

The components were dissolved in 900 ml dH_2O. The pH was adjusted to 7.0 with 1 M NaOH. The bottle was filled with dH_2O to a final volume of 1 L and the medium was filter sterilized using a 0.22 µm filter. Storage at 4°C.

Dichloran-Glycerol-Agar (DG18-Agar)

Agar ..... 15.0 g

K_3PO_4 ..... 1.0 g

$MgSO_4$ ..... 0.5 g

Dichloran ..... 0.002 g

Chloramphenicol ..... 0.1 g

Peptone from soya ..... 5.0 g

α-D (+) Glucose Monohydrate ..... 10.0 g

Dissolved in 1 L dH_2O, heated and stirred

Addition of 175 ml glycerol

Phosphate-buffered saline (PBS) Buffer (1X)

NaCl ..... 8.00 g

KCl ..... 0.20 g

KH_2PO_4 ..... 0.24 g

$Na_2HPO_4*2H_2O$ ..... 1.44 g

pH adjustment with HCl to 7.4

Tris-HCl Buffer (50 mM)

Tris ..... 6.057 g/L

pH adjustment with HCl to 7.0

NaCl solution (0.85%)

NaCl ..... 8.5 g/L

Glycerol stock

40% v/v in dH_2O

3 Results

3.1 Antibacterial activity

3.1.1 Time-kill kinetics – IBDM

The goal of this experiment was to examine the antibacterial activity of the pyrazine derivate IBDM. Thereby, the relationship between the applied concentration of IBDM, the cell concentration and the incubation time of the antibacterial assay was studied in more details.

A defined cell concentration of 10^7 *E. coli* cells was exposed to 0.05 – 0.3% IBDM and incubated for 2 – 6 h. After the treatment, the samples were plated out for the enumeration of viable cells. The mean values of the cell concentrations were calculated. The obtained results are listed in Table 8. The negative controls stayed on a stable cell concentration of 10^7 CFU/ml. A solution of 0.3% IBDM was able to kill 7-log10-units of *E. coli* cells in 2 h, whereas a 0.2% solution needed 4 h for the same effect. A 0.05% IBDM solution reduced the cell concentration after 6 h of incubation to $3.1*10^6$ CFU/ml. In comparison to it, an IBDM concentration of 0.1% decreased the cell concentration a 1-log10-unit more. The time-kill curves are displayed in Figure 3.

The antibacterial efficacy of IBDM increased with increasing incubation time and increasing application volume of this derivate. However, the concentration of IBDM had a greater impact on the antibacterial effect. It was more efficient to use a higher amount of IBDM, than extending the incubation time.

Table 8: Results of the antibacterial assay performed with the pyrazine derivate IBDM. 10^7 *E. coli* cells were treated with a PBS-solution containing 0.05 – 0.3% IBDM; incubation for 2 – 6 h at 30°C. The cell concentrations in the samples after the treatment are listed (mean values).

	Incubation [h]		2	4	6
Cell concentration [CFU/ml]	Negative controls		$1.3*10^7$	$1.3*10^7$	$1.3*10^7$
	IBDM	0.05%	$1.1*10^7$	$7.5*10^6$	$3.1*10^6$
		0.1%	$7.4*10^6$	$3.2*10^6$	$3.7*10^5$
		0.2%	$3.9*10^5$	0	0
		0.3%	0	0	0

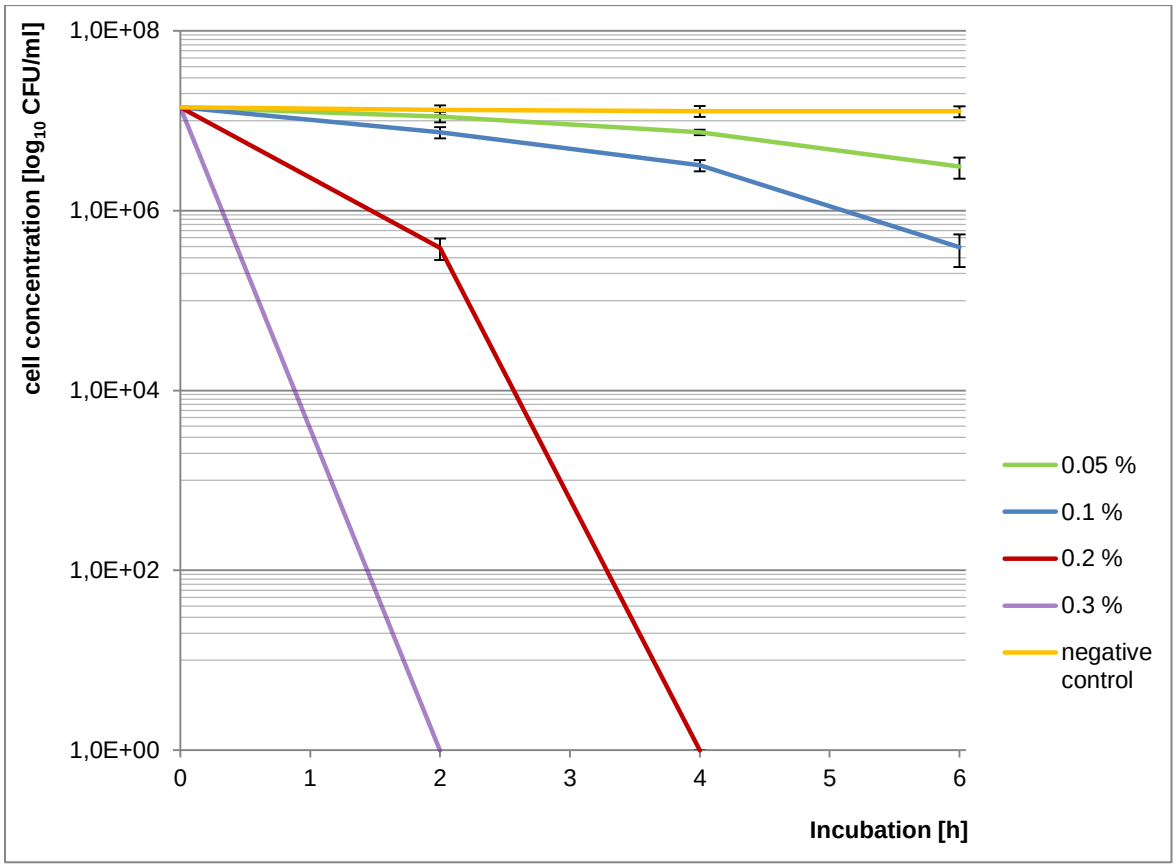


Figure 3: Results of the antibacterial assay performed with the pyrazine derivate IBDM. 10^7 *E. coli* cells were treated with a PBS-solution containing 0.05 – 0.3% IBDM for 2 – 6 h. The mean values of the cell concentrations and the standard deviations are shown. Detailed data can be obtained from Table 8. The x-axis is scaled logarithmically.

3.1.2 Antibacterial efficacy of IPM, IBM, IP and IB

The purpose of this experiment was to test if the pyrazine derivatives IBM, IPM, IB and IP exhibit antibacterial activity. The results should give information about the relation between structural composition of pyrazines and their bactericidal activity.

Defined cell concentrations of *E. coli* (10^5 - 10^7 CFU/ml) were treated with 0.3% of the pyrazine derivatives IBM, IPM, IB and IP. The incubation of 4 h was performed at 30°C. After the treatment, the samples were plated out to enumerate the number of viable cells (CFU/ml). In Table 9 the results are summarized. The mean values of the cell concentrations were calculated. In general it can be said that all of the pyrazine derivatives tested exhibit an antibacterial activity. The nutrient plates of the negative controls amounted to a stable cell number of 10^5 – 10^7 CFU/ml. All derivatives were able to kill 10^5 CFU/ml of *E. coli* cells during this treatment. The agar plates of these pyrazine treated samples did not show a

single colony. Moreover, 0.3% pyrazine-PBS solutions of IBM were lethal to 10^6 and 10^7 CFU/ml. The compound IP was able to reduce a cell concentration of 10^6 CFU/ml by more than half (54% died). Samples containing 10^7 *E. coli* were nearly decreased by half (46% died) due to IP. The derivate IPM reduced a cell number of 10^6 CFU/ml by 1-log10-unit (89% died) and 10^7 CFU/ml by 83%, respectively. IB decreased 10^6 CFU/ml by 3-log10-units and 10^7 CFU/ml by nearly 2-log10-units. Due to these data the following ranking regarding the antibacterial activity of the derivatives can be established: IBM, IB, IPM and IP. The time-kill data of the pyrazine compounds are graphically demonstrated in Figure 4.

Table 9: Results of the antibacterial assay performed with the pyrazine derivatives IBM, IPM, IB and IP. 10^5 - 10^7 *E. coli* cells were treated with a PBS-solution containing 0.3% pyrazine for 4 h. The cell concentrations in the samples after the treatment are listed (mean values).

Cell concentration [CFU/ml]				
Negative controls	IP	IPM	IB	IBM
$2.50*10^5$	0	0	0	0
$1.23*10^6$	$5.6*10^5$	$1.4*10^5$	$1.2*10^3$	0
$1.20*10^7$	$6.5*10^6$	$2.1*10^6$	$1.8*10^5$	0

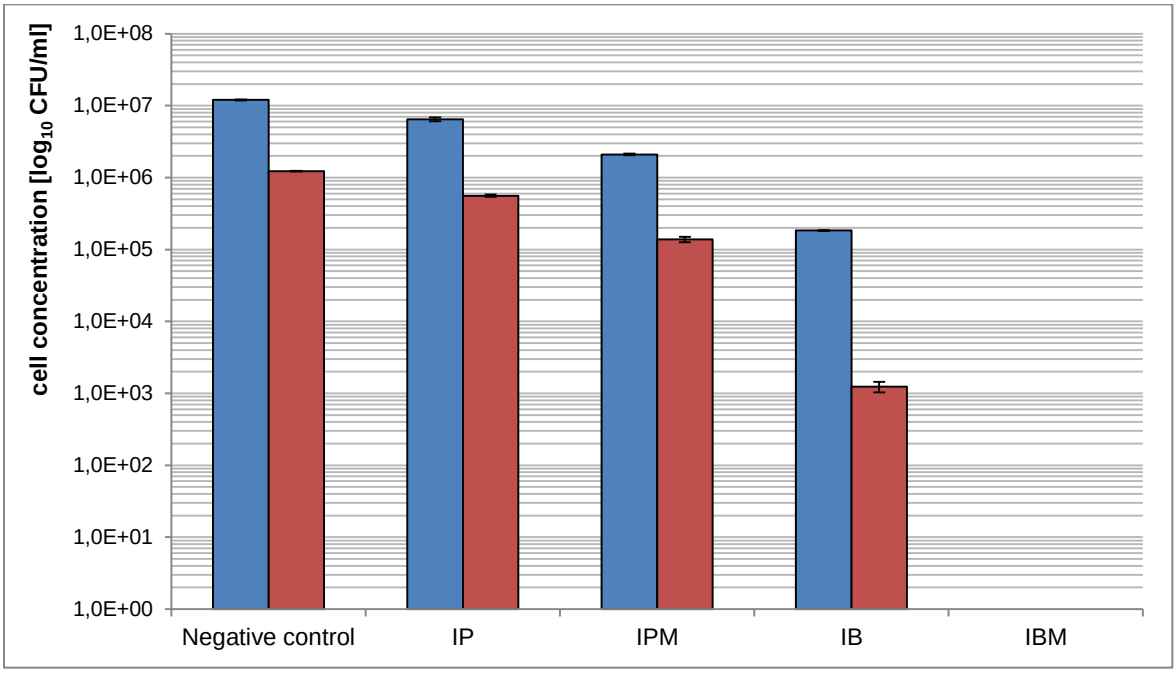


Figure 4: Results of the antibacterial assay performed with the pyrazine derivatives IBM, IPM, IB and IP. 10^6 and 10^7 *E. coli* cells were treated with a PBS-solution containing 0.3% pyrazine for 4 h. The mean values of the cell concentrations and the corresponding standard deviations are shown. Detailed data of the determined cell numbers can be obtained from Table 9. The x-axis is scaled logarithmically.

3.1.3 Antibacterial efficacy of 2,3- and 2,5-DMP

The goal was to compare the antibacterial efficacy of two pyrazine derivatives, which differ in the position of one methyl group, 2,5 and 2,3-dimethylpyrazine. The results should show the influence of the structural composition of pyrazines on their bactericidal activity.

A defined concentration of 10^3 *E. coli* cells was treated with 0.3, 0.6 and 1.2% of the pyrazine derivatives and incubated for 2 – 6 h at 30°C with agitation. After the treatment, the samples were plated out to determine the number of viable cells. The mean values of the cell concentrations were calculated. The obtained data are summarized in Table 10. The agar plates of the negative controls amounted to a stable cell concentration of 3.6 – 4.3*10^3 CFU/ml. 2,3-DMP caused no reduction in the viability of the cells when comparing with the negative controls. The samples showed a constant cell concentration of 3.7 – 4.9*10^3 CFU/ml. Due to this fact a bactericidal effect could not be detected within this experiment. However, 2,5-DMP showed an antibacterial activity in this assay. The effect was enhanced with increasing incubation time and increasing application volume of this derivate. A 0.3% solution was able to kill two third of the cells after an incubation time of 4 h. Samples containing 0.6% 2,5-DMP were reduced by 1-log10-unit in 2 h and after an incubation of 4 h the viability of the cells was decreased to 0. A 1.2% solution was able to kill 10^3 CFU/ml after an incubation of 2 h. The obtained data is graphically demonstrated in Figure 5.

Table 10: Results of the antibacterial assay performed with the derivatives 2,3-DMP and 2,5-DMP. 10^3 CFU/ml of *E. coli* were treated with a PBS-solution containing 0.3 – 1.2% pyrazine; incubation for 2 – 6 h at 30°C. The cell concentrations in the samples after the treatment are listed (mean values).

	Incubation [h]		2	4	6
Cell concentration [CFU/ml]	Negative controls		4.3*10^3	4.3*10^3	3.6*10^3
	2,3-DMP	0.3%	4.3*10^3	4.9*10^3	4.9*10^3
		0.6%	4.9*10^3	4.5*10^3	4.9*10^3
		1.2%	4.4*10^3	3.7*10^3	3.8*10^3
	2,5-DMP	0.3%	4.1*10^3	1.5*10^3	1.2*10^3
		0.6%	3.3*10^2	0	0
		1.2%	0	0	0

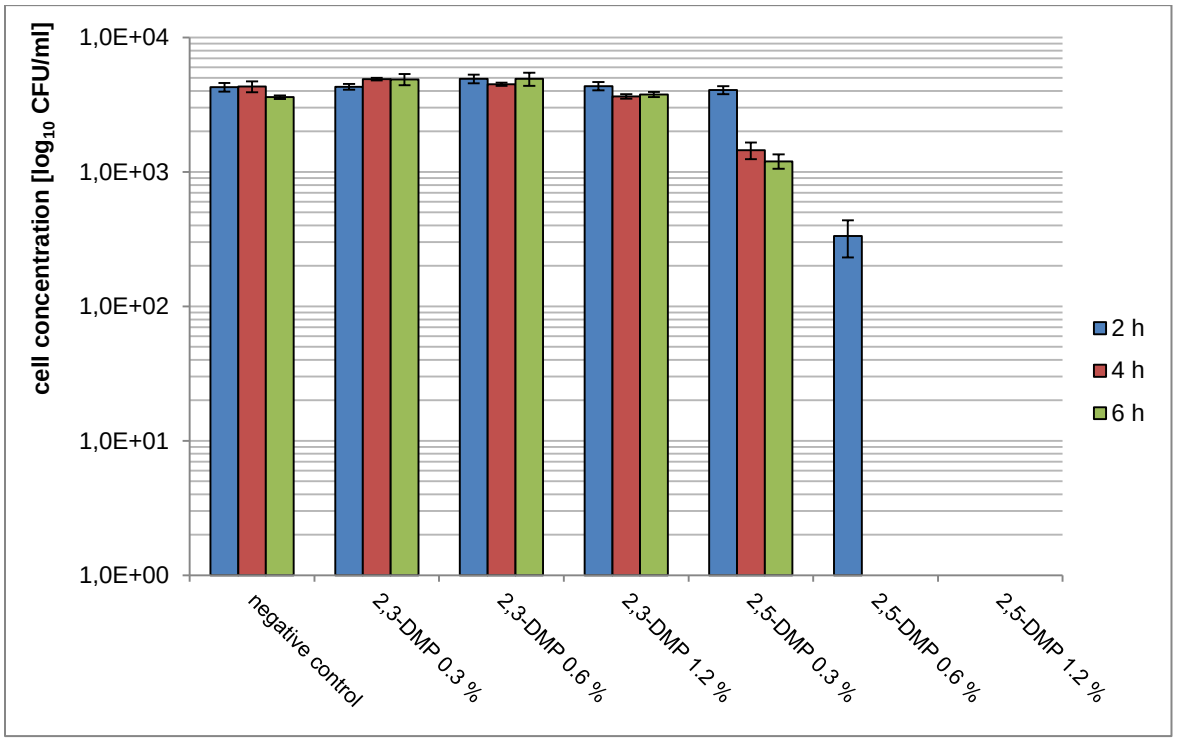


Figure 5: Results of the antibacterial assay performed with the derivatives 2,3-DMP and 2,5-DMP. 10^3 *E. coli* cells were treated with a PBS-solution containing 0.3 – 1.2% pyrazine for 2 – 6 h. The mean values of the cell concentrations and the corresponding standard deviations are shown. Detailed data of the determined cell concentrations can be obtained from Table 10. The x-axis is scaled logarithmically.

Comparing the antibacterial efficacy of 2,5-DMP with the activity of other pyrazine derivatives already tested, the compound appears much less bactericidal. In the previous experiment the derivate IP was found to exhibit the lowest antibacterial activity among the derivatives IBDM, IBM, IB and IPM. However, a 0.3% IP-solution was able to kill 10^5 CFU/ml after an incubation of 4 h.

3.1.4 Determination of MIC and MBC

The MICs and the MBCs were determined for the pyrazine derivatives IBDM, IPM, IP, IB and IBM. For the treatment the bacterial strains *E. coli* and *S. aureus* were used. Each sample was inoculated with approximately $5*10^5$ CFU/ml and the samples were incubated at 37°C for 18 h [89]. The obtained data of this experiment are summarized in Table 11. The derivatives are ordered regarding their antibacterial efficacy, ongoing with the most effective substance.

The MIC is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism under defined conditions [90]. The

MBC is defined as the lowest concentration of the agent required for 99.9% reduction of viable bacteria in the sample [91]. As can be seen in the table, the concentrations needed for all derivatives to reach the MICs and MBCs are between 2 and 17 mg/ml. The values are very close to each other. Furthermore, all derivatives need higher concentrations when treating *S. aureus*. For the pyrazines IBDM and IBM the MIC and the MBC are at the same concentration level, when treating *E. coli*. Although a narrow range of concentrations was used (difference of 1 mg/ml), it was not possible to define the amount of the substance which had only an inhibitory effect. Either the concentration of the derivate killed all the bacterial cells in the sample or the nutrient agar plates were overgrown. This means that the MIC and MBC are very close to each other.

Comparing the minimal concentrations, the pyrazine IBDM was the most effective substance. Although IBDM and IBM required the same concentrations for *E. coli*, IBDM needed lower amounts to inhibit the growth as well as to kill *S. aureus* cells. The following ranking regarding the bacteriostatic and bactericidal efficiency of the substances can be established: IBDM, IBM, IB, IPM and IP. Due to the fact that the ranking is consistent with both bacterial strains, it might be applied to the whole genera bacteria.

Table 11: Results of the MIC and MBC determinations of selected pyrazine derivatives. Comparison of the concentrations required for the bacterial strains *E. coli* and *S. aureus*. The derivatives are ordered regarding their bacteriostatic and bactericidal efficacy.

Pyrazine derivate	*E. coli*		*S. aureus*	
	MIC [mg/ml]	MBC [mg/ml]	MIC [mg/ml]	MBC [mg/ml]
IBDM	3	3	4	5
IBM	3	3	5	6
IB	4	5	8	9
IPM	5	6	10	13
IP	7	10	13	17

3.1.5 5 min bactericidal testing with IBDM

The bacterial strains *E. coli*, *S. aureus* and *P. aeruginosa* were exposed to different amounts of IBDM and incubated for 5 min. After the treatment, the samples were plated out to determine the number of viable cells. The mean values were calculated and the results are summarized in Table 12 - Table 14. In general it can be said that it was possible to achieve a bactericidal effect within an exposure time of 5 min. 4-log10-units of *E. coli* cells were killed with an IBDM concentration of 0.6%. For 10^5 and 10^6 CFU of *E. coli* the exposure to 0.9 and 1.2% IBDM respectively was lethal. The results of *P. aeruginosa* consisted exactly with the results obtained from the experiment with *E. coli*. In comparison to these two bacterial strains, *S. aureus* was more resistant to IBDM and higher amounts of the antibacterial agent were required. A treatment with 1.8% IBDM killed 4-log10-units *S. aureus* cells. In samples with 10^5 and 10^6 CFU the exposure to 2.4 and 3.0% respectively reduced the cell viability completely. *S. aureus* is a gram-positive bacterium, in contrast to *E. coli* and *P. aeruginosa.*

Table 12: Results of the 5 min-IBDM-treatment of *E. coli* cells. The cell concentrations of the negative controls are listed was well as the concentrations of IBDM required to cause complete absence of viability.

	Cell concentration [CFU/ml]	Lethal IBDM concentration [%]
Negative controls	$1.2*10^6$	1.2
	$1.3*10^5$	0.9
	$1.2*10^4$	0.6

Table 13: Results of the 5 min-IBDM-treatment of *P. aeruginosa* cells. The cell concentrations of the negative controls are listed was well as the concentrations of IBDM required to cause complete absence of viability.

	Cell concentration [CFU/ml]	Lethal IBDM concentration [%]
Negative controls	$2.7*10^6$	1.2
	$3.7*10^5$	0.9
	$2.0*10^4$	0.6

Table 14: Results of the 5 min-IBDM-treatment of *S. aureus* cells. The cell concentrations of the negative controls are listed was well as the concentrations of IBDM required to cause complete absence of viability.

	Cell concentration [CFU/ml]	Lethal IBDM concentration [%]
Negative controls	$1.9*10^6$	3.0
	$2.0*10^5$	2.4
	$1.5*10^4$	1.8

3.2 Antifungal activity

3.2.1 Determination of MIC and MFC

The MICs and the MFCs were determined for the pyrazine derivatives IBDM, IPM, IP, IB and IBM. For the treatment the fungus *C. albicans* was used. Each sample contained $2.5*10^5$ CFU/ml and the samples were incubated at 37°C for 24 h [93]. The obtained data of this experiment are summarized in Table 15. The derivatives are ordered regarding their antifungal efficacy, ongoing with the most effective substance. The MIC is defined as the lowest concentration of an antifungal agent that inhibits fungal growth under defined conditions [93]. The MFC is defined as the lowest concentration of the agent required for a 99% killing of fungal cells after a distinct exposure time and subculture to antibiotic-free medium [94]. As can be seen in the table, the concentrations needed for all derivatives to reach the MICs and MFCs are between 1 and 9 mg/ml. The values are very close to each other. For the pyrazine IBM the MIC and the MBC are at the same concentration level. The two concentrations are too close for defining the amount of the substance, which has only an inhibitory effect. Comparing the minimal concentrations, the pyrazine IBDM is shown to be the most effective antifungal substance. IBDM required the lowest amount to inhibit the fungal growth as well as to kill all fungal cells (MIC: 3 mg/ml, MFC: 4 mg/ml). The following ranking regarding the fungistatic and fungicidal efficiency of the substances can be established: IBDM, IBM, IB, IPM and IP. This ranking consists perfectly with the ranking experimentally determined for the antibacterial efficacy of these derivatives.

Table 15: Results of the MIC and MFC determinations of selected pyrazine derivatives for the fungus *C. albicans*. The derivatives are ordered regarding their fungistatic and fungicidal efficacy.

Pyrazine derivate	MIC [mg/ml]	MFC [mg/ml]
IBDM	3	4
IBM	4	4
IB	6	7
IPM	7	8
IP	8	9

3.2.2 5 min fungicidal testing with IBDM

The yeasts *S. cerevisiae* and *C. albicans* were treated with different amounts of IBDM and incubated for 5 min with agitation. After the treatment, the samples were plated out to determine the number of viable cells. The mean values were calculated and the results are summarized in Table 16 and Table 17. In general it can be said that it was possible to achieve a fungicidal effect within an exposure time of 5 min. 4-log10-units of *S. cerevisiae* cells were killed with an IBDM concentration of 0.6%. For 10^5 and 10^6 CFU of *S. cerevisiae* the exposure to 0.9 and 1.2% respectively caused complete absence of viability. In comparison, *C. albicans* was slightly more resistant to IBDM. A treatment with a 1.2% IBDM-PBS-Buffer solution killed 10^4 and 10^5 cells. For 6-log10-units of *C. albicans* cells the exposure to 1.8% IBDM was lethal.

Table 16: Results of the 5 min-IBDM-treatment of *S. cerevisiae* cells. The cell concentrations of the negative controls are listed was well as the concentrations of IBDM required to cause complete absence of viability.

	Cell concentration [CFU/ml]	Lethal IBDM concentration [%]
Negative controls	$2.3*10^6$	1.2
	$2.0*10^5$	0.9
	$1.7*10^4$	0.6

Table 17: Results of the 5 min-IBDM-treatment of *C. albicans* cells. The cell concentrations of the negative controls are listed was well as the concentrations of IBDM required to cause complete absence of viability.

	Cell concentration [CFU/ml]	Lethal IBDM concentration [%]
Negative controls	$2.4*10^6$	1.8
	$2.5*10^5$	1.2
	$2.2*10^4$	1.2

3.2.3 Antifungal assay – *Penicillium sp.* spores

In this experiment 4-log10-units of *Penicillium sp.* spores were treated with 3 and 6 µl of IBDM. The negative controls were mixed with NaCl. After 15 min of incubation, the samples were spread on DG18 agar plates. The growth of the *Penicillium sp*. on the nutrient plates was observed after 48, 72 and 96 h. In general it can be said that IBDM was able to inhibit fungal growth. The treated

samples showed less growth comparing to the negative controls, especially after the first 48 h the inhibiting effect was well observable. The sterility controls showed no fungal growth. Representative illustrations of the results are shown in Figure 6. An application volume of 6 µl IBDM achieved a higher inhibition of fungal growth (Figure 6, row B) comparing to the samples treated with 3 µl of IBDM (Figure 6, row A).

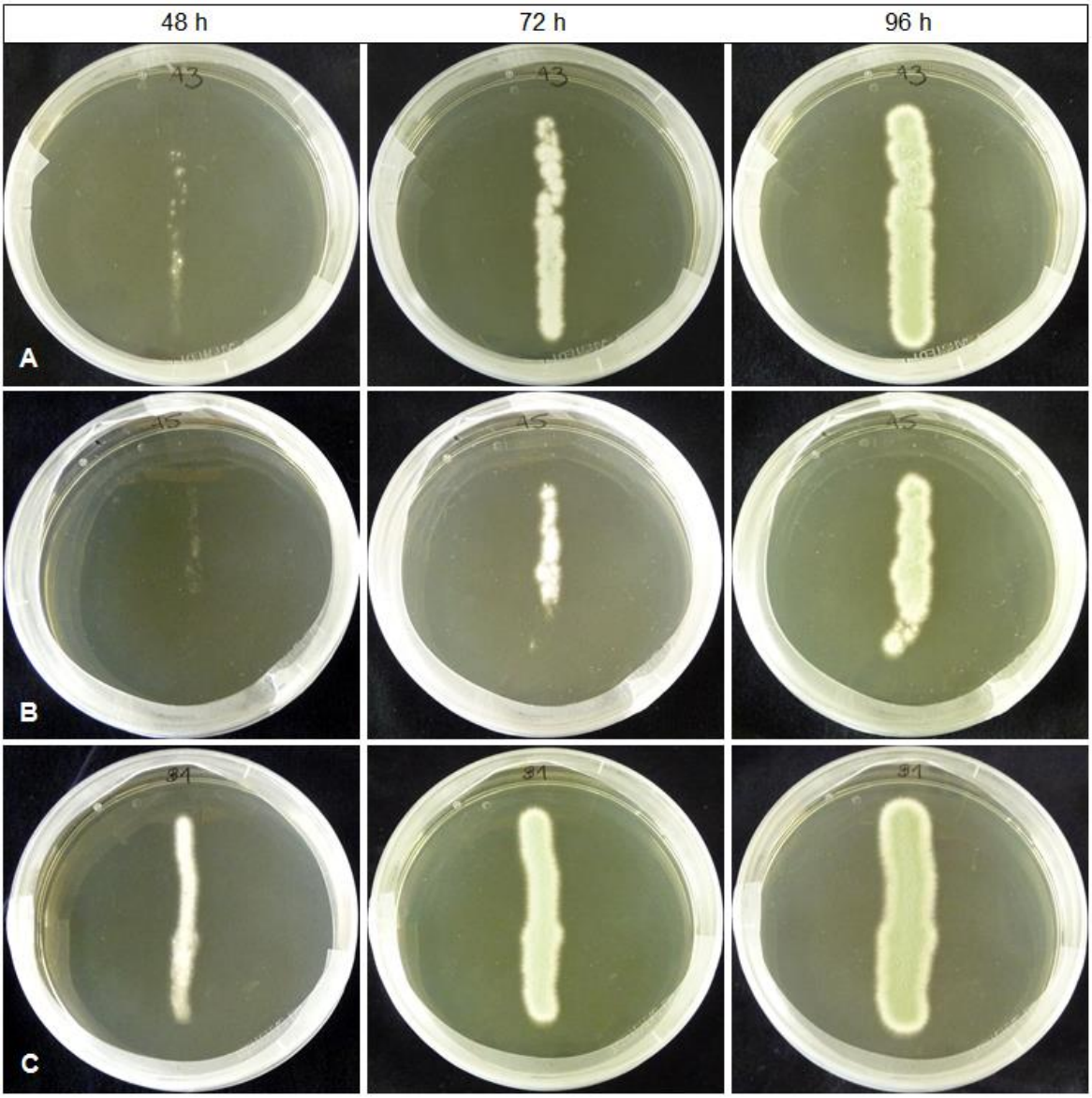


Figure 6: Antifungal activity of IBDM. 4-log10-units of *Penicillium sp.* spores were treated with: Row A: 3 µl of IBDM; Row B: 6 µl of IBDM; Row C: not treated, negative controls.

3.2.4 Growth kinetics of *S. cerevisiae* in the presence of IBDM

In this experiment the influence of IBDM on the growth behaviour of the yeast *S. cerevisiae* was examined. The incubation was carried out at 30°C for 12 h. The yeast cultures 1 and 2 were controls, cultures 3 and 4 were treated with 0.3% IBDM and cultures 5 and 6 had an IBDM concentration of 0.6%. The optical density of the cultures was measured every hour, over a period of 12 h. The mean values were calculated and illustrated in Figure 7. After 12 h of incubation culture 1 and 2 reached an OD_{600} of 11.4 and 10.9, respectively. For both cultures the optical density was increasing continuously with time and was constant in the last three to four hours of incubation. In culture 3 and 4 the optical density varied between 0.11 and 0.13 during the period of 12 h. In culture 5 and 6 the OD_{600} values stayed nearly constant over the whole time, they even dropped slightly.

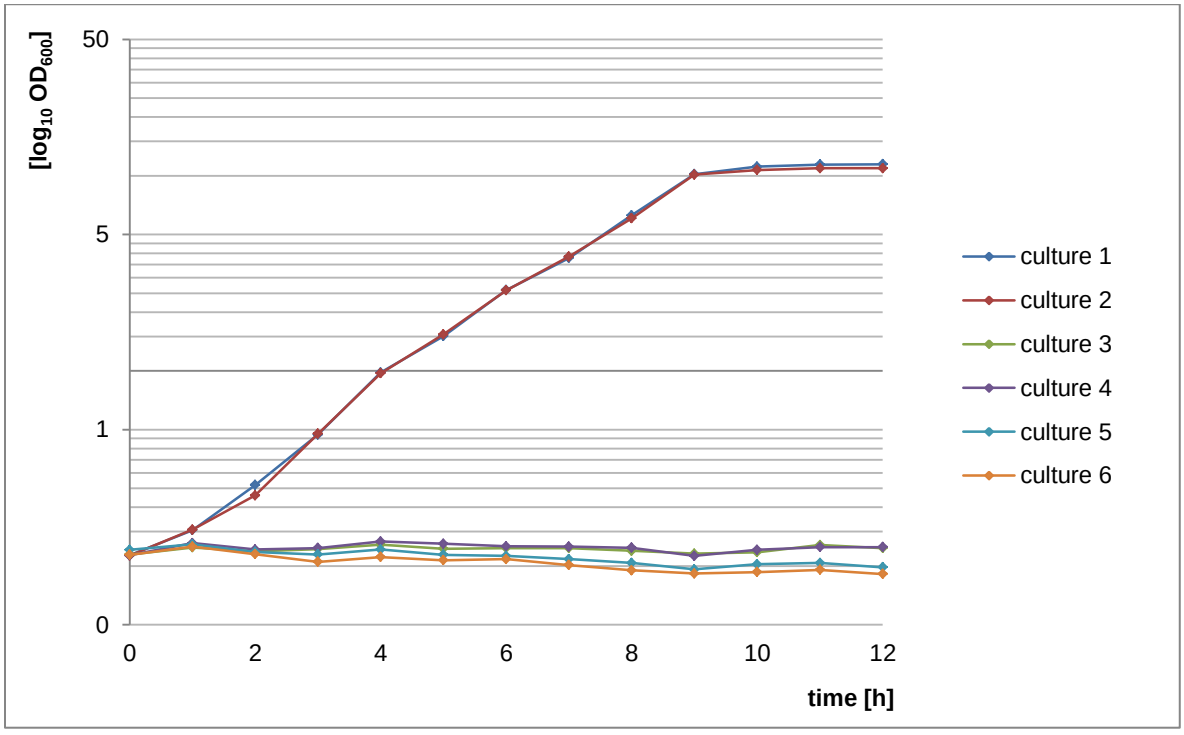


Figure 7: Growth curves of the *S. cerevisiae* cultures according their optical density (OD_{600}). The x-axis is scaled logarithmically. Cultures 1 + 2 were controls (no addition of pyrazine); cultures 3 + 4 were treated with 0.3% IBDM; cultures 5 + 6 were treated with 0.6% IBDM.

Moreover, the cell concentration (CFU/ml) in each culture was determined by plating out serial dilutions. The mean values were calculated and the growth curves are demonstrated in Figure 8. After 12 h of incubation culture 1 reached a cell number of $6.15*10^7$ CFU/ml and culture 2 achieved $5.70*10^7$ CFU/ml. The

cells in the control cultures needed 1 h to adjust themselves to the new environment (lag phase). In the next 8 h they grew exponentially (log phase). Between hour 9 and 10 the stationary phase was reached. Culture 3 started with $4.65*10^5$ CFU/ml. The number of viable cells dropped in the first two hours and reached a value of $1.50*10^5$ CFU/ml. This cell concentration level stayed nearly constant until the end of incubation. This inhibitory effect was also observed for culture 4, which started with $4.35*10^5$ CFU/ml. The number of viable cells was decreased in the first two hours to a value of $1.65*10^5$ CFU/ml. The cell concentration remained nearly constant until the end of incubation. The applied pyrazine dose of 0.3% had a fungistatic effect. In culture 5 and 6 the cell concentration was reduced to 0 after the first hour of incubation. In this case the applied pyrazine dose of 0.6% was lethal to all cells and consequently the effect was fungicidal.

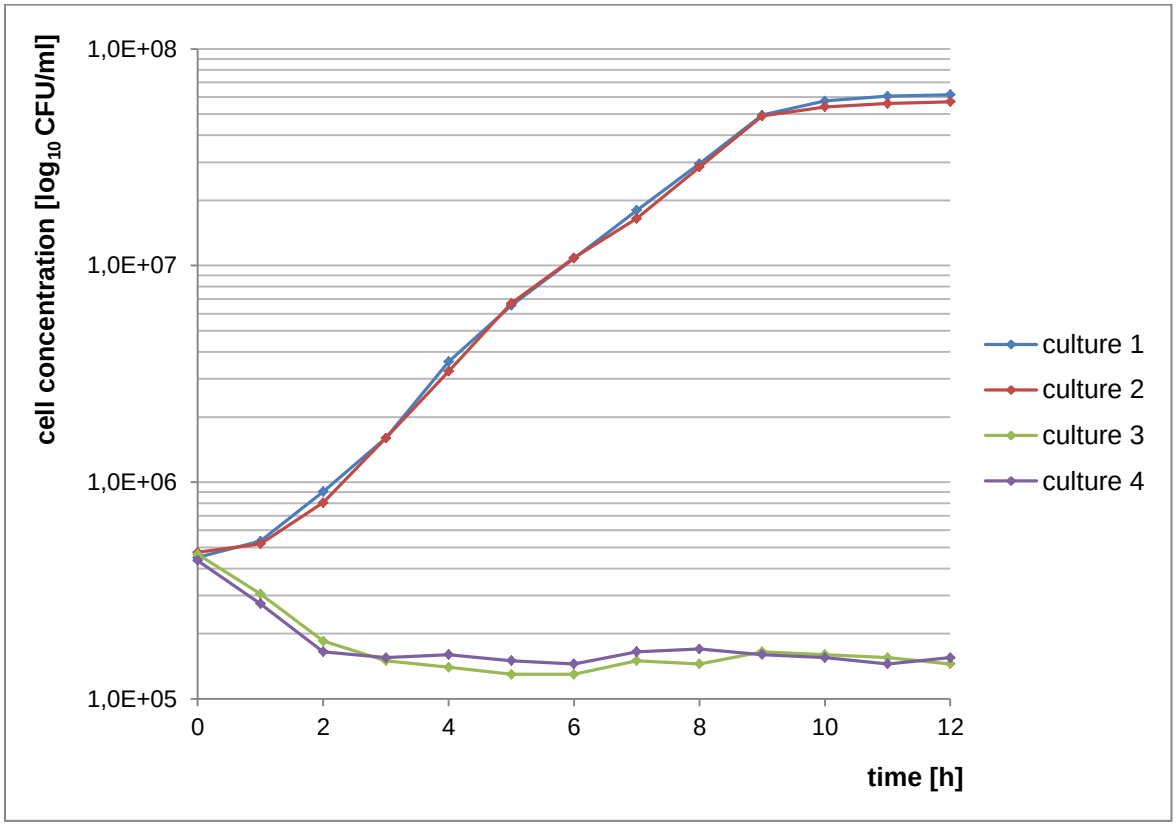


Figure 8: Growth curves of *S. cerevisiae* cultures according their cell concentrations. The x-axis is scaled logarithmically. Cultures 1 + 2 were controls (no addition of pyrazine); cultures 3 + 4 were treated with 0.3% IBDM; Due to the fact that the cell concentration of culture 5 + 6 was reduced to 0 after the first hour of incubation and the x-axis is scaled logarithmically, their growth curves cannot be illustrated.

3.3 Mode of action

3.3.1 LIVE/DEAD *Bac*Light Bacterial Viability Kit

The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit was used to examine if IBDM is acting by reducing the membrane integrity. The green-fluorescent nucleic acid stain (SYTO 9) stains all bacteria, with intact and damaged membranes. The red-fluorescent nucleic acid stain propidium iodide (PI) can only enter the bacterial cell if the membrane is damaged. The stain causes a reduction of SYTO 9 fluorescence. Accordingly, bacteria with intact cell membranes are considered alive and stain fluorescent green. Bacteria with damaged membranes are considered dead and stain fluorescent red. In general this test kit is used for bacterial enumeration and viability testing. However, the assay can also serve as rapid method for identifying membrane-active compounds [95 - 97].

The bacterial strain *E. coli* was treated with different concentrations of IBDM and 70% isopropanol as positive control. The obtained data of the fluorescence measurement are summarized in Table 18. The mean values and the standard deviations were calculated. The green/red fluorescence ratio demonstrates the degree of cells with intact membranes. Cell membrane damage is increasing with a decreasing ratio. The values are graphically demonstrated in Figure 9. In general it can be said that the treated samples showed a lower green/red ratio than the negative controls. The fluorescence ratio was reduced by increasing concentrations of IBDM, which demonstrates a reduction in membrane integrity [95]. The samples treated with 10, 20 and 40 mM IBDM had a mean green/red ratio of 1.96, 1.03 and 0.57, respectively.

Due to increased cell membrane permeability the uptake of propidium iodide was increased [97]. This can be attributed to a membrane–damaging effect of IBDM. The positive control, which was treated with 70% isopropanol, demonstrated also a low green/red ratio (0.66), but was higher than the ratio of the samples treated with 40 mM IBDM (~ 0.8% v/v).

Table 18: Results of the membrane damage assay. *E. coli* cells were treated with IBDM and isopropanol (positive control). Afterwards they were stained with SYTO 9 and PI. The mean values of the fluorescence intensities were used for calculating the green/red fluorescence ratio.

Samples	green/red fluorescence ratio
Negative control	4.09 ± 0.39
Isopropanol 70%	0.66 ± 0.03
IBDM 10 mM	1.96 ± 0.13
IBDM 20 mM	1.03 ± 0.13
IBDM 40 mM	0.57 ± 0.02

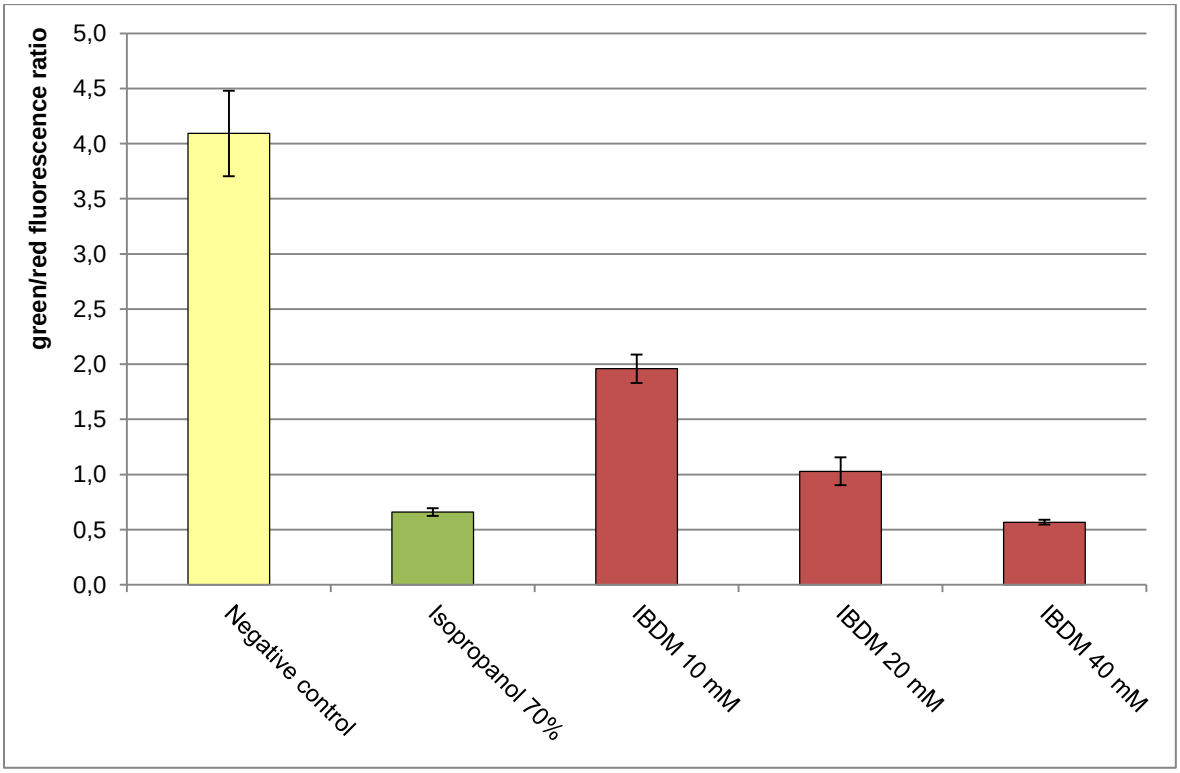


Figure 9: Results of the membrane damage assay. The green/red fluorescence ratio demonstrates the degree of cells with intact membranes [95]. The mean values and the corresponding standard deviations are shown. Detailed data can be obtained from Table 18.

3.3.2 *Bac*Light Bacterial Membrane Potential Kit

The Bacterial Membrane Potential Kit was used to investigate if IBDM causes alteration of the membrane potential. The fluorescence assay contains the carbocyanine dye $DiOC_2(3)$, which exhibits a green fluorescence in bacterial cells at low concentrations. In cells with an intact electrical potential gradient the dye becomes accumulated. The self-association of the dye molecules leads to a fluorescence shift from green to red. The proton ionophore CCCP (carbonylcyanide-3-chlorophenyl-hydrazone) is included in this kit as positive control. This compound breaks down the bacterial membrane potential by eradicating the proton gradient. Thereby the red/green fluorescence intensity ratio gets reduced [98].

For the test, *S. aureus* cells were treated with different concentrations of IBDM and IBM. The obtained data of the fluorescence intensity measurement are summarized in Table 19. The mean values and the standard deviations were calculated. Due to the fact that the amount of red and green fluorescence intensity depend on the cell size and aggregation, the ratio of red to green fluorescence is used as size-independent indicator of membrane potential. The lower the red/green fluorescence ratio, the higher was the depolarization effect on the membrane potential [98]. The values are visualized in Figure 10.

The red/green ratio of cells treated with IBDM or IBM was reduced significantly compared to the untreated cells (red/green ratio of 0.17). The ratiometric parameter decreased with increasing IBDM and IBM concentrations, which demonstrated a depolarization of the membrane potential [98]. Comparing the results of IBDM and IBM, IBDM had a higher effect on the membrane potential. The exposure to 5 mM IBDM reduced the red/green ratio to a value of 0.08, whereas 5 mM IBM decreased the ratio to 0.10. When treating the *S. aureus* cells with 20 mM IBM the red/green ratio was reduced to 0.03. This result correlates with the obtained result that IBDM has a slightly higher antimicrobial efficacy than IBM. The lowest ratio and for this reason the strongest alteration effect of the membrane potential was achieved with an IBDM concentration of 20 mM, leading to a ratio of 0.025. The positive control, which was treated with the proton ionophore CCCP, demonstrated a red/green ratio of 0.036, which was higher than the ratio of the samples treated with 10 and 20 mM IBDM and 20 mM IBM.

Table 19: Results of the membrane potential assay. *S. aureus* cells were stained with $DiOC_2(3)$ and treated with IBDM and IBM, respectively. CCCP was used as a positive control. The mean values of the fluorescence intensities were used for calculating the red/green fluorescence ratio.

Samples	red/green fluorescence ratio
Negative control	0.169 ± 0.0173
Positive control	0.036 ± 0.0017
IBDM 20 mM	0.025 ± 0.0060
IBDM 10 mM	0.033 ± 0.0023
IBDM 5 mM	0.079 ± 0.0012
IBM 20 mM	0.030 ± 0.0047
IBM 10 mM	0.055 ± 0.0027
IBM 5 mM	0.104 ± 0.0015

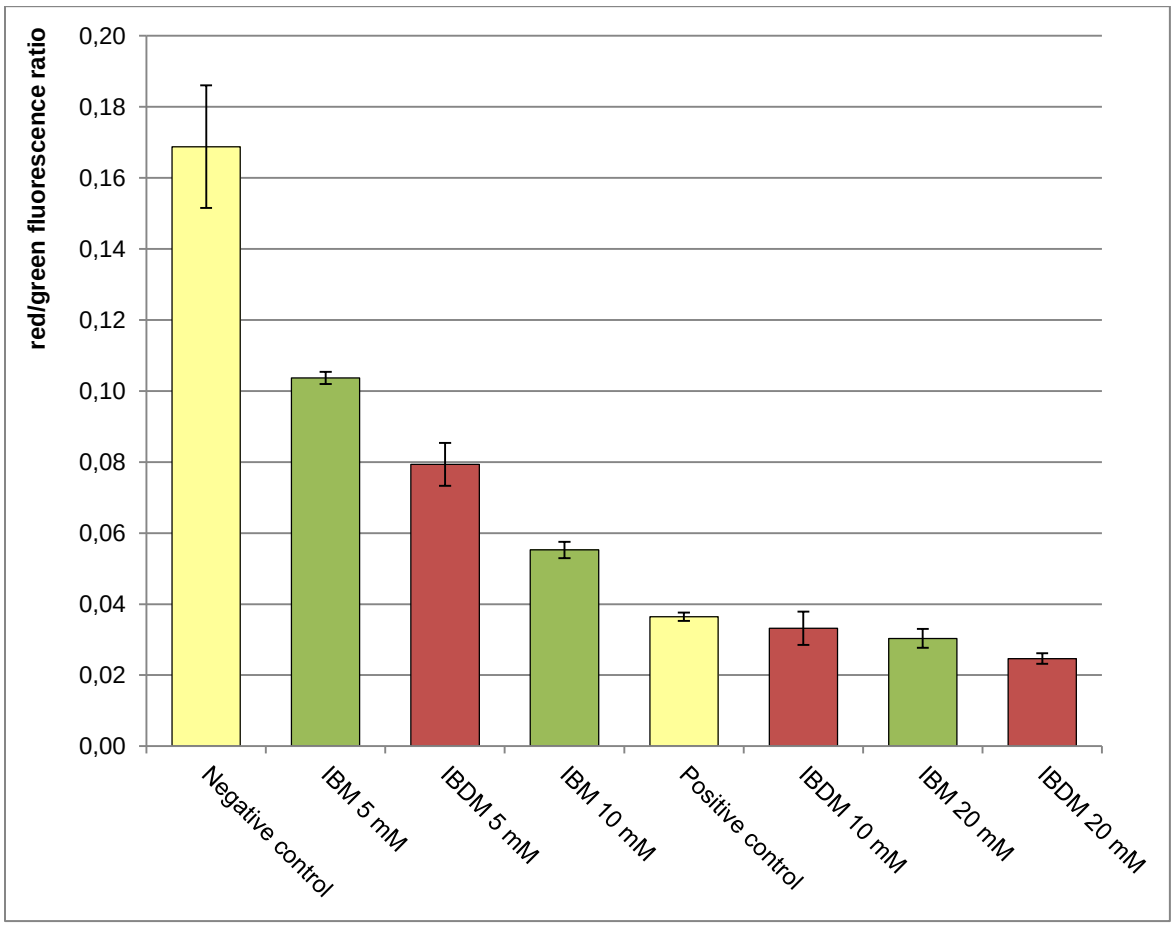


Figure 10: Results of the membrane potential assay. The ratio of red to green fluorescence intensity is used as size-independent indicator of the membrane potential [98]. The mean values and the corresponding standard deviations are shown. Detailed data can be obtained from Table 19.

3.3.3 β-Galactosidase assay – inner membrane permeabilization

It was verified if IBDM has a permeabilizing effect on the inner membrane of bacterial cells. Therefore, a β-galactosidase assay was performed. At first, *E. coli* cells were induced with IPTG to overexpress the cytoplasmic enzyme β-galactosidase. The cells were treated with serial concentrations of IBDM. The disruption of the inner membrane was detected by the leakage of β-galactosidase. The activity of the enzyme in the cell-free medium was determined by adding the substrate ONPG. The conversion of ONPG to the yellow product o-nitrophenol was quantified by measuring the absorbance at 415 nm [99, 100].

The mean values were calculated and the obtained data are summarized in Table 20. The absorbance value achieved for the negative control (untreated cells) was assumed as 100% (A_{415} 1.34). The absorbance of the treated samples was calculated in relation to the absorbance of the negative control. The higher the absorbance, the higher was the leakage of β-galactosidase and the higher was the permeabilization effect of the inner membrane [99]. The values are visualized in Figure 11. The positive control, which was treated with 70% isopropanol, demonstrated the highest absorbance value of 1.86. The second highest absorbance value (A_{415} 1.73) was achieved with the lowest concentration of IBDM (10 mM). The absorbance decreased with an increasing application volume of IBDM. The absorbance values of the samples treated with 50 and 100 mM IBDM were even lower than the absorbance of the negative control.

Table 20: Results of the β-galactosidase assay. IPTG-induced *E. coli* cells were treated with IBDM and isopropanol (positive control). The production of o-nitrophenol is quantified by measuring the absorbance at 415 nm. The mean values and the corresponding standard deviations are listed.

Samples	A_{415}	A_{415} relative to the negative control [%]
Negative control	1.34 ± 0.0153	100
Isopropanol 70%	1.86 ± 0.0059	139
IBDM 10 mM	1.73 ± 0.0050	130
IBDM 20 mM	1.60 ± 0.0344	120
IBDM 50 mM	1.27 ± 0.0092	95
IBDM 100 mM	0.87 ± 0.0142	65

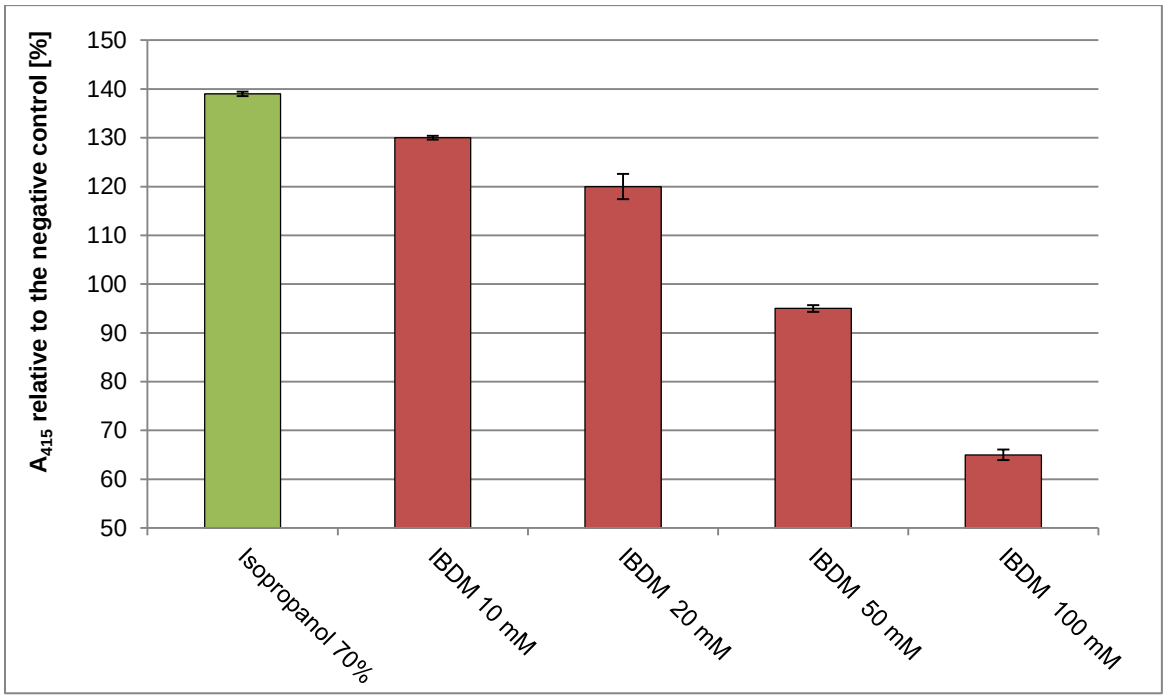


Figure 11: Results of the β-galactosidase assay. A high absorbance indicates a high permeabilization of the inner membrane [99]. The absorbance values in relation to the absorbance of the untreated cells and the standard deviations were calculated. Detailed data can be obtained from Table 20.

3.3.4 Interaction of pyrazine derivatives with proteins

Interaction of IBDM with intracellular proteins

A possible interaction of proteins with IBDM was examined. Therefore, cellular proteins of *E. coli* were obtained via ultrasonic. The protein solution was mixed with IBDM and incubated at 37°C for 5 min. The control samples did not show a change in their appearance. The samples stayed transparent. However, the protein mixtures with IBDM got turbid (milk-white) and showed a higher viscosity. To verify if IBDM was reacting with a certain protein or enzyme class of *E. coli* respectively, the proteins were separated by their size (<10 kD to ≥100 kD). The four protein fractions were mixed with IBDM, which generated a transparent solution. After incubation for 5 min at 37°C, all reaction mixtures got turbid (milk-white). The solution of the control samples, which did not contain IBDM, stayed transparent. However, the turbid samples got transparent too after some minutes at room temperature. The samples were incubated again at 37°C for 5 min and got turbid again. This result might indicate that IBDM is not interacting with a certain cellular protein, but with several. IBDM might have the ability to form reversible bonds with proteins at higher temperatures.

Interaction of pyrazine derivatives with BSA

The objective of this experiment was to verify if pyrazine derivatives undergo an interaction with the protein BSA, when they get in contact with it. The derivatives IBDM, IBM, IB, IPM and IP were used. 50 µl of a BSA-solution (10 mg/ml) were mixed with 2 µl of a derivate. After the samples were vortexed no alterations of the mixtures were visible. The samples were incubated at 37°C and 500 rpm for 5 – 25 min. After 5 min of incubation, samples with the derivatives IBDM and IBM showed a change in their visual appearance. The mixtures got turbid (milky-white) and showed a higher viscosity. In the sample with the compound IB a slight turbidity was noticeable. The rest of the derivatives had no visible effect on the BSA molecules. The samples stayed transparent. Moreover, all control samples did not show a change in their appearance. After further 10 min of incubation at 37°C, the samples with the derivatives IBDM, IBM, IB and IPM showed milky-white turbidity. Mixtures with the compound IP and the controls stayed unchanged. Further 10 min of incubation at 37°C caused no change in the results. BSA mixtures were prepared with the addition of 4 µl and 6 µl of IP. After 10 min of incubation at 37°C the samples with 4 µl of IP had no effect on the BSA solution. The samples with 6 µl of IP were slightly turbid and after vortexing them, their turbidity increased. To summarize the results of this experiment, a ranking of the reactivity of the derivatives with BSA can be established: (1) IBDM, IBM, (2) IB, (3) IPM and (4) IP.

Influence of BSA on the antibacterial efficacy of IBDM

The goal of this experiment was to examine if the additional presence of a protein in an antimicrobial assay has an influence on the efficacy of IBDM. Therefore, BSA was used as additive. *E. coli* cells were treated with a 0.3% IBDM-PBS-Buffer solution, with and without the addition of BSA (0.25 – 1 mM). The samples were incubated at 30°C with agitation for 2 h. After the treatment, the samples were plated out to determine the number of viable cells. The mean values of the cell concentrations were calculated. The results are summarized in Table 21 and demonstrated graphically in Figure 12.

Table 21: Results of the BSA-influence test. The cell concentrations of the control samples are compared to the cell concentrations of the samples treated with IBDM, with or without the addition of BSA.

BSA [mM]		1	0.5	0.25	-
Cell concentration [CFU/ml]	Negative controls	$8.3*10^4$	$8.4*10^4$	$7.3*10^4$	$5.0*10^4$
	IBDM-treated	$4.2*10^4$	$2.2*10^4$	$2.0*10^3$	0

The obtained data showed that the presence of BSA had a significant influence on the efficacy of IBDM. The antibacterial activity of IBDM got reduced with increasing BSA concentrations. The application of 0.3% IBDM, without the addition of BSA, caused a complete absence of viability, it was lethal to all the cells in the sample. In comparison, when adding 0.25 mM BSA, 2.7 ± 0.6% of the cells survived. Putting a twice as high concentration of BSA (0.5 mM) into the sample, led to the survival of the ten-fold amount (26 ± 3.9%). Half of the cells (49.7 ± 3.9%) survived by the addition of 1 mM BSA. Moreover, BSA had a growth promoting effect on *E. coli* cells. The negative controls, which contained BSA, had slight higher cell concentrations than the controls without BSA. Nevertheless, this positive influence could not explain the strong reduction of the antibacterial efficacy of IBDM. The antagonising effect of BSA is probably mediated by the formation of intermolecular bonds between the protein and aromatic compound.

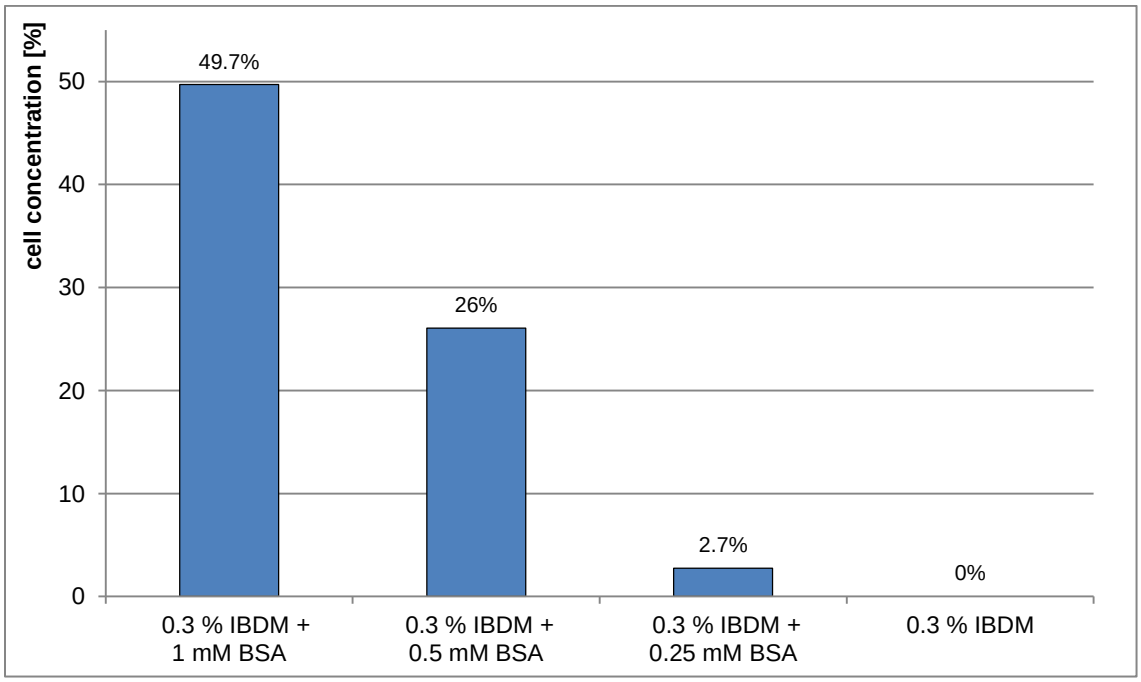


Figure 12: Influence of BSA on the antibacterial activity of IBDM. The reaction formulations, the corresponding cell concentrations after the treatment and the standard deviations are shown. Detailed data can be obtained from Table 21. The presence of BSA reduced the antibacterial activity of IBDM.

3.4 *E. coli* Keio Knockout Collection

3.4.1 Single-gene knockout mutants of *E. coli*

The Dharmacon *E. coli* Keio Knockout Collection is a library of mutants with single-gene deletions of non-essential genes. It contains a total set of 7980 knockout strains [101]. The whole knock out library was replicated to perform further experiments with the duplicate. The copy, which is demonstrated in Figure 13, consists of 90 microtiter plates and is stored at -80°C. Moreover, 233 different mutants were selected to carry out further experiments (see Table 6) and sorted in three microtiter plates.

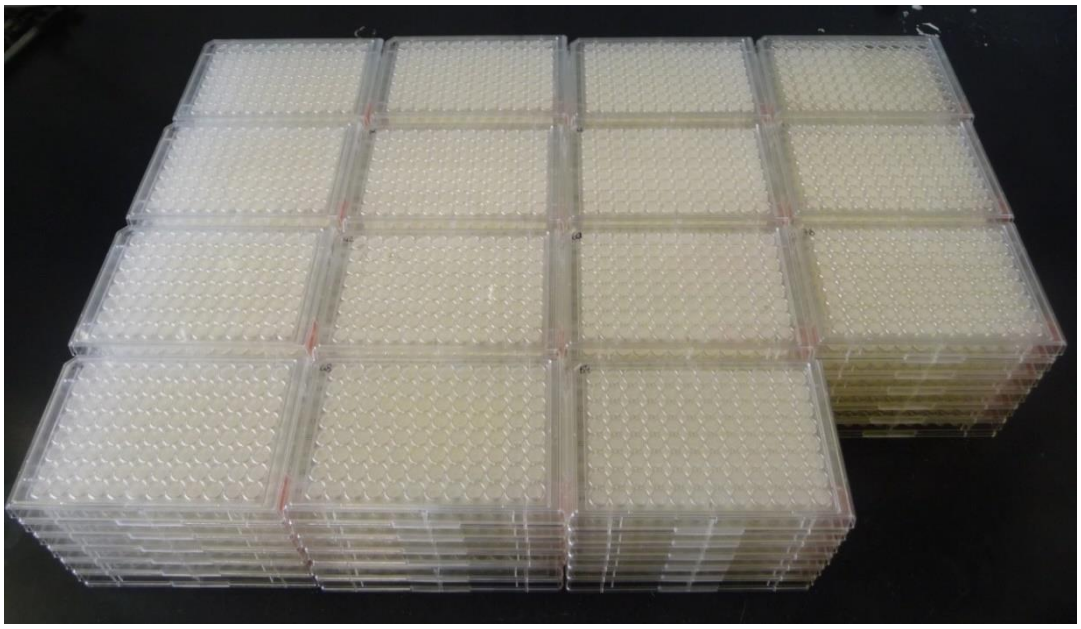


Figure 13: Copy of the *E. coli* Knockout Collection consisting of 90 96-well microtiter plates.

3.4.2 Susceptibility test with selected *E. coli* mutants – potential increased sensitivity or resistance to IBDM

In this experiment a selected group of mutants of the *E. coli* Knockout Collection was treated with the pyrazine derivate IBDM. Thereby, it was tested whether these clones are possibly more resistant or sensitive to IBDM compared to the wild-type strain. The results may give a better understanding of the mode of action of alkylated pyrazine derivatives. Due to limited working time, 42 mutants were tested. In regard to the function of the deleted gene, these mutants can be

assigned to six different categories (see Table 7) [104]. The clones as well as the wild-type strain were treated with three different IBDM concentrations (2, 3 and 4 mg/ml). The application of these three concentrations is based on the arising results of a previous experiment. The MBC of IBDM was found to be 3 mg/ml.

The obtained data of the experiment are listed in Table 22 and Table 23. The wild-type strain showed the following results: the untreated negative controls had an average cell concentration of $6*10^8$ CFU/ml. Samples, which were treated with 2 mg/ml, had an average cell number of $8.1*10^6$ CFU/ml. 3 and 4 mg/ml were bactericidal to all cells, the nutritive plates of those samples showed no growth after the treatment.

Mutants, which had distinct tolerance to IBDM when comparing with the wild-type strain, are highlighted in yellow. Seven of the 42 mutants showed an increased sensitivity. An IBDM concentration of 2 mg/ml had a lethal effect to these strains. There was a complete reduction of viable bacteria. As a consequence, a lower amount of IBDM is needed to kill those *E. coli* mutants. These results have been confirmed by repeating the experiment with the sensitive clones and the wild-type strain. Mutants with an enhanced resistance to IBDM have not been found. The sensitive mutants and the corresponding products of the deleted genes are listed in Table 24.

Five out of these seven clones belong to the group of rfa-genes, which are involved in the biosynthesis of the inner core region of lipopolysaccharides (LPS) [105]. The gmbH mutant, which showed also a reduced tolerance to IBDM, encodes an enzyme, which is involved in the biosynthesis of ADP-L-glycero-β-D-manno-heptose, a precursor of the inner core region of LPS [106]. Enhanced sensitivity was also observed for the pal-mutant. Pal is a peptidoglycan-associated lipoprotein [107].

Table 22: Results of the antibacterial susceptibility testing using the wild-type strain *E. coli* K-12. The cell concentration of the control samples is compared to the cell concentrations of the samples treated with IBDM (mean values).

	Cell concentration after the treatment [CFU/ml]			
	IBDM 0 mg/ml (NC)	IBDM 2 mg/ml	IBDM 3 mg/ml	IBDM 4 mg/ml
Wild-type strain	$6.0*10^8$	$8.1*10^6$	0	0

Table 23: Results of the antibacterial susceptibility testing using 42 mutants of the *E. coli* Keio Knockout Collection. Listed are the deleted genes, the corresponding categories (description see Table 7) and the cell concentrations of the treated and untreated samples (negative controls, NC). Mutants, which showed a higher sensitivity to IBDM, are highlighted in yellow.

Mutants			Cell concentration after the treatment [CFU/ml]			
Nr.	Cat.	Gene	IBDM 0 mg/ml (NC)	IBDM 2 mg/ml	IBDM 3 mg/ml	IBDM 4 mg/ml
1	1	recA	$3.0*10^8$	$9.0*10^6$	0	0
2	1	recB	$2.3*10^8$	$8.4*10^6$	0	0
3	1	ruvC	$3.0*10^8$	$8.8*10^6$	0	0
4	2	acrB	$4.2*10^8$	$2.5*10^7$	0	0
5	2	argO	$4.5*10^8$	$9.6*10^6$	0	0
6	2	cpxA	$6.1*10^8$	$9.0*10^6$	0	0
7	2	csgG	$6.3*10^8$	$8.8*10^6$	0	0
8	2	envC	$2.2*10^8$	$8.0*10^6$	0	0
9	2	envZ	$6.0*10^8$	$1.1*10^7$	0	0
10	2	mdtJ	$4.5*10^8$	$1.9*10^7$	0	0
11	2	mrcB	$2.1*10^8$	$1.2*10^7$	0	0
12	2	ompF	$6.6*10^8$	$1.1*10^7$	0	0
13	2	ompT	$5.6*10^8$	$1.3*10^7$	0	0
14	2	pal	$5.6*10^8$	0	0	0
15	2	rfaC	$6.3*10^8$	0	0	0
16	2	rfaD	$5.9*10^8$	0	0	0
17	2	rfaE	$7.1*10^8$	0	0	0
18	2	rfaG	$3.8*10^8$	0	0	0
19	2	smpA	$5.6*10^8$	$1.2*10^7$	0	0
20	2	surA	$5.0*10^8$	$1.5*10^7$	0	0
21	2	ybfB	$6.5*10^8$	$1.3*10^7$	0	0
22	2	ybjO	$5.2*10^8$	$8.3*10^6$	0	0
23	2	ybjP	$6.2*10^8$	$1.4*10^7$	0	0
24	2	yheL	$7.1*10^8$	$1.3*10^7$	0	0
25	3	rimK	$3.3*10^8$	$9.1*10^6$	0	0
26	3	rplA	$3.6*10^7$	$9.9*10^6$	0	0
27	3	ydgD	$4.8*10^8$	$1.2*10^7$	0	0
28	3	yheN	$5.1*10^8$	$1.1*10^7$	0	0
29	3	yibK	$5.4*10^8$	$1.0*10^7$	0	0
30	4	aceE	$4.9*10^8$	$1.1*10^7$	0	0
31	4	dapF	$4.7*10^8$	$1.2*10^7$	0	0
32	4	gmhB	$7.7*10^8$	0	0	0
33	4	nudB	$4.9*10^8$	$9.0*10^6$	0	0
34	4	pykF	$5.1*10^8$	$9.0*10^6$	0	0
35	5	ihfA	$4.9*10^8$	$9.3*10^6$	0	0
36	5	pdhR	$5.0*10^8$	$1.1*10^7$	0	0
37	5	rfaH	$4.9*10^8$	0	0	0
38	5	seqA	$4.9*10^8$	$9.1*10^6$	0	0
39	5	xapR	$4.8*10^8$	$9.6*10^6$	0	0
40	6	yfjR	$5.0*10^8$	$9.2*10^6$	0	0
41	6	ylcG	$6.0*10^8$	$1.2*10^7$	0	0
42	-	cyoA	$7.5*10^8$	$1.5*10^7$	0	0

Table 24: Sensitive mutants and role of the deleted genes [104].

Gene	Function
pal	Peptidoglycan-associated lipoprotein precursor
gmhB	D,D-heptose 1,7-bisphosphate phosphatase (yaeD)
rfaC	Lipopolysaccharid-heptosyltransferase-1
rfaD	ADP-L-glycero-D-manno-heptose-6-epimerase
rfaE	Heptose 1-phosphate-adenyltransferase
rfaG	Lipopolysaccharid-glucosyltransferase-1
rfaH	Transcriptional antiterminator

3.5 Stability Tests – Effect of abiotic factors on alkylpyrazines

3.5.1 Influence of temperature

The objective was to verify the stability of IBDM in relation to the influence of different temperatures. Samples of 0.3% IBDM solutions were stored for 24 h at -20°C, 2°C, 22.5°C, 37°C and 60°C. Afterwards, the IBDM concentration was measured with a spectrophotometer at A_{280}. The percentage of IBDM loss was calculated and is listed in Table 25. Samples, which were incubated at -20°C, 2°C or room temperature for 24 h, showed no significant loss of IBDM, the concentration stayed constant. Samples, which were incubated at 37°C and 60°C, lost 35.8% and 77.1%, respectively due to evaporation. Furthermore, the anti-bacterial efficacy of the temperature treated IBDM solutions were compared by treating *E. coli* cells. After an incubation of 6 h, the samples were plated out to determine the cell concentrations. The mean values were calculated and are listed in Table 25. The negative controls, which were not treated with IBDM, had a cell concentration of $8.40*10^3$ and $8.25*10^4$ CFU/ml, respectively. The 0.3% IBDM mixtures, which were stored for 24 h at temperatures between -20°C and 37°C, were lethal to all cells in the samples. Nevertheless the 37°C sample lost 35.8% of IBDM, the concentration was high enough to kill all the *E. coli* cells. After exposure to the IBDM solution, which was incubated at 60°C, the cell concentration was at the same level as in the negative controls. This result could be attributed to the high evaporation of IBDM. The cells were treated with the remaining 23% of IBDM, which was insufficient.

However, a destabilizing effect of the molecule structure and thereby of the antibacterial efficacy of IBDM, due to storage at different temperatures, was not detected.

Table 25: Results of the temperature-stability test of IBDM. 0.3% IBDM-PBS-Buffer mixtures were stored at different temperature for 24 h. The percentage of IBDM loss was determined by using a spectrophotometer. The cell concentrations in the negative controls are compared to the cell concentrations of the IBDM treated samples (NC – negative control; n.c. – not countable).

Temperature	-20°C	2°C	22.5°C	37°C	60°C	- (NC)
Loss of IBDM [%]	1.2	0.85	0	35.8	77.1	-
Cell concentration [CFU/ml]	0	0	0	0	n.c.	$8.25*10^4$
	0	0	0	0	$8.18*10^3$	$8.40*10^3$

3.5.2 Influence of light

The aim was to verify the stability of IBDM- and IPM-molecules in relation to light exposure. Therefore, one fraction of the pyrazine derivatives was placed directly under an UV-lamp for 24 h and the other fraction was placed under an average light source for four days. The antibacterial efficacy of these light-exposed pyrazine samples was compared to the activity of the control pyrazines (non-light-exposed). After the light exposure, defined concentrations of *E. coli* cells were treated with 0.3% of the compounds and the samples were plated out to determine the cell number (CFU/ml). The mean values were calculated. The cell concentrations in the untreated samples (negative controls) were assumed as 100%. The percentage of viable cells after the treatment was calculated relative to the 100% in the untreated samples (see Table 26). The obtained data show that all three IBDM samples, so the control (original agent) as well as the bulb-light exposed and the UV-light exposed IBDM solutions, reduced the cell concentration to 0. In all cases, an IBDM concentration of 0.3% was lethal to 10^5 and 10^6 CFU of *E. coli*. The results of the IPM samples showed no significant differences in their efficacy. A cell concentration of $2.2*10^5$ CFU/ml was reduced to 22.7% with the control IPM, to 18.2% with the UV-light exposed IPM solution and to 25.0% with the bulb-light exposed IPM sample. In the case of $2.2*10^4$ *E. coli* cells, the percentage of viable cells was decreased to 25.0% with the control, to 20.5% with the UV-light exposed IPM and to 20.5% with the bulb-light exposed IPM.

Table 26: Results of the light-stability test with the derivatives IBDM and IPM. The cell concentrations of the negative controls (non-treated) are compared to the cell concentrations of treated samples. The control pyrazines were not exposed to light (original agent).

Cell concentration - negative controls		Cell concentration, survived [%]		
[CFU/ml]	[%]	Control - IBDM	UV-light-exposed IBDM	Bulb-light-exposed IBDM
$2.2*10^6$	100	0	0	0
$2.2*10^5$	100	0	0	0
[CFU/ml]	[%]	Control - IPM	UV-light-exposed IPM	Bulb-light-exposed IPM
$2.2*10^5$	100	22.7	18.2	25.0
$2.2*10^4$	100	25.0	20.5	20.5

4 Discussion

Pyrazine compounds, especially alkylated derivatives, are almost ubiquitously distributed in nature. They are found in vegetables and heated food, where they highly contribute to their flavour [2, 6, 7]. Pyrazine molecules are also widespread among animals such as insects and terrestrial vertebrates, where they are used as semiochemicals for intra- or interspecific communication [8]. Due to their broad aroma spectrum and their low olfactory thresholds, they are used as flavour ingredients in various food products [2, 64]. However, there is almost no information about an antimicrobial activity of alkylated pyrazines or the mode of action causing these effects. The aim of this thesis was to investigate the antimicrobial activity of alkylated pyrazine derivatives on facultative pathogenic microorganisms. Thereby, the focus was set on the understanding of the structure-activity relationship and the metabolic effects initiated by those pyrazine derivates.
Within this work, seven alkylated pyrazine derivatives were used (see Table 3), whereby the compound 5-isobutyl-2,3-dimethylpyrazine (IBDM) was used in several experiments as a model for the activity of these structural group. For studying the antimicrobial potential of the pyrazine derivatives, the bacterial strains *E. coli, S. aureus* and *P. aeruginosa* as well as the fungal strains *C. albicans*, *S. cerevisiae* and a *Penicillium sp.* were used.

4.1 Antibacterial activity of alkylpyrazines

Firstly, the objective was set on getting a deeper insight into the antibacterial activity of IBDM when treating *E. coli* cells. Thereby the relationship between the applied concentration of IBDM, the cell concentration and the incubation time of the antibacterial assay was studied in more details. The obtained results showed that the antibacterial efficacy of IBDM was enhanced with increasing application volume and increasing incubation time. However, it was observed that the concentration of IBDM had a greater impact on the bactericidal effect in the assay. The application of higher amounts of IBDM was more efficient than extending the incubation time. An IBDM concentration of 0.1% reduced 10^7 *E. coli* cells by a 1-log10-unit after an incubation of 6 h. In comparison, a solution of 0.2% IBDM was able to kill 7-log10-units of *E. coli* cells in 4 h and a 0.3% solution needed only 4 h for the same lethal effect.

In the next experiment it was examined if the pyrazine derivatives IBM, IPM, IB and IP exhibit antibacterial activity. Therefore, defined cell concentrations of *E. coli* (10^5 – 10^7 CFU) were treated for 4 h with solutions of 0.3% pyrazine. The findings revealed that all compounds tested in this assay showed a bactericidal effect. They were all able to kill 5-log10-units of *E. coli* cells during this treatment. The derivate IBM caused in all samples (10^5 – 10^7 CFU) a complete absence of viability and thus was the most active agents in this assay, followed by IB, IPM and IP.

4.2 Antifungal activity of alkylpyrazines

Spores of the fungus *Penicillium sp.* were treated with IBDM for 15 min, before they were transferred onto nutritive agar plates. The obtained results showed that plates with treated samples had less fungal growth comparing to the controls. These findings might be attributed to an inhibition of the *Penicillium* germination. Fungi like *Penicillium* are a big problem in food industry, because they spoil several food products such as baked goods. Moreover, some species of *Penicillium* produce harmful toxins and are able to provoke allergic reactions [109]. Due to the fact that IBDM showed an antifungal activity in this assay, it might be possible to use it for the minimization of fungal contamination and proliferation in food.

Additionally, the influence of IBDM on the growth behaviour of *S. cerevisiae* was examined. The incubation was carried out at 30°C for 12 h. A concentration of 0.3% of IBDM killed two-thirds of the yeast cells after 2 h of incubation. One third survived and this cell concentration stayed constant until the end of incubation. The applied pyrazine dose had a fungistatic effect. The remaining third of the cells was inhibited in growth by the residual concentration level of IBDM. The exposure to the double amount of IBDM (0.6%) caused a complete absence of viability after 1 h of incubation. In this case the applied pyrazine dose was lethal to all cells and consequently the effect was fungicidal.

4.3 Alkylpyrazines as potential disinfectants

It was tested if IBDM exhibits bactericidal and fungicidal activity within a contact time of 5 min. The most important facultative pathogens of nosocomial infections, which are hospital-acquired infections, are *S. aureus, E. coli, C. albicans* and *P. aeruginosa* [108]. Due to this fact, those microorganisms as well as the yeast

S. cerevisiae were used in this assay. In general it can be said that it was definitely possibly to achieve bactericidal and fungicidal effects within an exposure time of 5 min. By using a 1.2% IBDM solution, 10^6 cells of *E. coli, P. aeruginosa* and *S. cerevisiae* were killed. In comparison, for the gram-positive bacterium *S. aureus* a solution of 3.0% IBDM was required for the same effect. This fast-acting effect demonstrates that alkylpyrazines are potential candidates for disinfectants.

4.4 Comparison of the antimicrobial efficiency of selected alkylpyrazines

For a standardized evaluation and for more precisely comparison of the already tested pyrazine derivatives, the MIC and MBC for the bacteria *E. coli* and *S. aureus* as well as the MIC and MFC for the fungus *C. albicans* were determined. The MIC is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism under defined conditions [90]. The MBC is defined as the lowest concentration of the agent required for a 99.9% reduction of viable bacteria in the sample [91]. The MFC is defined as the lowest concentration of the agent required for a 99% killing of fungal cells after a distinct exposure time and subculture to antibiotic-free medium [94]. The pyrazine derivatives IBDM, IPM, IP, IB and IBM were tested. In general it can be said that the values were very close to each other. The effective concentrations ranged from 2 to 17 mg/ml. The following ranking regarding the antimicrobial efficiency of the investigated substances can be established: the pyrazine IBDM is the most effective derivate, followed by IBM, IB, IPM and IP. According the obtained data, the ranking is consistent with both bacterial strains tested as well as with the results of *C. albicans*.

4.5 Structure-activity relationship of alkylpyrazines

The ranking of the pyrazine derivatives may be explained by the hydrophobicity of these compounds. To compare the hydrophobicity of the derivatives, the log P values of the substances are listed in Table 27 [110]. The partition coefficient (log P) is a physicochemical parameter and defined as the logarithm of the ratio between the equilibrium concentrations of a substance dissolved in a two-phase system consisting of a polar, aqueous and non-polar, organic phase, usually water and 1-octanol [111]. The log P value is used for displaying the polarity of the

compound. A positive log P value indicates higher solubility of the tested substance in the hydrophobic phase than in the aqueous phase. As a consequence, the hydrophobicity increases with increasing log P value [112]. The pyrazine IBDM, which was the most effective derivate in this study, has the highest log P value and the derivate IP, the less effective derivate, has the lowest log P value [110]. The ranking regarding the antibacterial efficacy of the pyrazine derivatives perfectly consists with the ranking of the log P values.

Figure 14 demonstrates the correlation between the log P value of the compounds and their antimicrobial efficacy. The higher the log P values of the alkylpyrazines, the lower are the concentrations required to achieve the MIC for the respective microorganisms.

Table 27: Log P values of the pyrazine derivatives. The value is a measure of lipophilicity. The higher the value, the higher is the hydrophobicity of the substance [110, 112].

Pyrazine derivate	Log P value
IBDM	2.320
IBM	1.960
IB	1.715
IPM	1.626
IP	1.281

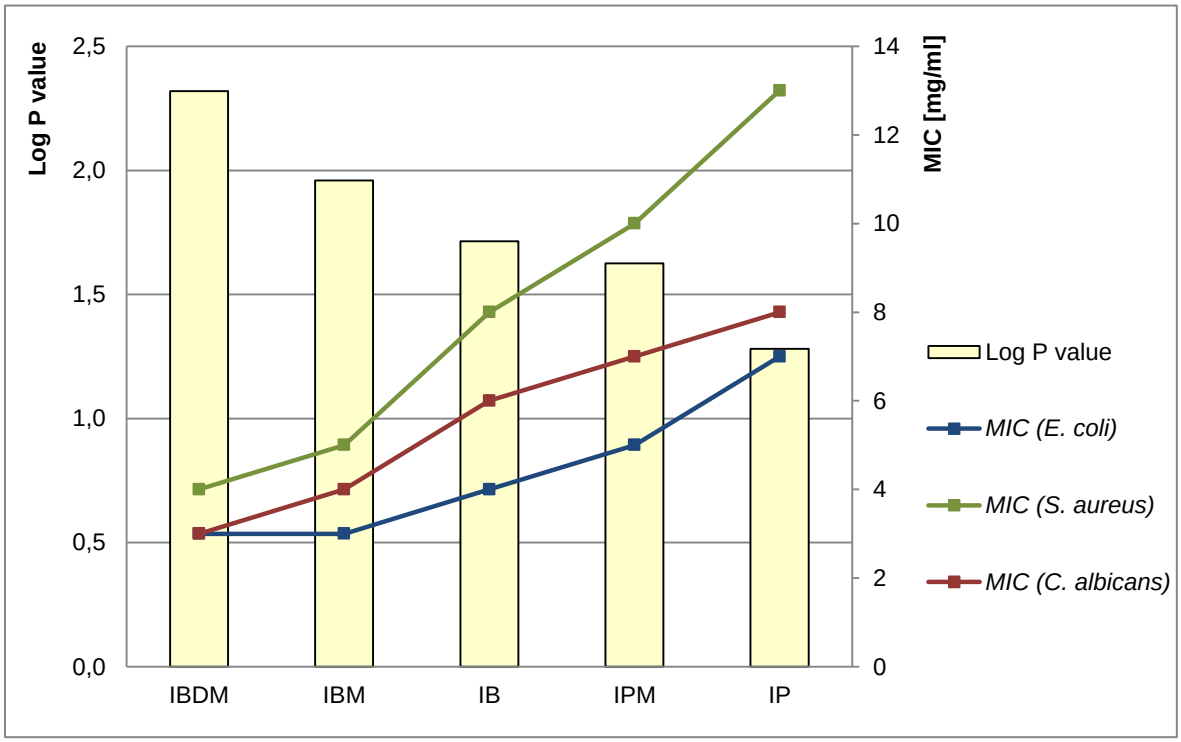


Figure 14: Correlation between the log P value of the pyrazine derivatives and their antimicrobial efficacy. The antimicrobial activity is demonstrated by the MIC values needed for the microorganisms *E. coli*, *S. aureus* and *C. albicans*.

1-octanol is supposed to be a good simulator of the polarity of a biological membrane lipid. Due to the hydrocarbon part of the alcohol it has an aliphatic character. Hydrophobic compounds diffuse easier into lipid-rich media such as cell membranes [112]. This fact might indicate that the cellular target of alkylated pyrazine derivatives might be narrowed to the membrane. Moreover, the derivatives required higher concentrations when treating *S. aureus*. This might be attributed to the fact that *S. aureus* is a gram-positive bacterium and *E. coli* is a gram-negative bacterium [113]. Gram-positive bacteria have a thicker and more rigid cell wall, which might make them more resistant to the penetration of the derivatives into the membrane [114].

Furthermore, the two derivatives 2,5- and 2,3-dimethylpyrazine were studied in more detail. 2,5-DMP is a rat pheromone and exhibits inhibitory effects on estradiol and thus on the uterus of rats [115]. Due to the fact that the compounds differ only in the position of one methyl group, the influence of structural composition of pyrazine derivatives on their antimicrobial activity was examined. 2,3-DMP exhibited within the experiment no bactericidal effect, in contrast to 2,5 DMP. However, the test was performed with a low *E. coli* concentration of 10^3 CFU/ml. Comparing the antibacterial efficacy of 2,5-DMP with the activity of other pyrazine derivatives already tested, the compound appears much less bactericidal. The low activity and the difference of the antibacterial effect of 2,3- and 2,5 DMP might be attributed to the log P value of the compounds. The values are relatively low, whereby the log P value of 2,5-DMP is slightly higher than the log P value of 2,3-DMP [110]. This fact might be the reason for the low but still detectable antibacterial activity of 2,5-DMP.

It is known that Tetramethylpyrazine (TMP) exhibits hematoencephlic barrier penetrability [116], and *in vitro* permeation across porcine buccal mucosa [117]. Due to this fact, a previous study has examined the interaction between pyrazine derivatives and the biological phospholipid membrane. Therefore, they generated a model of a membrane phospholipid bilayer and simulated the diffusion process of the pyrazines into the membrane. Due to the hydrophobic character of alkylated pyrazine derivatives, they could easily be dissolved in membrane lipid bilayers. Pyrazine molecules have an extended hydrophobic surface, which depends on the substitution pattern. The tested 2-methylpyrazine, 2,5-DMP, 2,3-DMP and TMP penetrated the bilayer up to a certain depth and stayed there. Depending on their

hydrophobicity they were located at different depth positions in the membrane. The unsubstituted pyrazine was found nearby the membrane surface, whereas the most hydrophobic pyrazine molecule (TMP) was staying at the deepest position. It was positioned in the bilayer center, where the free volume was at its maximum. These findings showed that the point of accumulation and lateral diffusion of pyrazine molecules depend on the number and length of substitutes on the pyrazine ring and on the free volume distribution in the bilayer [118]. According these results, the pyrazine molecules tested in this thesis should also have a large lateral mobility and a large diffusion coefficient for the penetration of biological membranes.

4.6 Cell membrane as potential target of alkylpyrazines

To verify if IBDM is a membrane-active antibacterial agent, different tests targeting the bacterial cell membrane were performed. Firstly, the *Bac*Light Bacterial Viability Kit was used. This kit is usually applied on bacterial enumeration and viability tests. However, the assay can also serve as rapid method for identifying membrane-active compounds [97]. The obtained results indicated that IBDM has an effect on the bacterial cell membrane. The higher the concentration of IBDM, the lower was the green/red fluorescence intensity ratio, which demonstrates a reduction in membrane integritiy. Due to increased cell membrane permeability, the uptake of propidium iodide is increased [95]. The fact that 0.8% IBDM caused a higher uptake of propidium iodide than the membrane-active isopropanol (70%) indicates that IBDM has membrane–damaging properties.

An important characteristic of bacterial cell membranes is the maintenance of a membrane potential. Due to the selective permeability and the concentration gradients of ions, such as sodium, potassium, chloride and hydrogen, a difference in electric potential between the inside and outside of a cell is generated. This membrane potential is about 100 mV or more and interior-negative. Due to the fact that it reflects the metabolic energy state and the integrity of the cytoplasmic membrane of bacteria, the influence of pyrazine derivatives on the membrane potential was examined [119, 120]. It has to be mentioned that treatments, which exhibit membrane-permeabilizing activity, do not necessarily affect the membrane potential [121]. The findings of the *Bac*Light Bacterial Membrane Potential assay showed that the compounds IBDM and IMB have an effect on the bacterial cell

membrane potential. The higher the concentration of IBDM or IMB used, the stronger was the depolarization of the membrane potential [98]. A depolarization occurs if the membrane potential drives from a negative value to zero [120]. The depolarization effect was higher when treating the cells with IBDM. Eliminating the proton gradient and thereby changing the membrane potential might be one mechanism of the antimicrobial activity of alkylated pyrazine derivatives.

Additionally, it was verified if IBDM has a permeabilization effect on the inner membrane of bacterial cells. Therefore, a β-galactosidase assay was performed. In this test the disruption of the inner membrane is detected by the leakage of the cytoplasmic enzyme β-galactosidase. The activity of this enzyme is determined by the conversion of ONPG into the yellow product o-nitrophenol, which is detected at 415 nm [100]. The positive control (70% isopropanol) demonstrated the highest absorbance value. The results of the IBDM treated samples provided two findings. On the one hand, a permeabilizing effect was achieved with an IBDM concentration of 10 and 20 mM. The absorbance values were higher than the absorbance of the negative control (untreated cells). On the other hand, IBDM concentrations of 50 and 100 mM had lower absorbance values than the negative control. The higher the IBDM concentration used, the lower was the amount of o-nitrophenol. This result might be attributed to a lower conversion rate of the reaction due to enzyme inhibition. IBDM was probably permeabilizing the inner cell membrane and additionally, it was inhibiting the enzyme. IBDM concentrations of 10 and 20 mM were possibly high enough to achieve a disruption of the inner membrane, but too low to inhibit most of the β-galactosidase enzymes. This might resulted in a higher production of o-nitrophenol. Although it is thought that IBDM concentrations of 50 and 100 mM had a permeabilizing effect, it probably could not be detected, due to the fact that most of the β-galactosidase enzymes were inhibited.

4.7 Interaction of alkylpyrazines with proteins

On this basis, the role of proteins was studied in more details. Therefore, a possible interaction of IBDM with cellular proteins of *E. coli* was tested. Thereby, the proteins were separated in four size fractions to verify if IBDM is reacting with a certain protein or enzyme class of *E. coli,* respectively. All protein mixtures with

IBDM showed the same visual changes. These observations suggest that IBDM is not interacting with a certain cellular protein, but with several.
Next, it was verified whether the pyrazine derivatives IBDM, IBM, IB, IPM and IP undergo an interaction with BSA. At room temperature no effects were visible. When incubating the samples at 37°C with agitation, a reaction occurred. For each derivate the samples got milky-white turbid and the viscosity of the mixtures was increased. However, the reaction occurred for the different derivatives varyingly strong and fast. A ranking regarding the reactivity with BSA can be established: (1) IBDM, IBM, (2) IB, (3) IPM and (4) IP. Due to the heat, the proteins got probably slightly unfolded and the derivatives are able to bind reversibly to these structures. The turbidity might be attributed to the formation of these pyrazine-protein complexes. Interestingly, the ranking regarding the reactivity degree with BSA is consistent with the ranking of the antimicrobial efficacy of the derivatives, which depends most likely on the hydrophobicity of the derivatives.

Moreover, it was tested if the presence of BSA has an influence on the efficacy of IBDM. Therefore, an antibacterial assay was performed by adding different BSA concentrations to the IBDM reaction mixtures. The obtained data showed that the application of BSA had an effect on the antibacterial activity of IBDM. The efficacy of IBDM got reduced with increasing BSA concentrations. This antagonising effect might be attributed to the formation of intermolecular bonds between the protein and IBDM. IBDM possibly gets caught by the proteins and only the unbound, free compound might be able to penetrate through the cell wall. The binding of a pyrazine molecule by BSA was also observed in a previous study with pyrazinamide (PZA), an anti-tuberculosis drug. The MIC of PZA and its active derivate pyrazinoic acid for *Mycobacterium tuberculosis* was increased due to the presence of BSA. However, the interaction was not studied in more details [122].

4.8 Mode of action of alkylpyrazines

It is known that lipophilic compounds such as terpenes, alkanes, alcohols and phenols accumulate due to their physical properties in biological membranes. They interact with the membrane by affecting the phospholipid bilayer and/or membrane proteins. This can lead to a decrease of the membrane integrity and an increase of the permeability, respectively. These effects are able to cause alterations of the

membrane potential. The membrane damage mechanism can be based on the disturbance of hydrophobic interactions between lipids and proteins. Membrane-bound and –embedded proteins can be affected directly, if the compound is interacting with hydrophobic parts of the proteins or indirectly by alterations of the membrane structure. Thereby, significant enzymes like transport proteins, ATPases, transferases, oxidoreductases or signal-transducing enzymes could be inactivated [123]. For alcohols it was observed that their potential of disrupting the membrane integrity and thereby their degree of toxicity is directly correlating with their hydrophobicity [124].

Further studies dealt with the effects of the alkylpyrazine derivatives tetramethyl-, triethyl- and tetraethylpyrazine on biological membranes. Within the experiments plasma membrane-enriched microsomal fractions isolated from the smooth muscle of dog aorta and model systems with synthetic phospholipids were used. It was observed that the membrane fluidity increased due to the dissolution of the pyrazine molecules in the lipid bilayer. Moreover, they suggested that pyrazine molecules are interacting with membrane proteins, such as ion channels and membrane receptors, since TMP is affecting ATP-dependent K^+ channels, voltage dependent Ca^{2+} channels and Na^+ channels. It was proposed that the effects on these membrane proteins are intensified with increasing bulkiness and hydrophobicity of the pyrazine derivatives [125, 126]. However, an antimicrobial activity of those three pyrazine compounds was not mentioned or studied in any regard.

The findings and data obtained in the performed experiments of this master thesis lead to the assumption that alkylated pyrazine derivatives are targeting the cell membrane. Due to their physico-chemical properties and hydrophobic character they can easily penetrate into the bilayer of cell walls [118]. It was observed that gram-positive bacteria are more resistant to the penetration. Testings regarding the structure-activity relationship showed that the antimicrobial efficiency of the pyrazine molecules is directly proportional to their log P value. The higher their hydrophobicity, the larger is their lateral mobility and diffusion coefficient for the penetration of biological membranes and the bigger is their degree of toxicity [118]. Inserted in the membrane, the pyrazine compounds might interact with membrane proteins such as ion channels and pumps, membrane receptors or other enzymes. Results in this study showed that the reactivity degree of the derivatives interacting

with proteins such as BSA is correlating with their hydrophobicity too. Moreover, it was interpreted that IBDM inhibited the enzyme β-galactosidase in the β-galactosidase assay. The interference with membrane proteins may cause a disorder of the membrane structure and organization, leading to a deformation of the membrane integrity and an increase of the permeability. Consequently, these effects might increase the passive flux of protons across the membrane and lead to the dissipation of proton motive force. These membrane-damaging effects might kill the cells after a short exposure to alkylated pyrazine derivatives.

4.9 Enhanced sensitivity of *E. coli* mutants to IBDM

Furthermore, the Dharmacon *E. coli* Keio Knockout Collection, which consists of 7980 knockout strains, was used in this study. Therefore, the whole library was replicated first. The experiment was carried out with a selected group of mutants. A defined cell concentration was treated with different concentrations of the derivate IBDM. Thereby, it was examined whether these clones were more resistant or sensitive to IBDM compared to the wild-type strain. Seven out of the 42 tested mutants showed an increased sensitivity to IBDM (see Table 23 and Table 24). While the wild-type showed a cell concentration of $8*10^6$ CFU/ml after an exposure to 2 mg/ml IBDM, the number of viable cells of the sensitive mutants was reduced to 0. Five of the seven less tolerant mutants belong to the group of rfa-genes. The products of these genes are involved in the biosynthesis of the inner core region of lipopolysaccharides (LPS). LPS are glycolipids, which are situated in the outer membrane of gram-negative bacteria. The outer membrane serves as a diffusion barrier for hydrophobic molecules and thus has a protective function. Mutations, which affect the LPS structure, lead to an alteration of the integrity of the outer membrane and thereby the permeability is increased. As a consequence, the outer membrane diffusion of hydrophobic molecules like IBDM got easier and the mutated bacterium reacted hypersensitive to the compound [105]. Furthermore, it was experimentally shown that the gmhB-mutant had a lower tolerance to IBDM. The gene gmbH encodes an enzyme, which is involved in the biosynthesis of ADP-L-glycero-β-D-manno-heptose. This component is a pre-cursor of the inner core region of LPS. The deletion of the gene gmhB initiates modifications of the LPS structure, which led to an increased sensitivity to

IBDM [106]. Enhanced sensitivity was also observed for the pal-mutant. Pal is a peptidoglycan-associated lipoprotein and positioned in the outer membrane. The deletion of this gene leads to a destabilization of the integrity of the outer membrane. As a consequence, bacterial cells reacted hypersensitive to IBDM [107].

Data obtained from this experiment might be helpful for further product development. A possible strategy could be the combination of organic volatile compounds with agents, which function as permeabilizer of the outer membrane. Thereby, the sensitivity of gram-negative human pathogens like *Escherichia coli* or *Pseudomonas aeruginosa* to these aromatic compounds could be increased. As a consequence, the antimicrobial efficacy of the volatiles would be enhanced. For the future it would be interesting to test the whole *E. coli* Keio Knockout Collection, to get complete sensitivity and resistance profiles. The results might give a more detailed understanding of the mode of action of alkylated pyrazine derivatives. Moreover, these profiles might be a supportive resource for the development of product formulations and application approaches.

4.10 Effect of abiotic factors on alkylpyrazines

In addition, the influence of the abiotic factors temperature and light on the stability of pyrazine molecules was examined. To test the effect of temperature, IBDM solutions were stored for 24 h at different temperatures. A destabilizing effect of the molecules structure and thereby a reduction of the antibacterial efficacy of IBDM, due to storage at different temperatures, was not detected. Samples, which were stored at -20°C, 2°C, 22.5°C and 37°C, had the same antibacterial efficacy. The IBDM sample, which was kept at 60°C for 24 h, showed no antibacterial activity, because 77% of IBDM evaporated during the storage. For further experiments or applications, the evaporation of the volatile pyrazine derivatives at higher temperature should be kept in mind.

The stability of pyrazine molecules in relation to light exposure was also verified. Pyrazine solutions were placed under an UV-lamp for 24 h or under an average light source for four days. The antibacterial efficacy of these samples was compared to the activity of the non-light-exposed pyrazines. The obtained data revealed that all pyrazine samples had the same antibacterial efficacy. These facts

indicated that the given lighting conditions had no observable destabilizing effects on the molecules of those two pyrazine derivatives. The exposure to light did not influence the antibacterial efficacy of the compounds. To confirm the results, further experiments could be carried out, in which the exposure to light is intensified.

4.11 Future prospects

Within this thesis it could be proved that naturally occurring alkylated pyrazines possess promising antimicrobial activities, which open them the doors to a broad field of application. Their ability to kill the most important facultative pathogens of nosocomial infections within an exposure time of 5 min, make them suitable candidates for applying them as disinfectants. There was a maximum of 3.0% IBDM solution required to kill 6-log10-units of the microorganisms tested. The application as disinfectant could be investigated in more details by performing specific surface-disinfection experiments.

Furthermore, potential antifungal capacity against *Penicillium sp.*, *C. albicans* and *S. cerevisiae* was demonstrated by using IBDM. Due to the fact that fungi are the most common spoilers in food products such as baked goods [109], it could be contemplated to use alkylpyrazines as food preservatives. Thereby, the incorporation of the volatile compounds in the food package, called active packaging, could be a possibility. On the one hand, the pyrazine molecules might prolong the shelf-life of food products by controlling microbial growth. On the other hand, it may be possible to use them additionally as flavour ingredients, due to the fact that pyrazines are applied as flavour additives in numerous foods, including baked good and beverages [64]. As a consequence, two birds could be killed with one stone. Natural occurring alkylated pyrazine compounds might be an interesting alternative to the use of synthetic preservatives such as sorbate or propionic acid [127].

Moreover, the potential of using alkylpyrazines as antimicrobial pharmaceuticals has to be further investigated by carrying out *in vivo* experiments as well as toxicity studies.

For the further exploration of the mode of action of alkylated pyrazine derivatives, it would be interesting to examine the expression profile of treated cells via transcriptome analysis. Additionally, the use of GFP-tagged membrane proteins or the performance of electron microscopy could be promising approaches. Another possibility could be the labelling of an alkylpyrazine with isotopic tracers to follow its pathway and interactions in the cell.

Furthermore, the attainment of complete sensitivity and resistance profiles by testing the whole *E. coli* Keio Knockout Collection might be a supportive resource for the development of product formulations and application strategies.

The study revealed that the antimicrobial efficiency of alkylated pyrazines increases with their hydrophobicity. This information could be highly useful for the identification of other naturally occurring, antimicrobial active alkylpyrazines as well as for the engineering of new derivatives. The chemical synthesis of higher alkylated pyrazine molecules would be a promising possibility.

5 Conclusion

In conclusion, this study enhanced the knowledge of the antimicrobial potential of natural occurring alkylated pyrazine derivatives. It was demonstrated that these compounds exhibit bactericidal as well as fungicidal activity against facultative pathogenic microorganisms including *S. aureus, E. coli, P. aeruginosa* and *C. albicans.* Furthermore, the structure-activity relationship was studied in more details. It was shown that the antimicrobial efficiency of the investigated derivates increases with their hydrophobicity, which is reflected by their log P values. The reactivity degree of the derivatives interacting with proteins such as BSA is also correlating with their hydrophobicity. Additionally, the bactericidal efficiency could be reduced due to the presence of BSA. Moreover, it was interpreted that IBDM has enzyme inhibiting activities. Fluorescence assays revealed that alkylated pyrazines exhibit membrane-damaging effects by disrupting the inner plasma membrane and by depolarizing the membrane potential. All these findings lead to the assumption that alkylated pyrazines are targeting the cell membrane. Due to their hydrophobic character and their relatively small molecule dimension, they penetrate easily into the bilayer of cell membranes [118]. Inserted in the membrane, the compounds might interact directly with membrane proteins such as ion channels and pumps, membrane receptors or other enzymes, or indirectly by altering the lipid environment. This interference may cause a deformation of the membrane integrity and an increase of the permeability, leading to an increased passive flux of protons and thus to the disruption of the membrane potential. These membrane-damaging effects might be lethal to bacterial and fungal cells after a short exposure to alkylated pyrazines. In addition, the study revealed that alkylpyrazines are promising antimicrobial agents for the application as disinfectants, food preservatives or antimicrobial pharmaceuticals.

6 References

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7 Appendix

7.1 List of abbreviations

%	percentage
µ	micro
BSA	bovine serum albumin
CFU	colony-forming unit
dH_2O	distilled water
DMP	dimethylpyrazine
DMSO	dimethylsulfoxid
e.g.	exempli gratia
etc.	et cetera
g	gram
h	hour
HCl	hydrochloride
IB	2-isobutylpyrazine
IBDM	5-isobutyl-2,3-dimethylpyrazine
IBM	2-isobutyl-3-methylpyrazine
IP	2-isopropylpyrazine
IPM	2-isopropyl-5-methylpyrazine
K_3PO_4	tripotassium phosphate
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
l	liter
LB	Luria-Bertani broth
m	milli
M	molar
MBC	minimum bactericidal concentration
MFC	minimum fungicidal concentration
$MgSO_4$	magnesium sulphate
MHB	Müller-Hinton-Broth
MIC	minimum inhibitory concentration
min	minute

n	nano
$Na_2HPO_4*2H_2O$	disodium hydrogen phosphate dihydrate
NaCl	sodium chloride
NaOH	sodium hydroxide
OD	optical density
ONC	overnight culture
PBS	phosphate-buffered saline
rcf	relative centrifugal force
rpm	rounds per minute
RT	room temperature
TMP	tetramethylpyrazine
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

7.2 List of tables

7.3 List of figures