Isolation and Characterization of New Triethylamine and Ethyl Acrylate Degrading Bacteria

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In Loving Memory of My Father

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INTRODUCTION

1.1 The revolution of biotechnology

The foundation for what has since been named biotechnology was established by the geneticist and evolutionary biologist JBS Haldane more than half a century ago, a visionary polymath (Prentis, 1989). The term "biotechnology" is frequently refered to in contemporary biology (Brown *et al*., 1988) but is variously defined as the application of biological organisms, systems or processes to the manufacturing and service industries (Smith, 1990); and as the controlled application of simple biological agents, such as living or dead cells, or cell components, to technically useful operations, either in manufacture or as service operations (Bu'lock and Kristiansen, 1987). Biotechnology integrates biology, microbiology, biochemistry, chemical engineering and process engineering, together with other disciplines, to optimize the exploitation of their potential (Bu'Lock and Kristiansen, 1987; Smith, 1990). The European Federation of Biotechnology (EFB) has defined biotechnology as "the integration of natural sciences and engineering in order to achieve the application of organisms, cells, parts thereof and molecular analogues for products and services" (EFB General Assembly, 1989; Qoura, 2006).

Examples of biotechnology can be traced back thousands of years. Wine and beer are known to have been produced using microbes (yeast extract) prior to 6000 BC, and leavened bread was similarly produced using yeast extract by 4000 BC (Prentis, 1989; Smith, 1990). A more recent example from the sixteenth century is the harvesting of algae from lakes to be used for food by the Aztecs (Smith, 1990; Qoura, 2006).

A first step in the understanding of biotechnology was made in the late 16th and early 17th centuries, when microbes were first observed by Antoni van Leeuwenhoek using his newly designed microscope. Later, during the 18th century, Louis Pasteur, who is known as the father of microbiology, demonstrated the use of microorganisms in fermentation and could also be considered as the father of biotechnology. In the early 20th century Eduard Buchner,

earned the Nobel Prize in chemistry for his further work on fermentation and his demonstration that the enzymes extracted from yeast extract could convert sugar into alcohol (Prentis, 1989; Smith, 1990; Qoura, 2006).

In the first half of the 20th century, the application of microbes to large-scale sewage purification was established, Alexander Fleming discovered penicillin, and the double helix structure of DNA was revealed. Later, and particularly during the seventies, genetic engineering experiments were first undertaken, marking the beginning of applied genetics and recombinant DNA technology. In addition, the technology for the production of monoclonal antibodies evolved (Prentis, 1989; Smith, 1990; Qoura, 2006).

Over the previous decades, biotechnology has been developing very rapidly and has been applied to all aspects of life. The importance of biotechnology lies in: Livestock husbandry and animal health, the production of food crops, chemical and pharmaceutical industries, the accurate diagnosis and prevention of human diseases, the conversion of biomass into energy, the transformation of wastes and agricultural and industrial bio-products, pollution control and environmental sanitation (Bull *et al*., 1982; Hacking, 1987; Yanchinski, 1985; Zimmerman, 1984a,b; Qoura, 2006).

1.2 Odour and odorous compounds

1.2.1 Definitions

Odour may be defined as a physiological stimulus of the olfactory cells in the presence of specific molecules. The nature and concentration of molecules detected by olfactory cells varies between individuals and with environmental conditions, such as humidity, pressure and temperature. According to this definition, the term odour includes volatile organic compounds (VOCs) as well as some inorganic compounds (VICs) (Kennes and Veiga, 2001; Rappert and Müller, 2005b).

Without a clear understanding of what odour is, how to measure it, and where it originates, it will be difficult to control the odour (Mackie *et al*., 1998; Zhu, 2000).

Moeser *et al.* (2003) reported that the response to the odour depends on individual odour perception (Rappert and Müller, 2005a).

Poor odour control and prevention of environmental problems are related to a lack of knowledge of the fundamental nature of odour and its production. A quantitative description

of environmental odour exposure is limited both by the complexity of chemical mixtures and by the sensitivity of the human nose (Rappert and Müller, 2005a).

1.2.2 Sources

Generation of odours is a complex process that involves many bacterial species (Rappert and Müller, 2005a).

Biosolids are an abundant source of food for microorganisms, including proteins, amino acids, and carbohydrates. The microorganisms degrade these energy sources, and odorous compounds are formed (Rappert and Müller, 2005a).

Dimethyl disulfide (DMDS) is produced by many bacteria and fungi found in waste water (Tomita *et al*., 1987; Sunesson *et al*., 1995). The methylation of sulfide may be responsible for its emission (Tomita *et al*., 1987; Kelly *et al*., 1994; Ginzburg *et al*., 1999; Rappert and Müller, 2005a).

Trimethylamine (TMA) is always present in the highest concentration compared with other atmospheric amines (ca. 7-folds). This is possibly because of the fact that trisubstituted amines are less readily attacked by the microorganisms during the protein catabolism than monoamines (Rosenfeld *et al*., 2001). Trimethylamine (TMA) is frequently found in effluents of fish meal manufacturing processes (Sandberg and Ahring, 1992; Hwang *et al*., 1994; Rappert and Müller., 2005a).

1.2.3 Food processing

Odours generated in food processing plants are usually a low concentration mixture of various organic and inorganic compounds. Most of these compounds are reduced carbon, nitrogen, and/or sulfur compounds, such as aldehydes, ketones, alcohols, acids, ammonia, amines, sulfides, mercaptans and hydrogen sulfide, which are easily biodegraded. In some cases the odours may also be caused by volatile organic compounds, which are less biodegradable (Rappert and Müller, 2005a).

Amines, nitriles, hydrocarbons and aldehydes result from decarboxylation and deamination of amino acids at very high temperatures, significantly above the boiling point of water (Mottram, 1991; Rappert and Müller, 2005a).

A number of volatile compounds in food may be produced by microbiological processes. Microorganisms (fungi, yeast extracts, bacteria) produce volatile compounds during both

primary and secondary metabolisms from a wide variety of starting compounds (e.g., acetate, amino acids, fatty acids, and keto acids) as de novo biosynthesis products and secondary metabolites (Rappert and Müller, 2005a).

Objectionable odours in the food industry are generally a result of the physical processing of foods (such as heating, drying, or smoking of food). Typical odorous compounds encountered in food processing include aldehydes, ketones, lactones, alcohols, acids, esters, ammonia, amines, pyrazines, sulfides, mercaptans, and hydrogen sulfide, which are not toxic and easily biodegradable (Rappert and Müller, 2005a).

An ester's odour is described as fruity, berrylike, and floral, whereas an amine's odour is described as fishy (Rappert and Müller, 2005a).

1.2.4 Volatile organic compounds

VOCs are organic hydrocarbons that can volatilize to form an organic vapour in air. Hydrocarbons are molecules that consist of hydrogen and carbon. Hydrocarbons with 1 to 4 carbon atoms exist in the gas phase at ordinary temperature and pressure. Hydrocarbons with 5 to 12 carbon atoms exist in the liquid or solid phases at ordinary temperature and pressure. Hydrocarbons with more than 12 carbon atoms do not volatilize sufficiently to reach atmospheric concentrations in the gas phase (Waldbott, 1973; Rafson, 1998).

1.2.5 Exposure to VOCs

Exposure to volatile organic compounds (VOCs) occurs in the workplace. They are also the most common accidental release in the outdoor environment. By definition, it is the volatility of these organic compounds which renders them most susceptible to inhalation by exposed workers. This most commonly occurs in manufacturing or other industries which rely on the VOCs to perform a number of functions, including degreasing, lubricating, fueling, stripping or thinning paint, and cleaning (Rafson, 1998).

Because of their lipid solubility, VOCs are readily absorbed by the lung, gastrointestinal tract, and, in liquid form, the skin. They may bioaccumulate in lipid tissues in the body. In general, most of the internal dose is eliminated quickly, although a small fraction is eliminated more slowly. Cigarette smoking is a major source of VOCs found in the body and is the major confounder in studies of workers and potentially exposed citizens (Ashley *et al*., 1996).

Most chemical solvents and all the VOCs are known to cause both acute and chronic Central Nervous System (CNS) disease. In addition, the fat-soluble VOCs easily move into the

airways and across the lung tissue into the bloodstream. Many of them have a local, irritating effect on the airways and can cause irritation of the respiratory tract, leading to (1) throat irritation and coughing in mild exposure, (2) irritation of the airways, leading to spasm and wheezing which may be transient or permanent, or (3) damage to the lung itself, leading to leakage of fluid into lung tissue and impairing diffusion of oxygen across the tissue (Rafson, 1998).

VOCs may also cause liver disease. In some cases, the liver may create metabolic products which are more toxic than their parent compounds. All the VOCs can be irritating to the skin, particularly when contacted in liquid form. Formaldehyde has been shown to cause an allergy-based contact dermatitis. Because of their fat solubility, and after damaging protective layers of skin, the VOCs are readily absorbed through the skin into the bloodstream (Rafson, 1998).

1.2.6 Odour treatment

The basic principles for controlling odours are reduction of odours at the generation sources and removal of odours from collection air-stream before the odours are discharged into the atmosphere. The major techniques for removing odorous compounds from the exhaust air stream include mist filtration, absorption (scrubbing), adsorption, thermal oxidation/incineration, chemical oxidation, and biological oxidation (biofiltration, biotrickling filtration, bio scrubbing) (Rappert and Müller, 2005a).

Although many technologies exist, biofiltration still is the most attractive method due to its low maintenance and operating costs (Sheridan *et al*., 2003). Biofilters are generally used to clean the air exiting the building through exhaust fans. However, biofilters have only been efficient at treating low concentrations of odorants from waste exhaust air. Aeration, the basic principle of this treatment is to provide, by whatever means, enough dissolved oxygen to aerobic bacteria so they can actively decompose the odorous compounds; hence achieving odour reduction (Rappert and Müller, 2005a).

A biofiltration system consists mainly of a reactor packed with solid materials (*e.g*. peat, perlite, compost, wood chips) on which a biofilm is formed, given the proper microbial population. When a contaminated air stream passes through the reactor, the pollutants are transferred to the biofilm where they are biodegraded to simple end products such as water and carbon dioxide (Adler, 2001). A consortium of microbial populations is known to play an important role in this process, but current understanding of the mechanisms and specific microbial enzymes involved is limited (Burgess *et al*., 2001**;** Torkian *et al*., 2005).

Studies on biofiltration over the last several decades have primarily been focused on odorous compounds such as hydrogen sulphide, ammonia, mercaptanes, etc. (Strikauska *et al*., 1999; Wani *et al*., 1999; Busca and Pistarino, 2003). However, adoption of stricter emission policies in recent years has greatly increased the inventory of compounds subject to regulation. Furthermore, increasing costs of chemicals and disposal of hazardous wastes have provided further incentive for development and optimization of biological treatment methods. Biofiltration has emerged as one of the cost effective biological air pollution control technologies for treatment of volatile organic compounds (VOCs) emitted from chemical and process industries (Yoon and Park, 2002; Torkian *et al*., 2005).

1.2.7 Analysis technique

Gas chromatography (GC) is the technique most commonly applied to separate and identify volatile and gaseous samples. This method provides the accurate concentration of specific compounds in a sample and can be used on-site and for continuous assessment (Rappert and Müller, 2005a).

1.3 Ethyl acrylate

1.3.1 Properties

Ethyl acrylate ($C_5H_8O_2$) is a colourless organic compound with a sharp, acrid penetrating odour. The odour threshold for ethyl acrylate ranges from 0.001 to 0.005 parts per million (ppm) parts of air. The vapour pressure for ethyl acrylate is 40 mm Hg at 26 ºC, and its log octanol/water partition coefficient (log K_{ow}) is 1.33. It is also called carbonyl ethylene, 1-propenoic acid ethyl ester, 2-propenoic acid ethyl ester, ethyl-2-propenoate, ethyl propenoate (IUPAC name), ethyl acrylic ester, acrylic acid ethyl ester and ethoxycarbonyl ethylene (Online 1; 2; 6; 7; 12; 15).

Ethyl acrylate is flammable, highly reactive and slightly soluble in water and is completely miscible with ether, chloroform and alcohol. Elevated temperatures may cause polymerization and contact of ethyl acrylate with oxidizers, peroxides, polymerizers, strong alkalies, or moisture causes a violent reaction. When heated to decomposition, ethyl acrylate emits smoke and acrid fumes (Online 1; 2; 4).

Ethyl acrylate is used in the production of polymers including resins, plastics, rubber and denture material. It has been produced commercially since the early 1930s. Occupational exposure occurs in the manufacture of ethyl acrylate and in the manufacture and use of its

emulsion polymers. It is also used as a synthetic flavouring substance and fragrance adjuvant in consumer products, but it should be noted that its use as a food additive has been steadily decreasing since the early 1980s (Online 2; 3; 10).

1.3.2 Occurrence

Ethyl acrylate occurs naturally in some fruits: Blackberries, raspberries, pineapples and yellow passion fruit (BUA, 1995). Ethyl acrylate levels in these fruits are very low, with pineapples having ethyl acrylate concentrations of 0.77 mg/kg (IARC, 1986; Online 6).

1.3.3 Applications

Ethyl acrylate is used to form paint coatings that are resistant to water, sunshine and weather. These coatings retain flexibility even at low temperatures. Ethyl acrylate is also used in industrial finishes and coatings for cans and coils. Fabrics gain texture and durability when ethyl acrylate is added during their manufacture. Ethyl acrylate also imparts dirt resistance, improves abrasion and binds pigments to fabric. Paper is coated with ethyl acrylate to make it water-resistant. Magazines, books, business paper, frozen-food packaging and folding boxboards have such coatings, making them resistant to water, grease and oil. Ethyl acrylate is also used in adhesives for envelopes, labels and decals. Caulk, glazing and various sealants also contain ethyl acrylate. Leather products, such as automotive upholstery, furniture, clothing and shoes contain ethyl acrylate so that topcoatings do not migrate. Ethyl acrylate is also used as a fragrance additive in various soaps, detergents, creams, lotions, perfumes and as a synthetic fruit essence (IARC 1986). Ethyl acrylate is also found in such household items as nail mending kits and in medical items that assist with the binding of tissues, sealing wounds and ileostomy appliances (Online 6).

1.3.4 Preparation

Ethyl acrylate can be prepared by several industrial methods. Acrylonitrile can be reacted with ethanol using sulphuric acid as a catalyst to produce ethyl acrylate. It may also be prepared from acetylene, carbon monoxide and ethanol (Online 2).

The direct esterification of alcohol with acrylic acid is the only significant route for the production of esters (Figure 1.1). Some smaller volume esters are produced through transesterification. It is catalyzed by an acid such as sulphuric acid or a sulfonated styrene

DVB copolymer. To optimize the catalyst performance different degrees of crosslinking and porosities are used to ensure high selectivity and conversion rate (Online 17).

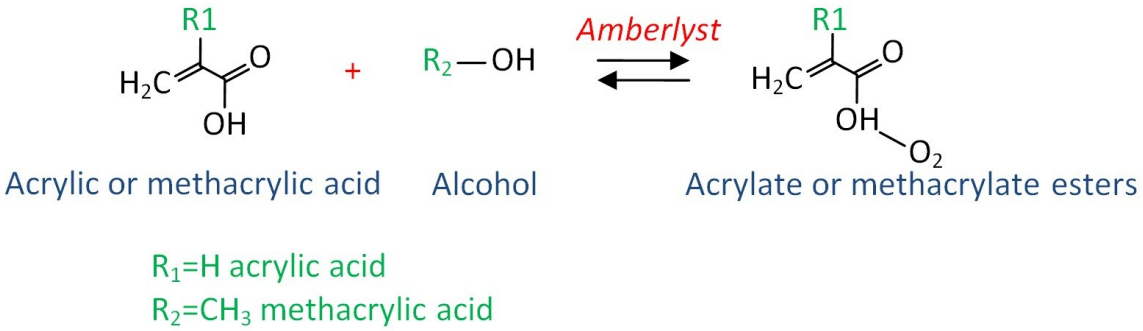

Figure 1.1: Direct esterification acrylic acid with sulphonated styrene DVB copolymer (Online 17)

Process for production of ethyl acrylate: A process is provided for the continuous production of ethyl acrylate from ethylene and acrylic acid in the presence of sulphuric acid. The reaction is believed to involve the formation of intermediate sulphates from the reaction of ethylene with sulphuric acid. These sulphates further react with acrylic acid to form ethyl acrylate. The process includes the removal of impurities from the reaction system by passing minor portions of sulphuric acid residue from the process through a wiped-film evaporator whose initial sections are heated to a specific temperature range and whose last or withdrawal sections are cooled to a specific temperature range. The treated sulphuric acid residue is withdrawn from the reaction system (Online 13).

The addition of a polymerization inhibitor is generally desirable when producing or purifying ethyl acrylate. Such inhibitors are known, and can be materials absolute in the reaction medium or soluble in the product obtained from the recovery distillation tower. Suitable polymerization inhibitors include hydroquinone, phenothiazine, the methyl ether of hydroquinone, quinine and the like (Online 13).

1.3.5 Exposure

Widespread human exposure to multifunctional acrylates is of concern, due to their inherent reactivity and irritating properties. Trimethylolpropane triacrylate (TMPTA) and pentaerythritol triacrylate (PETA) are industrially important representatives of multifunctional acrylates (Online 14).

Exposure to ethyl acrylate can occur through inhalation, ingestion and eye or skin contact. It is a strong irritant of the eyes, skin, mucous membranes, respiratory system and gastrointestinal tract in humans (Clayton and Clayton, 1981-1982; Hathaway *et al.* 1991). At a concentration of 50 ppm, for a period of exposure described only as “prolonged”, exposure to ethyl acrylate caused drowsiness, headache, and nausea (ACGIH 1991; Hathaway *et al.* 1991). Ethyl acrylate causes sensitization in some exposed individuals; a 4 percent concentration in petroleum jelly caused skin sensitization in 10 of 24 volunteers (ACGIH 1991; Hathaway *et al*. 1991; Online 1).

Acute exposure to ethyl acrylate vapour may cause irritation of the eyes, nose, and throat, with tearing, runny nose and burning of the throat. While chronic exposure to ethyl acrylate may cause skin sensitization, with redness, swelling and itching of the affected areas (DFG, 1994; Potokar *et al*., 1985; Online 1; 16).

Following inhalation exposure, ethyl acrylate is hydrolysed by carboxylesterases to acrylic acid in the nasal cavity (Frederick *et al.*, 1994). Resorption is higher in the upper respiratory tract than in the lower respiratory tract (Stott and McKenna, 1984). After oral administration (gavage) ethyl acrylate is rapidly absorbed and distributed into all major tissues of rats. The major route of excretion after oral application is exhalation of CO_2 (about 70% of the administered dose) followed by urinary excretion of mercapturic acids, degradation products of GSH conjugates (Ghanayem *et al.*, 1987; Online 16).

Carcinogenicity: Ethyl acrylate was first listed in the National Toxicology Program (NTP) Fifth Annual Report on Carcinogens as reasonably anticipated to be a carcinogen based upon a gavage study resulting in dose-related forestomach benign and malignant neoplasms in rats and mice (NTP 1998; Online 6; 7).

It was reported that an ex**c**ess of colon and rectum cancers ($p = 0.0001$) was found in a group of 3934 workers exposed to ethyl acrylate prior to 1946 compared with unexposed workers. However, this study is confounded by the fact that there was concurrent exposure to a number of other chemicals (unquantified); ethyl acrylate comprised approximately 10% of all the chemicals manufactured at the plant (Online 10).

No data are available to evaluate the reproductive effects or prenatal toxicity of ethyl acrylate to humans and also no case report or epidemiological study is available to evaluate the carcinogenicity of ethyl acrylate to humans. On the other hand, there is sufficient evidence for the carcinogenicity of ethyl acrylate in experimental animals (Online 3).

In one experiment in rats, oral administration of ethyl acrylate produced signs of embryotoxicity and foetotoxicity at mildly maternally toxic doses but did not increase foetal malformation. It was not embryotoxic, foetotoxic or teratogenic to rats at an airborne concentration that produced slight maternal toxicity (Online 9).

Ethyl acrylate was not mutagenic to *Salmonella typhimurium* in the presence or absence of an exogenous metabolic system, nor was it mutagenic to *Drosophila melanogaster*. It induced chromosomal aberrations in Chinese hamster lung cells *in vitro* and micronuclei in the bone marrow of mice treated *in vivo* (Online 9).

The US government and the National Toxicology Programs (NTP) removed ethyl acrylate from its list of potential cancer-causing agents in 2000. This is the first time that the NTP has removed a substance from its list, but this does not change the current need to reference ethyl acrylate on Material Data Safety Sheets (MSDSs). Although ethyl acrylate induces tumors in animals, it only does so when the chemical is given by mouth at such high concentrations that there is severe persistent injury to the stomach. Comparable “significant chronic human oral exposure to (comparably) high concentrations of ethyl acrylate” was deemed by the government to be “unlikely” (Online 5; 11).

Environmental exposure: Ethyl acrylate enters the environment mainly as a result of spills and industrial discharges. Human exposure to ethyl acrylate occurs mostly through inhalation of ethyl acrylate vapours, but it may also result from skin contact or drinking contaminated water. Ethyl acrylate is highly soluble in water and is slightly persistent (half-life of 2-20 days). However, the majority of ethyl acrylate will dissipate and mix with the air (91%). Ethyl acrylate also bioaccumulates in fish; with fish tissues analyzed having about the same average concentrations as the water they inhabit (EPA, 1998; Online 6).

1.3.6 Toxicity

Genotoxicity: The genotoxicity of ethyl acrylate has been investigated extensively in both *in vitro* and *in vivo* assays. The *in vitro* assays demonstrate that ethyl acrylate can induce DNA damage including chromosomal aberrations and gene/point mutations. When tested *in vivo*, ethyl acrylate was found to be nonmutagenic in systems measuring both the induction chromosomal damage and induction of gene/point mutations. The lack of mutagenicity *in vivo* is consistent with data in rats on its rapid metabolism by hydrolysis to acrylic acid (IARC 1986). Thus, ethyl acrylate has mutagenic potential for the induction of chromosomal damage that is not fulfilled *in vivo* due to its rapid metabolism. In conclusion, the *in vitro* and

in vivo data on the genotoxicity of ethyl acrylate are consistent with the interpretation that ethyl acrylate should be considered non-genotoxic to exposed human populations (Online 6).

1.3.7 Metabolism

The major metabolite of ethyl acrylate is acrylic acid ($C_3H_4O_2$). It is a clear colourless liquid and is soluble in water, DMSO, 95% ethanol and acetone (Miller *et al*., 1981; Online 6).

Ethyl acrylate is metabolized by carboxylesterases (Silver and Murphy 1981; Stott and McKenna 1985; Udinsky and Frederick 1989) and by conjugation with glutathione (GSH) (Hashimoto and Aldridge 1970; Frederick *et al*., 1992). The mercapturic acid of ethyl acrylate has also been shown to be a minor urinary metabolite (deBethizy *et al*., 1987). It has also been proposed that ethyl acrylate binds to proteins and lipids *in vivo* (Ghanayem *et al*., 1987; Online 6).

Ethyl acrylate biodegrades faster in air than in water. In the atmosphere, it undergoes photo oxidative reduction with OH-radicals, and its half-life has been calculated at 13.7 hours. Ethyl acrylate has also been qualitatively detected in the air of a landfill in the United States. Ethyl acrylate can be readily absorbed into the ground, making it a very mobile compound (BUA 1995; Online 6).

1.3.8 Sampling and analysis

Ethyl acrylate vapour sampling is the best method for determining environmental ethyl acrylate concentrations. National Institute of Occupational Safety and Health (NIOSH) approves of various collection tubes, with the best being a carbon disulfide tube. The tubes are then analyzed by gas chromatography. Biomarkers are not used because they cannot accurately be analyzed (NIOSH, 1981; Online 6).

1.4 Amines

1.4.1 Definition

General Description Of Amine: Amine is a group of basic organic compounds derived from ammonia (NH_3) by replacement of one (primary amines), two (secondary amines), or three (tertiary amines) hydrogen atoms by alkyl, aryl groups or organic radicals. Amines, like ammonia, are weak bases because the unshared electron pair of the nitrogen atom can form a coordinate bond with a proton. Amines react with acids to give salts and with acid

anhydrides (or ester) to form amides. They react with halogenoalkanes to form longer chains (Online 22).

Many amines are not only bases but also nucleophiles that form a variety of electrophile compounds. They are important intermediates for chemical syntheses due to the basic functionality of the nitrogen atom and electrophilic substitution at nitrogen (Online 22).

Amines, in general, are difficult to collect and analyse because of their reactivity. For many tertiary amines, NIOSH (National Institute for Occupational Safety and Health) and OSHA (Occupational Safety and Health Administration) do not presently have validated sampling and analysis methods. The most suitable methods for validation for triethylamine were considered to be those of (Andersson and Andersson, 1989). These methods for sampling and analysis of triethylamine involve collection on a charcoal tube and analysis by a gas chromatograph equipped with a flame ionization detector (FID) or a thermionic specific detector (TSD). The charcoal tubes must be stored under freezer conditions as soon as possible after sampling. Ideally, they should be analysed within 15 days (Verma *et al*., 1998).

1.4.2 Preparation

Synthetic amines are made mostly by reaction of alcohols with ammonia, catalyzed by metals (nickel or copper) or metal oxide at high temperature (Online 22).

Primary amines contain the functional group $–NH_2$ (amino group) and are converted into secondary and tertiary amines if heated with alkyl or aryl iodides. Tertiary amines combine with one molecular proportion of an alkyl iodide to form quaternary ammonium salts in which a central nitrogen atom is joined to four organic radicals and one acid radical. Quaternary ammonium salts are used as corrosion inhibitor, emulsifying and antiseptic agents. Aliphatic amines which have the lowest carbon content are water-soluble gases or liquids of low boiling point, also readily soluble in water in the case of the next lowest carbon content. But aliphatic amines which have the high carbon content are odourless solids of high boiling point and are insoluble in water. They are all bases and easily form salts with the mineral acids and double salts with the halogenoalkanes. Amine salts are crystalline substances that are readily soluble in water. If alkali (sodium hydroxide) is added to solutions of such salts the free amine is liberated (Online 22).

1.4.3 Applications

Amines are used as reducing agents for the recovery of precious metals. They are versatile intermediates. They have active applications in organic synthesis for polymerization catalyst,

chain extender in urethane coatings, agrochemicals, pharmaceuticals, photographic, heat stabilizers, polymerization catalysts, flame-retardants, blowing agents for plastics, explosives and colorants. Alkyl tertiary amines are used as fuel additives and preservatives. They have similar applications with long chain alkyl amines. Aromatic amines also exist, such as phenylamine, which are important for the production of diazonium salts. They dissociate in water (some very weakly). Aromatic amines are much weaker bases than the aliphatics. One of the most important aromatic amines is aniline, a pale brown liquid boiling at 184 °C, melting at – 6 °C (Online 22).

Amines are widely used as catalysts in casting operations. They are also the major pollutants in the gaseous emissions of chemical manufacturing factories (Torkian *et al.*, 2005). Tertiary amines, such as triethylamine, are the main gaseous catalysts comprising the majority of nitrogenous emissions (Borger *et al*., 1997). Previous studies have suggested suitable biodegradation potential of amines (Tang *et al.*, 1996; Chou and Shiu, 1997). As such, biofiltration seems to be an appropriate method to treat waste gases containing these pollutants (Torkian *et* al., 2005).

1.5 Triethylamine

1.5.1 Properties

Triethylamine is the colourless chemical compound with the formula $N(CH_2CH_3)_3$, commonly abbreviated Et_3N. It has a strong fishy ammonia-like odour, with an odour threshold of 0.48 ppm (Online 21). Triethylamine exhibits golden yellow colour on long standing (Online 32). The vapour pressure for triethylamine is 54 mm Hg at 20 °C and its log octanol/water partition coefficient (log K_{ow}) is 1.45 (Online 21). It is also called N,N-diethylethanamine, TEA, TEN, di(ethylamino)ethane or diethylaminoethane and N,N,N-triethylamine. It is commonly encountered in organic synthesis probably because it is the simplest symmetrically trisubstituted amine, i.e. a tertiary amine, that is liquid at room temperature. Diisopropylethylamine (Hünig's base) is a widely used relative of triethylamine. Triethyamine is also the smell of the hawthorn plant, and semen, among others (Lloyd, 2006; Online 18; 19; 21; 32).

Triethylamine is stable, moderately soluble in water and extremely flammable (Online 19). It is also soluble in acetone, benzene and chloroform (Online 31). It readily forms explosive mixtures with air and is incompatible with strong oxidizing agents, strong acids, ketones, aldehydes and halogenated hydrocarbons (Online 19; 31).

Triethylamine is heavier than air and may travel a considerable distance to a source of ignition (Online 27).

Triethylamine is a high volume chemical with production exceeding 1 million pounds annually in the U.S. It is used in consumer products, building materials or furnishings that contribute to indoor air pollution and is ranked as one of the most hazardous compounds (worst 10%) to ecosystems and human health (Online 20).

1.5.2 Applications

Triethylamine is commonly used as a catalyst in the making of foundry sand molds, phenolic resins, polycarbonates, polyesters and engineered plastics. It has a variety of pharmaceutical applications where it is used as an extraction solvent and as an intermediate in the manufacture of Antibiotics like Penicillin, Ampicillin, Amoxycillin, Cephalexin and other derivatives (Online 32). It is also used in the synthesis of pesticides, pharmaceuticals, paints and coatings, corrosion inhibitors (Online 28) and as a catalyst in the esterification reaction to manufacture perfume (Online 32).

Triethylamine is used as a catalytic solvent in chemical syntheses; as an accelerator activator for rubber; as a corrosion inhibitor; as a curing and hardening agent for polymers; as a propellant; in the manufacture of wetting, penetrating, and waterproofing agents of quaternary ammonium compounds; and for the desalination of seawater (Online 21).

Triethylamine was found to catalyze the reaction of Phenols benzoylation in dioxane at 25 °C. This acylation reaction was studied for substituted nitrophenols (R = 2,4,6-$(NO_2)_3$, 2,5-$(NO_2)_2$, 2,4-$(NO_2)_2$, 4-(NO_2), 4-NO, 4-PhN=N) (Belousova *et al.,* 1999).

Triethylamine is commonly employed in organic synthesis as a base, most often in the preparation of esters and amides from acyl chlorides (Sorgi, 2001). Such reactions lead to the production of hydrogen chloride which combines with triethylamine to form the salt triethylamine hydrochloride, commonly called triethylammonium chloride. This reaction removes the hydrogen chloride from the reaction mixture, which is required for these reactions to proceed to completion (R, R' = alkyl, aryl) (Online 18).

$$R_2NH + R'C(O)Cl + Et_3N \rightarrow R'C(O)NR_2 + Et_3NH^+Cl^-$$

Like other tertiary amines, it catalyzes the formation of urethane foams (Albrecht and Stephenson, 1988) and epoxy resins and is used as a corrosion inhibitor for polymers (Nelson and Bull, 1990). It is also useful in dehydrohalogenation reactions and Swern oxidations (Online 18). Triethylamine is one of the amines emitted from cattle feedlots (Mosier *et al*., 1973) and in the gas phase it can react with nitric acid to form amine nitrates that become part of atmospheric particulates (Online 31).

Triethylamine is readily alkylated to give the corresponding quaternary ammonium salt (Online 18):

$$RI + Et_3N \rightarrow Et_3NR^+I^-$$

Triethylamine is ideal for HPLC separation and analysis of peptides. It is an ion-pairing reagent that alters selectivity in reverse-phase HPLC separations. By pairing with peptides, it effectively sharpens peaks, resulting in improved peak resolution (Online 26).

In 1996, C7 gas mask filters entered service. They contain a dual bed of carbon with the inlet bed (i.e., upper bed) being 7 percent triethylamine (TEA) impregnated base carbon (known as BPL) and the outlet bed (i.e., lower bed) ASC carbon with 3 percent TEDA (triethylenediamine). The C7 filter contains a total of 250 ml of adsorbent with the inlet and outlet bed each containing 125 ml. Triethylamine was selected as the impregnant of choice because of its superior chemisorptive properties (Verma and Eckstein, 1998).

1.5.3 Exposure

Triethylamine is corrosive – causes burns, is harmful by ingestion, inhalation and if absorbed through the skin. Chronic exposure may cause liver damage and it is very damaging to the mucous membranes. Triethylamine is also a lachrymator (tear gas) (Online 19; 31).

Environmental data: Triethylamine is harmful to aquatic organisms (Online 29). When released into the soil, this material may leach into groundwater and may evaporate to a moderate extent. And when released into water, it may evaporate to a moderate extent. Triethylamine has an estimated bioconcentration factor (BCF) of less than 100 and is not expected to significantly bioaccumulate. When released into the air, it is expected to be readily degraded by reaction with photochemically produced hydroxyl radicals and be readily removed from the atmosphere by wet deposition (Online 30).

Occupational exposure to triethylamine may occur primarily via inhalation and dermal contact during its manufacture and use. Exposure to 200 ppm is immediately dangerous to life and health. Triethylamine can irritate the lungs and repeated exposure may cause bronchitis to develop with cough, phlegm and/or shortness of breath. The general population may be exposed to triethylamine from ingesting contaminated food; triethylamine has been identified in broiled beef (Bethesda, 1993; Online 21; 25).

Acute (short-term) exposure of humans to triethylamine vapour causes eye irritation, corneal swelling and halo vision. People have complained of seeing “blue haze” or having “smoky vision.” These effects have been reversible upon cessation of exposure. Acute exposure can irritate the skin and mucous membranes in humans (Online 21).

Amines have been noted to have other effects on the eye, including mydriasis and cycloplegia (Albrecht and Stephenson, 1988; Reilly *et al*., 1995).

In a study carried out to determine the effects of experimental exposure to triethylamine on vision and the eye, it was found that triethylamine caused a marked oedema and microcysts in corneal epithelium but only minor increases in corneal thickness. The effects may be mediated by the lacrimal fluid owing to its high triethylamine concentration. Four hour exposure to a triethylamine concentration of 3.0 mg/m^3 seemed to cause no effects, whereas exposure to 6.5 mg/m^3 for the same period caused blurred vision and a decrease in contrast sensitivity (Järvinen, 1998**;** Järvinen *et al*., 1999).

In another study carried out in a foundry in Japan, complaints of visual disturbances were found to be prevalent among workers exposed to triethylamine. This study suggested that visual disturbances could develop even at about 1 ppm, but as reported by all workers, these symptoms were transient and improved within several hours after discontinuation of work exposed to triethylamine (Yoshida *et al*., 2001).

The ocular toxicity observed with triethylamine is most likely not due to a specific property of the chemical, but rather to the alkaline nature of aliphatic amines. It is a sensitive effect in humans, with minimal effects occurring at around 10 mg/m^3 (Warren and Selchan, 1988; Åkesson *et al*., 1988; Online 23).

A study was carried out by Järvinen to determine if the exposure to triethylamine provokes headaches and elevated blood pressure among the workers of three different foundries (Järvinen, 1998). As stated earlier by Diamond and Baltes, many chemicals can produce headaches by vasodilatation (*e.g*., nitrates) or hypoxia (*e.g*., carbon dioxide or carbon monoxide). In addition, other factors, such as odours and noise, can cause headaches

(Diamond and Baltes, 1967). Some reports have suggested a relationship between amine exposure and headaches. Theoretically headaches can develop from the possible histamine releasing and vasodilating effects of the amines (Albrecht and Stephenson, 1988). In the clinical classification of headaches (traction headache, vascular headache and muscle tension headache), triethylamine induced headache is likely to be of the vascular type (Diamond and Baltes, 1967). The results of Järvinen suggest, however, that triethylamine exposed workers more regularly have work-associated mild headaches and it seems unlikely that triethylamine exposure is associated with elevated blood pressure (Järvinen, 1998).

Acute animal tests, such as the LC_{50} and LD_{50} tests in rats, mice and rabbits, have demonstrated triethylamine to have moderate acute toxicity from inhalation, moderate to high acute toxicity from oral exposure and high acute toxicity from dermal exposure (Online 21).

Chronic (long-term) exposure of workers to triethylamine vapour has been observed to cause reversible corneal edema (Online 21).

Chronic inhalation exposure has resulted in inflammation of the nasal passage in rats. Thickening of the interalveolar walls of the lungs, mucous accumulation in the alveolar spaces of the lungs, and haematological effects have also been reported in rats chronically exposed by inhalation (Online 21).

Chronic inhalation exposure of rabbits has been reported to cause irritation of the lungs, edema, moderate peribronchitis, vascular thickening and eye lesions (Online 21).

The inhalation Reference Concentration (RfC) for triethylamine is 0.007 mg/m^3 based on inflammation of the nasal passages in rats (Lynch *et al*., 1990). It considers toxic effects for both the respiratory system (portal-of-entry) and for effects peripheral to the respiratory system (extrarespiratory effects). In general, the RfC is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily inhalation exposure of the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime (Online 21; 23; 31).

There is no direct evidence in the literature to quantify a differential effect of triethylamine in infants and children. However, it is a respiratory irritant and thus has the potential to exacerbate asthma. In addition, other alkylamines are known to be associated with occupational asthma (Bernstein *et al.*, 1999). There is some concern that triethylamine could have a similar effect (Online 31)

No information is available on the reproductive, developmental or carcinogenic effects of triethylamine in humans or animals. And EPA (Environmental Protection Agency) has not classified triethylamine with respect to potential carcinogenicity (Online 21; 24).

1.5.4 Metabolism

Although the knowledge of the metabolism of triethylamine in humans is important for the use of biological monitoring as a means to control exposure and risk, it was not studied until 1988 (Åkesson *et al*., 1988). The metabolism of a similar amine, trimethylamine (TMA), has been studied extensively, both in animals and in man. Trimethylamine is, to some extent, excreted unmetabolized in the urine. In addition, trimethylamine-N-oxide (TMAO), is formed which is excreted into the urine. The fractional oxygenation of triethylamine is much less than that observed for trimethylamine (Strack *et al*., 1963). Neither the calculations of absorbed amounts of triethylamine nor the chromatograms obtained at the analysis of plasma and urine indicated any dealkylation of triethylamine (formation of diethylamine). Had it been formed, it would appear in the urine, as excretion of diethylamine and monoethylamine has been shown in man after ingestion of these compounds (Rechenberger, 1940; Åkesson *et al*., 1988).

1.6 Degradation of environmental pollutants

Public awareness and concern about the quality of our environment is currently a strong driving force for improved pollution control. A highly effective and economically viable alternative method of pollution control is the microbial control of pollution. Bioremediation is becoming the technology of choice for the remediation of many contaminated environments. For the treatment of various waste effluents and contaminated environments many processes are currently used that rely enormously on microbial activity. Although microbial control of pollution is not a new concept and a number of biological treatment technologies have been developed, some pollution problems, such as the odour problem, remain to be solved (Rappert and Müller, 2005b).

Biological treatment is an environmentally safe technology, treatment is generally performed at ambient temperatures and it does not generate nitrogen oxides nor secondary waste streams (Deshusses, 1997). Organic pollutants are generally converted to carbon dioxide and water under the metabolic action of growing or resting microorganisms (Deshusses, 1997; Lewandowski and DeFilippi, 1998; Rappert and Müller, 2005b).

Microorganisms can transform virtually any organic compound, whether manmade or naturally occurring, if the environmental conditions (oxygen content, chemical composition, temperature, etc.) are suitable and the compounds are not toxic to microorganisms (Lewandowski and DeFilippi, 1998; Rappert and Müller, 2005b).

Biodegradation is dependent on three major factors: (1) the presence of microorganisms that can degrade the specific chemical structure, (2) environmental conditions that allow the microorganisms to grow and release their degradation enzymes, and (3) good physical contact between the organic substrate and the organism (Rappert and Müller, 2005b).

Biodegradation mostly results in the mineralisation of the target or parent compound into inorganic compounds containing C, N, P, and S (Alexander, 1999). Considering the mineralisation of hazardous compounds, microorganisms are very often the only means of converting these chemicals into inorganic products. Not to be neglected in this context is the fact that there are compounds in existence, which are converted by microorganisms into other organic substances, which in turn accumulate in the environment. These substances can be more toxic than the parent compound (Otto, 2001).

Because microorganisms play the major role in successful biological odour treatment system, the understanding of microbial degradation of the key odorants is very important. Some of the key odorous compounds selected are sulphides, esters, amines, and pyrazine compounds (Rappert and Müller, 2005b).

Air pollutants that are detected most easily are those related to odour or smell problems. Other contaminants leading to air pollution problems, such as carcinogenic compounds, are more difficult to detect although they are not less harmful. Their effect is often visible only after several years (Kennes and Veiga, 2001; Rappert and Müller, 2005b).

Odorous waste gases are a special kind of air pollutant (Hunter and Oyama, 2000). Odour emissions are important sources of air pollution in many industrial plants, in particular in livestock production, food processing plants, composting plants, waste water treatment facilities, rubber processing, pharmaceutical processing, pulp and paper processing, petroleum refining, paint finishing plants and chemical production (Valentin and North, 1980; Glowiak *et al.*, 1985; Fouhy, 1992; Passant *et al*., 1992; Williams and Miller, 1992; Luch, 1994; Kapahi and Gross, 1995; Both, 2001; Rappert and Müller, 2005b).

1.6.1 Biodegradation of amines

Amines are compounds derived from ammonia with one or more of the hydrogen atoms replaced by alkyl groups. Examples include methylamine, dimethylamine, and trimethylamine (Rafson, 1998).

Amines, such as dimethylamine (DMA), trimethylamine (TMA), diethylamine (DEA), and triethylamine (TEA), are the major pollutants in the gaseous emissions of chemical industries, food industries, and agricultural operations (Tang *et al.*, 1996; Van Agteren *et al.*, 1998; Chang *et al.*, 2004; Rappert and Müller, 2005b). The characteristics of these compounds are shown in Table 1.1. The occurrences, toxic properties and microbial degradation of each of these compounds are described below (Rappert and Müller, 2005b).

Table 1.1: Odour thresholds of amines and microorganisms degrading these amines[a] (Rappert et al., 2005b)

Compound	**Odour threshold (µg/l)**	**Odour description**	**Microorganisms**
Dimethylamine (DMA)	0.13[12]	Putrid, Fishy	*Arthrobacter* sp.[3,6], *Bacillus* sp.[1,5], *Hyphomicrobium* sp.[2,8,15], *Methylobacterium* sp.[16], *Pseudomonas aminovorans*[4,16], *Mycobacterium* sp.[16], *Paracoccus denitrificans*[16], *Methylophilus methylosporus*[11], *Micrococus* sp.[7], *Pseudomonas* sp.[3,15,19], *Paracoccus* sp. T231[21]
Trimethylamine (TMA)	0.00044[12]	Ammonical, Fishy	*Aminobacter aminovorans*[20,22], *Paracoccus* sp. T231[21], *Paracoccus aminovorans*[16], *Pseudomonas aminovorans*[4,17], *Hyphomicrobium* sp.[2,15], *Micrococus* sp.[7]
Diethylamine (DEA)	30[18]	Ammonical, Fishy	*Hyphomicrobium* sp.[9], *Pseudomonas* sp.[10,13], *Candida utilis*[14], *Hansenula polymorpha*[14]
Triethylamine (TEA)	0.48[12]	Ammonical, Fishy	Not yet determined

[a] Sources: [1]Myers and Zatmann (1971); [2]Attwood and Harder (1972); [3]Colby and Zatmann (1973); [4]Boulton *et al.* (1974); [5]Colby and Zatmann (1975); [6]Loginova and Trotsenko (1975); [7]Tate and Alexander (1976); [8]Meiberg and Harder (1978); [9]Meiberg (1979); [10]Claus and Kutzner (1981); [11]Large and Haywood (1981); [12] Amoore and Hautala (1983); [13]Ghisalba and Kuenzi (1983); [14]Zwart and Harder (1983); [15]Ghisalba *et al.* (1985); [16]Urakami *et al.* (1990); [17]Gamati *et al.* (1991); [18]Lundqvist *et al.* (1992) ; [19]Shirkot *et al.* (1994) ; [20]Lobo *et al.* (1997) ; [21]Kim *et al.* (2001) ; [22]Rappert *et al.* (2004).

1.6.2 Dimethylamine

Dimethylamine (DME) is mainly used as an accelerator in rubber vulcanization processes, during tanning of leather, and for production of detergents and pesticides. It is also formed during synthesis of solvents (N,N-dimethylformamide) and pesticides (tetramethylthiuram disulfide, TMTD) (Urakami et al., 1990; Shirkot et al., 1994). Dimethylamine is also formed during the degradation of trimethylamine by several algae and microorganisms (Meiberg and Harder, 1978; Lundstrom and Racicot, 1983; Van Agteren *et al*., 1998; Kim *et al*., 2001). Dimethylamine has a malodorous character and is important because of its role as precursor of the carcinogenic compound N-nitrosodimethylamine (Lundstrom and Racicot, 1983). Therefore, prompt treatment is necessary (Rappert and Müller, 2005b).

In agricultural soil dimethylamine was found to be rapidly metabolized by microorganisms and did not accumulate (Ayanaba et al., 1973; Smith and Aubin, 1992). The metabolism of dimethylamine in the environment under aerobic conditions starts with conversion to methylamine and formaldehyde (Van Agteren et al., 1998). Many microorganisms that degrade dimethylamine under aerobic conditions have been isolated and are summarized in Table 1.1. The hypothetical pathway for the aerobic microbial degradation of dimethylamine follows the same route as the degradation of trimethylamine, which is summarized in Figure 1.2 (Rappert and Müller, 2005b).

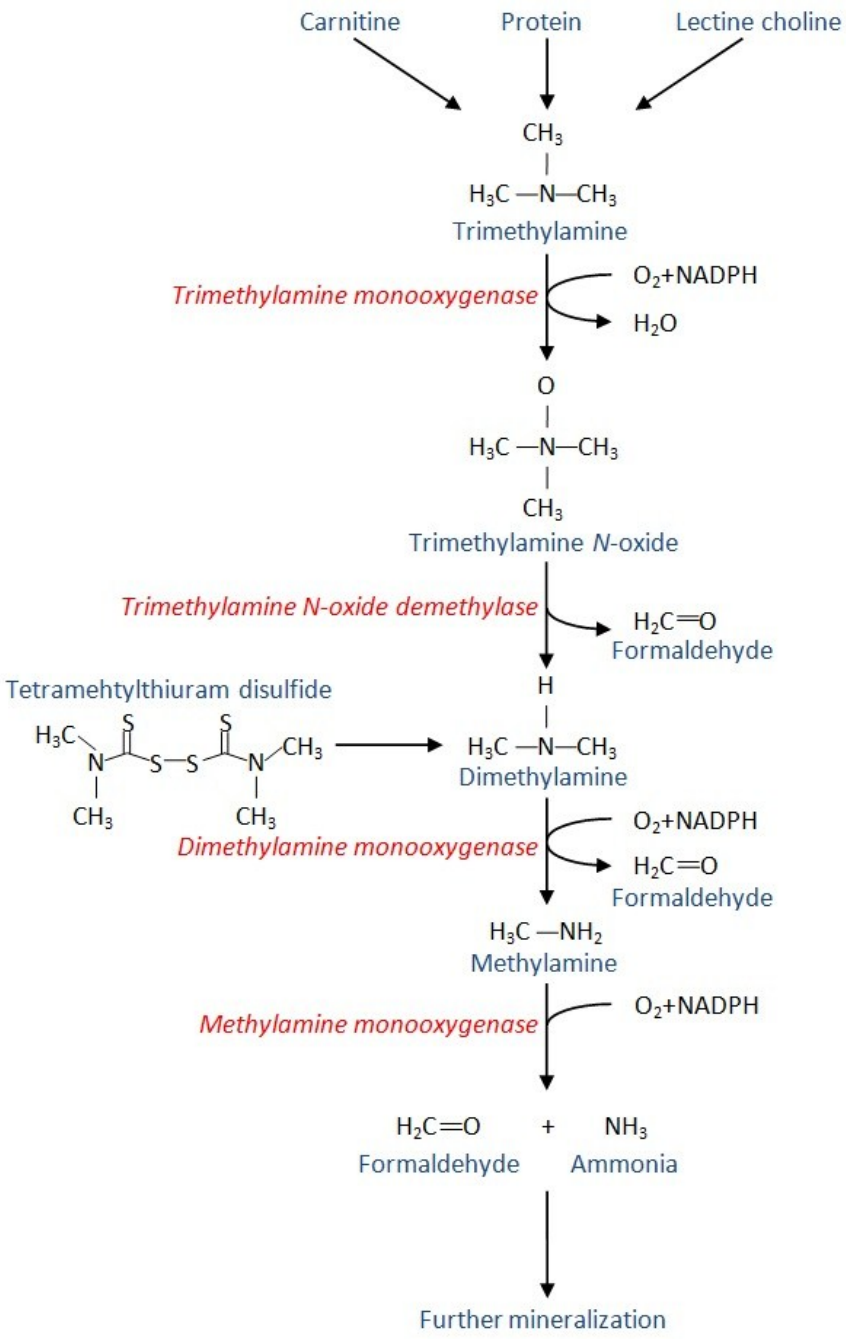

Figure 1.2: The proposed formation and degradation pathways for trimethylamine by microorganisms in the environment under aerobic conditions. The enzymes of the pathways are shown in red (Van Agteren et al., 1998; Kim et al., 2001; Rappert and Müller, 2005b).

1.6.3 Trimethylamine

Trimethylamine is a malodorous compound and is usually produced by microbial activities from choline, betaine or trimethylamine N-oxide present in marine fish (King, 1984; Barrett

and Kwan, 1985; Lin and Hurng, 1989; Mouné *et al*., 1999; Lopez-Caballero *et al*., 2001). In addition, trimethylamine is a toxic compound because of its tissue-corrosive and penetrative properties. It inhibits the biosynthesis of macromolecules, such as DNA, RNA, proteins, and has a teratogenic effect on mouse embryos (Guest and Varma, 1992). Trimethylamine is frequently found in effluents of fish-meal manufacturing processes (Sandberg and Ahring, 1992; Hwang *et al*., 1994; Rappert and Müller, 2005b).

Microbial degradation of trimethylamine under aerobic conditions has been intensively studied (Urakami *et al*., 1990; Ohara *et al*., 1990; Lobo *et al*., 1997; Kim *et al*., 2001). Many microorganisms that are able to degrade trimethylamine aerobically have been isolated and are summarized in Table 1.1. The rate of trimethylamine consumption by *Aminobacter aminovorans* was 1.79 mM h^{-1} measured in a bubble column continuous fermentation system (Lobo *et al*., 1997). Rappert and Müller found that in a shaking culture of *A. aminovorans* containing mineral medium 1.10 M supplemented with 5.0 mM trimethylamine > 95% of the trimethylamine was utilized after 24 h (Rappert and Müller, 2005b).

The degradation pathway of trimethylamine by *A. aminovorans* has been proposed. The strain oxidizes trimethylamine to dimethylamine by the action of the enzyme trimethylamine dehydrogenase, then to monomethylamine by the activity of dimethylamine monooxygenase and finally to formaldehyde and ammonia by the action of enzyme monomethylamine dehydrogenase before carbon incorporation in the serine pathway (Large, 1983). The same degradation pathway has been found with a newly isolated denitrifying bacterium, *Paracoccus* sp. T231 (Kim *et al*., 2001). The biological degradation pathway of trimethylamine under aerobic conditions is summarized in Figure 1.2 (Rappert and Müller, 2005b).

1.6.4 Diethylamine

Diethylamine is irritating to the skin and to mucous membranes. It is used in the rubber industry, in pharmaceutical products, in resin and in colouring materials (Van Agteren *et al.*, 1998; Rappert and Müller, 2005b).

Little is known about the physiology and microbial degradation of diethylamine, unlike dimethylamine and trimethylamine. In 1979, Meiberg isolated *Hyphomicrobium* strains that could grow on dimethylamine and diethylamine at a concentration of 1 g l^{-1}. Several groups have isolated *Pseudomonas* strains that are able to grow on diethylamine up to 5 g l^{-1} (Ghisalba and Kuenzi, 1983; Ghisalba *et al*., 1985; Claus and Kutzner, 1981; Rappert and Müller, 2005b).

Not only bacteria can degrade diethylamine, some yeast extracts such as *Candida utilis* and *Hansenula polymorpha* were found to degrade diethylamine and use it as a nitrogen source (Zwart and Harder, 1983). No information was found about the biodegradation of diethylamine in the environment, soil or water samples (Rappert and Müller, 2005b). Pietsch *et al*. (2001) reported that under conditions similar to drinking water treatment plants, dimethylamine had higher biodegrability than diethylamine. The degradation pathway of diethylamine under aerobic conditions is expected to be similar to that of dimethylamine (Van Agteren *et al*., 1998). Microorganisms that degrade diethylamine under aerobic conditions are showns in Table 1.1 (Rappert and Müller, 2005b).

1.6.5 Triethylamine

Triethylamine is used as an intermediate in the production of various chemicals, including pharmaceuticals. It is also widely used as catalyst for polymerisation reactions (in urethane and epoxy resin systems) and as solvent and corrosion inhibitor (Åkesson *et al*., 1988). Exposure to triethylamine may cause adverse health effects such as visual disturbances and asthma (Belin *et al*., 1983; Åkesson *et al*., 1985). In addition, triethylamine has a very low odour threshold, as shown in Table 1.1. Therefore, an effective treatment of this compound is necessary (Rappert and Müller, 2005b).

The metabolism of triethylamine in man has been studied (Åkesson *et al*., 1988). However, little is known about microbial degradation of triethylamine. Under anaerobic conditions, it was reported that triethylamine was not degraded in a biodegradation test (Kawahara *et al*., 1999). In contrast to the results obtained under anaerobic conditions, Tang *et al*. (1996) reported that 100% removal efficiency of triethylamine (with the triethylamine loads up to 140 g $(m^3\ h)^{-1}$) was observed in a laboratory-scale reactor. This bioreactor contained a mixture of sieved compost particles (equivalent diameter: 1.2-2.5 mm) and chaff particles (equivalent diameter: 4-5 mm), as the filter material, and activated sludge, obtained from the wastewater treatment plant of a fertilizer factory where amines were the major pollutant treated, as the inoculum. The removal efficiency of the biofilter decreased as the amount of triethylamine introduced into the biofilter increased above the maximum loading capacity (140 g $(m^3\ h)^{-1}$) (Rappert and Müller, 2005b).

The experimental results of the deodorization of triethylamine carried out in three different natural beds were shown by Palica and Walus (1998). It was found that the most effective natural bed for the deodorization was crumbled bark from deciduous trees. The use of processed material from mushroom production as a biofilter medium was less effective and the use of wheat straw was the least effective. The moisture content of the air was the most

decisive parameter influencing the deodorization, whilst the feeding and contact times were the least decisive parameters. It has been proven over a wide range of odour concentrations and air flow rates that full removal of triethylamine in process air is attained when using a biofilter medium of crumbled bark from deciduous trees (Palica and Walus, 1998; Rappert and Müller, 2005b).

There are no present published data on biodegradation of triethylamine by pure microbial strains.

1.7 Aim of the study

Biological degradation has recently emerged as an efficient method to treat odorous compounds. Two common industrial waste products, triethylamine and ethyl acrylate, were chosen for study here due to their exceedingly bad odour. The aims of this study were:

1. To isolate bacteria that can degrade triethylamine and ethyl acrylate.
2. Characterization and identification of the isolated strains.
3. To determine the biodegradability of these substrates alone, and also in the presence of other odorous compounds. The latter resembles the situation in nature and in industry.
4. Determination of the optimum temperature, pH and degradable substrate concentrations.
5. To determine the mechanism of degradation of these strains and suggest possible degradation pathways for each compound.
6. To extract, purify and characterize the enzyme of one of the strains isolated.

Chapter 2

MATERIALS AND METHODS

2.1 Overview

In this work, several bacterial strains were isolated from a variety of environmental samples and tested for their potential to degrade two odorous compounds: Triethylamine and ethyl acrylate. The environmental samples used here were screened from a large number of sources that were believed to be contaminated with the target compounds. Sources included biofilter material, bio-scrubber “biowäscher”, scrubber “wäscher” and soil. The two bacterial strains demonstrating the most efficient degradation of each odorous compound were chosen for further analysis. Gas chromatography was used to assess the substrate-degradation potential of each bacterial strain. Temperature, pH and concentration were varied to find the optimum conditions for degradation. Finally, one of the strains was selected and its enzyme was extracted and was purified and characterized.

2.2 Equipment, chemicals and media

The experimental work (if not indicated elsewhere) was carried out using the following equipment:

Table 2.1: List of equipment

Procedure	**Equipment**	**Company**
Sonication:	Branson Sonifier 450	Branson Inc.
Centrifugation: Minifuge T Biofuge A	M/E Centrifuge (J14/21 Rotors)	Beckmann Heraeus Heraeus
HPLC (purification):	HPLC Pump 2150 HPLC Controller 2152 Variable Wavelength Detector 2155 Fraction collector Superfrac XK 16/20 columns packed with: Q-Sepharose fast flow	LKB, Bromma LKB, Bromma LKB, Bromma Pharmacia Pharmacia Pharmacia
HPLC (analytic):	HPLC Pump 2248 Dilutor 401 Autosampler 231 Dioden array detector 994	Pharmacia Gilson/Abimed Gilson/Abimed Millipore
Ultrafiltration:	10, 30 and 70 kDa ultrafiltration kits	Amicon, Millipore
Buffer exchange:	PD 10; Sephadex G-25 M	Pharmacia
Photometer:	Spectralphotometer UV-160 A Uvikon 860 HP 8453 diode array spectrophotometer DW-2000 double beam spectrophotometer	Schimadzu Kontron, Germany Hewlett Packard Aminco
pH measurement:	pH-Meter CG 812 pH 535 Multical	Schott WTW, Weilheim
Cultivation of the cells:	Shaker	HL-Infos AG, Switzerland
Incubation:	Incubator	Köttermann, Germany
Cultivation, screening, etc…:	Flasks and glassware	Schott, Germany
Water supply:	Milli-Q Water purification system	Millipore, Germany
Microscopy:	Microscope Axiolab	Zeiss, Germany
Gas chromatography:	Model GC 6000 Vega Series 2	Carlo Erba Instruments, Milan, Italy
Headspace Sampler	HS MOD.250	Carlo Erba Instrument
Flame ionization detector & fused silica capillary column	DB-624	J&W Scientific, Folsom, CA, USA
Fermentation:	30 Litre Fermenter	BioEngineering AG, Wald, Switzerland
Cell count:	Neubauer Chamber	Paul Marienfeld GmbH&Co.KG, Lauda-Königshofen, Germany

All chemicals, of the highest purity available, were obtained from Sigma, Aldrich or Merck.

2.2.1 Growth media and solutions

Medium 1.10 M (+YE) (pH adjusted to 6.5)	
K_2HPO_4	2.5 g
KH_2PO_4	1.0 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4.7H_2O$	0.2 g
Yeast extract	0.1 g
H_2O	1000 ml

Medium 1.10 M (-YE) (pH adjusted to 6.5)	
K_2HPO_4	2.5 g
KH_2PO_4	1.0 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4.7H_2O$	0.2 g
H_2O	1000 ml

Medium 1.10 M (-YE – N) (pH adjusted to 6.5)	
K_2HPO_4	2.5 g
KH_2PO_4	1.0 g
$(NH_4)_2SO_4$	0.0 g
$MgSO_4.7H_2O$	0.2 g
H_2O	1000 ml

Medium 6a Med (+YE) * (pH adjusted to 6.6)	
NH_4Cl	1.0 g
$MgCl_2.6H_2O$	0.5 g
$KH_2PO_4.3H_2O$	0.4 g
$K_2HPO_4.7H_2O$	0.6 g
$CaCl_2.2H_2O$	0.2 g
$FeCl_3.6H_2O$	20.0 mg
Na_2-EDTA	50.0 mg
Yeast extract	0.1 g
H_2O	1000 ml

* (Reichert *et al.*, 1998)

Medium 6a Med (-YE) * (pH adjusted to 6.6)	
NH_4Cl	1.0 g
$MgCl_2.6H_2O$	0.5 g
$KH_2PO_4.3H_2O$	0.4 g
$K_2HPO_4.7H_2O$	0.6 g
$CaCl_2.2H_2O$	0.2 g
$FeCl_3.6H_2O$	20.0 mg
Na_2-EDTA	50.0 mg
H_2O	1000 ml

*(Reichert *et al.*,1998)

Medium 3 *	
NaCl	25.0 g
NH_4Cl	0.2 g
$CaCl_2.2H_2O$	0.225 g
KCl	0.2 g
$MgCl_2.6H_2O$	0.2 g
KH_2PO_4	0.02 g
Na_2CO_3	2.0 g
H_2O	1000 ml
Supplemented with:	
Vitamin B_{12} (filter sterile)	20.0 µg
$FeSO_4.7H_2O$ (filter sterile)	1.0 mg
Trace elements solution** (filter sterile)	1.0 ml
H_2O	1000ml

*(Visscher and Taylor, 1993a)

*Trace elements solution	
HCl 25%	10.0 ml
$FeCl_2.4H_2O$	1.5 g
$CoCl_2.6H_2O$	190.0 mg
$MnCl_2.4H_2O$	100.0 mg
$ZnCl_2$	70.0 mg
H_3BO_3	62.0 mg
$Na_2MoO_4.2H_2O$	36.0 mg
$NiCl_2.6H_2O$	24.0 mg
$CuCl_2.2H_2O$	17.0 mg
H_2O	1000 ml

*(Widdel and Pfennig, 1981)

At first, the $FeCl_2.4H_2O$ was dissolved in HCl solution, while the other components were added after addition of sterile distilled water. pH was adjusted to 7.5.

***Luria-Bertani medium (LB)**	
Luria-Bertani (LB) broth	
NaCl	10.0 g
Yeast extract	5.0 g
Peptone	10.0 g
H_2O	1000ml

*The pH of the solution was adjusted to 7.0 with NaOH (Sambrook and Russel, 2001)

LB agar plates

LB medium was prepared as above, but 15 g/l agar was added before autoclaving.

2.2.2 Buffers

The water for the buffers used was deionized in a water purification plant, Milli-Q Water Purification Systems, (Millipore, Germany). The buffers were used for chromatography, washing of cells, production of crude extract, dialysis and other methods.

Phosphate buffer (Long, 1961)

Stock solutions:

Na_2HPO_4	0.5 M
KH_2PO_4	0.5 M

The buffer was prepared at the needed molarity and pH according to the table in Cyril Long.

Glycine-sodium glycinate buffer (Long, 1961)

Stock solutions:

Glycine	2 M
NaOH	1 M

The buffer was prepared at the needed molarity and pH according to the table in Cyril Long.

2.3 Bacteria that degrade triethylamine

2.3.1 Isolation procedure

In order to isolate bacteria with the potential to degrade triethylamine, several environmental samples were collected from different sources, such as biofilter, biowäscher “bioscrubber” and wäscher “scrubber”, from the fish-meal producing company See Loewe in Cuxhaven, Germany. Using 100-ml serum bottles, 2 g of biofilter material were suspended in 20 ml of different media including 1.10 M (+YE), 1.10 M (-YE), 6a Med (+YE), 6a Med (-YE) and Medium 3. Media were adjusted to different pH values (6.5, 7.0 and 7.5). Same was done using 2 ml of scrubber or bioscrubber material. Finally 0.5 mM triethylamine were added as a carbon and energy source; then samples were sealed with a butyl rubber septum and an aluminium crimp seal to prevent the loss of volatile substrates. Cultures were stirred at 110 rpm while incubated at 25 °C. Samples showing turbidity were selected and 2 ml were transferred into a 20 ml fresh medium with the addition of 0.5 mM triethylamine. This process of inoculation was repeated several times and then 50 µl from the enrichment culture were spread on 1.10 M (-YE) mineral medium agar plates and incubated in a sealed jar, which was saturated with vapour of triethylamine. The jar was kept for several days at 25 °C and plates were monitored for growth of bacterial colonies. Single colonies were transferred onto fresh plates and this process was repeated till each plate only contained one type of colonies. At the end, a total of sixty one pure strains were isolated and then tested for the ability to degrade 0.5 mM triethylamine. Degradability was measured using a head-space GC and cell count was carried out simultaneously.

2.3.2 Anaerobic growth

The potential of RA1 and RA2 to degrade triethylamine under anaerobic conditions was also studied. Two anaerobic media were prepared: 1.10 M (+YE) and 1.10 M (-YE) (Section 2.2.1). To a one litre medium, an amount of 100 µl of a 1% Rezazurin solution was added before boiling and sterilization. The medium was boiled gently for approximately one hour using a heating mantle and a dark pink colour evolved. The growth experiments for each strain were carried out using 100-ml serum bottles (Section 2.3.1). Continuous purging with N_2 was performed to completely free the medium of O_2 during which the bottles were sealed and then sterilized. To each bottle containing 19 ml medium, a 1 ml innoculum was added to which an amount of 0.1 ml of a 5% Na_2S solution was added to detect any amount of O_2 that may still have been present in the solution. A complete disappearance of the pink colour confirmed the total absence of O_2 in the aneorobic medium. Finally, 0.5 mM triethylamine

was added to each bottle and cultures were stirred at 110 rpm while incubated at 25 °C where the growth of bacteria was monitored for 21 days.

2.4 Bacteria that degrade ethyl acrylate

2.4.1 Isolation procedure

In order to isolate bacteria with the potential to degrade ethyl acrylate, ten soil samples were collected in April 2000 from different sites of Aussenmühle Park in Harburg, Germany. In a 100-ml serum bottle, 2 g of a soil sample were suspended in 20 ml of 1.10 M (+YE), adjusted to different pH values (6.5, 7.0 and 7.5). 2.0 mM ethyl acrylate were added as a carbon and energy source and bottles were sealed with a butyl rubber septum and an aluminium crimp seal to prevent the loss of volatile substrates. Cultures were shaken at 110 rpm while incubated at 25 °C. Samples showing turbidity were selected and 2 ml of each were transferred into 20 ml fresh medium with the addition of 2.0 mM ethyl acrylate. This process of inoculation was repeated several times and then 50 µl from the enrichment culture were spread on LB-medium plates and incubated at 25 °C and were monitored for growth of bacterial colonies. Single colonies were transferred onto fresh plates and this process was repeated till each plate only contained one type of colonies. At the end, a total of two pure strains were isolated and then tested for the ability to degrade 2.0 mM ethyl acrylate. Degradability was measured using a head-space gas chromatograph and cell count was carried out simultaneously.

2.4.2 Anaerobic growth

The potential of 2C and 2Cbei to degrade ethyl acrylate under anaerobic conditions was also studied. Two anaerobic media were prepared as described in Section 2.3.2 where 2.0 mM ethyl acrylate was used (instead of 0.5 mM triethylamine) as an energy and carbon source. Finally, cultures were stirred at 110 rpm while incubated at 25 °C where the growth of bacteria was monitored for 21 days.

2.5 Analytical procedures

Bacterial growth was monitored routinely by recording the optical density at 600 nm, and the total cell count was determined by counting under the microscope using a Neubauer counting chamber (depth of 0.020 mm and a small square area of 0.0025 mm^2). Substrate concentrations of the odorous compounds were measured by gas chromatography (model GC 6000 Vega Series 2, Carlo Erba Instruments, Milan, Italy). One millilitre of the culture

medium was transferred into a 5-ml sample vial sealed with butyl rubber septa and aluminium crimp seal, and incubated in the water bath at 30 °C for 30 min. For amine compounds, in order to release free amines from their salts, 0.1 ml of 2.5 M NaOH was added to 1 ml of sample before incubation in the water bath (Kim *et al.*, 2001). A sample of 500 μl of the gas phase was taken by a Headspace Sampler (HS MOD.250, Carlo Erba Instrument) and injected into the GC equipped with a flame ionization detector and a J&W fused silica capillary column DB-624 (J&W Scientific, Folsom, CA, USA). The temperatures of the oven, of the injection port, and of the detector were 100, 60, and 200 °C, respectively. Nitrogen gas was used as carrier gas, at a flow rate of 20 ml/min.

2.6 Enzyme assay

2.6.1 Spectrophotometric assay with p-nitrophenyl acetate (ρ-NPA) as substrate

The activity of the esterase from 2Cbei was measured with various para-nitrophenyl esters. The yellow colour developed by the release of the nitrophenol was measured at 405 nm. For the calculation of the activity, a molar extinction coefficient of 9500 $L.mol^{-1}.cm^{-1}$ was used (Böckle, 1994). Enzyme activity was calculated according to the following formula:

Enzyme Activity (U/ml) = $\frac{\Delta E / \Delta t}{d \cdot e} \cdot \frac{Vk}{Vp}$

ΔE – difference of absorption

Δt – difference of time (min)

d – breadth of the cuvette (cm)

e – the molar extinction coefficient (cm^2/mole)

Vk – the general reaction volume (ml)

Vp – the volume of the enzyme (ml)

2.7 Purification and characterization of esterase from 2Cbei cells

2.7.1 Bacterial strain cultivation and native crude enzyme extraction

Cells of *Defluvibacter lusatiensis* 2Cbei were harvested from 2, 5 and 10 litre cell culture samples by centrifugation at 9,000 g for 90 min (producing ~ 0.2 g wet weight cell mass), washed 3 times with phosphate buffer of pH 7, and resuspended in same buffer in a constant ratio of 1:4 wet weight cell mass (g) to buffer volume (ml). The resuspended cells were disrupted with an ultrasonicator (Branson Sonifier 450, Branson Inc., USA) ~ 10 times and the suspension was centrifuged at 25,000 g for 80 min at 4 °C. The supernatant produced was filter sterile and used as the native enzyme crude extract.

2.7.2 Purification of esterase from 2Cbei cells

All purification steps were carried out at room temperature. The native esterase crude extract from ~ 50 g (wet weight) of *Defluvibacter lusatiensis* cell mass was dialyzed against 100 volumes of 20 mM phosphate buffer, pH 7. The crude extract was fractionated by the addition of ammonium sulfate. The enzyme precipitated between 40 and 60 % saturation. The precipitate was dissolved in 20 mM phosphate buffer of pH 7. After dialysis against the same buffer, the enzyme was loaded onto a column of Q-Sapharose (mono Q^{TM} 5/50 GL). The enzyme was eluted with a linear gradient from 0 to 1 M NaCl. Active fractions were pooled, dialyzed and loaded onto a column of Superdex G200. The column was run with 20 mM phosphate buffer of pH 7. Active fractions were pooled and concentrated with Microcon YM-10.

2.7.3 Influence of pH and temperature on esterase activity and thermostability

The optimum conditions for the enzyme activity were determined. The activity was measured under standard conditions at temperatures from 25 to 80 °C with increments of 5 °C and at pH 6.0, 6.5, 7.0, 7.5 and 8.0 in phosphate buffer and at pH 8.0, 8.5, 9.0, 9.5 and 10.0 in Glycine/NaOH buffer.

2.7.4 Effect of various compounds on esterase activity

The effect of various substances on esterase activity was examined using enzyme assay after pre-incubation of the purified esterase with various reagents in different concentrations at 20 °C for 120 min. For examination of the enzyme's residual activity, 100 µl of pre-incubation mixture was mixed with 900 µl of substrate in 50 mM phosphate buffer of pH 7.0. The reaction was carried out for 10 min at 60°C (unless stated otherwise). The enzyme's activity without additional compounds was defined as 100%. Four groups of the various compounds (organic solvents, inhibitors, surfactants and metal ions) were studied for their effect on esterase activity. The compounds are listed in Table 2.2 –Table 2.4.

Table 2.2: Organic solvents tested.

Organic solvent	**Concentration in pre-incubation mixture (v/v)**	**Dissolved in**
Tert-butanol	50%, 99%	dH_2O
Ethanol	50%, 99%	dH_2O
Isopropanol	50%, 99%	dH_2O
Acetone	50%, 99%	dH_2O
Methanol	50%, 99%	dH_2O
N,N-Dimethyl formamide	50%, 99%	dH_2O
Dimethylsulfoxide (DMSO)	50%, 99%	dH_2O
Pyridine	50%, 99%	dH_2O
n-Hexadecane	99%	-
n-Heptane	99%	-
Isooctane	99%	-
n-Amyl alcohol	99%	-
Toluene	99%	-
Benzene	99%	-
n-Hexane	99%	-
Acetonitrile	99%	-
n-Decyl alcohol	99%	-

Table 2.3: Inhibitors.

Inhibitor	Concentration in pre-incubation mixture	Inhibitory effect	Dissolved in
B-mercaptoethanol	10 mM	Disulfide bond reducing agent	dH_2O
Dithiotreitol (DTT)	10 mM	disulfide bond reducing agent	dH_2O
ρ-chloro-mercuric-benzoate (PCMB)	10 mM	reagent masking SH-groups	dH_2O
Guanidine hydrochloride	10 mM	denaturing reagent	dH_2O
Urea	10 mM	denaturing reagent	dH_2O
Sodium 2-iodoacetate	10 mM	Cysteinproteases	dH_2O
Ethylenediamine tetraacetic acid (EDTA)	10% (w/v)	Cation chelating reagent	dH_2O
Phenylmethylsulfonyl fluoride (PMSF)	10 mM	serine-modifying reagent	96% (v/v) EtOH
Pefabloc	10 mM	Serinproteases	dH_2O

Table 2.4: Surfactants.

Detergent	Concentration in pre-incubation mixture	Dissolved in
(3-[3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)	10% (w/v)	dH_2O
Sodium dodecyl sulphate (SDS)	10% (w/v)	dH_2O
Triton X-100	10% (v/v)	dH_2O
Tween-80	10% (v/v)	dH_2O
Tween-20	10% (v/v)	dH_2O
Polyvinyl alcohol	10% (w/v)	dH_2O
Ethylenediamine tetraacetic acid (EDTA)	10% (w/v)	dH_2O
(3-[Cyclohexylamino]-1-propanesulfonic acid) (CAPS)	10% (w/v)	dH_2O

Metal ions:

The salts containing the ions of Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Rb^{+}, Ag^{+}, Mn^{2+}, Co^{2+}, Sr^{2+}, Ni^{2+}, Al^{3+}, Fe^{3+}, Fe^{2+}, Zn^{2+}, Cr^{3+}, Hg^{2+} were tested with a concentration of 1 mM in the incubation mixture.

2.7.5 Substrate specificity

The enzyme specificity was studied with ρ-nitrophenyl esters of varying alkyl chain lengths from C2 to C18 as described in the enzyme assay. The reactions were carried out at 60°C for 10 min.

The following substrates were tested: ρ-nitrophenyl acetate (C2), ρ-nitrophenyl butyrate (C4), ρ-nitrophenyl caprylate (C8), ρ-nitrophenyl caprate (C10), ρ-nitrophenyl laurate (C12), ρ-nitrophenyl myristate (C14), ρ-nitrophenyl palmitate (C16), ρ-nitrophenyl stearate (C18).

2.8 Biochemical methods

2.8.1 Determination of protein concentration

(Bradford, 1976)

Protein concentration was determined by a modified Bradford method (BioRad protein assay) and by UV spectroscopy measurement at 280 nm. The BioRad protein assay is based on the differential colour change of a dye (acidic solution of Coomassie brilliant blue G-250) in response to various protein concentrations. Protein standard solutions of bovine serum albumin (BSA) were prepared in the range of 1 – 25 μg/ml. 0.2 ml of the concentrated BioRad dye was added to 0.8 ml of each of the protein solutions in sterile test tubes. The blank contained only the dye solution. After 15 minutes the absorbance of the samples was measured at 590 nm (corrected for the blank). By using the standard curve the protein concentration in the samples was extrapolated.

For a rough estimation of the protein concentration, the UV-spectra of the samples were recorded between 200-350 nm. Protein concentration was calculated according to the following equation:

$$\text{Protein (mg/ml)} = 1.55\ A_{280\ nm} - 0.76\ A_{260\ nm} \qquad \text{(Layne, 1957)}$$

2.9 Disposal of waste materials

Waste materials and residues were disposed of through the university's waste-water disposal network. Toxic reagents were only used when no environmentally safer alternatives are available. All other organic reagents (chloroform, acetone, hexane, etc.) were first pooled in large waste containers and disposed of through the university's technical service. The plastic waste, such as pipette tips were disposed of through the university's waste disposal network.

Chapter 3

RESULTS

3.1 Bacteria that degrade triethylamine

3.1.1 Isolation procedure

In order to isolate bacteria with the potential to degrade triethylamine, 2 ml of material taken from the scrubber “Wäscher” of the fish-meal producing company Seelöwe in Cuxhaven, Germany, were suspended in 20 ml of medium 1.10 M (-YE) with 0.5 mM triethylamine as a sole carbon and energy source. After 4 days, the triethylamine had disappeared and 2 ml were transferred into 20 ml of fresh medium. This procedure was repeated. After ~10 transfers, 50 µl were spread onto 1.10 M (-YE) mineral medium agar plates and incubated in a sealed jar containing triethylamine as a sole carbon and energy source. The strain was purified by consequent colony picking; different colonies were transferred onto fresh plates. After ~10 transfers, the bacteria obtained were checked for purity on nutrient broth agar plates. From the plates showing only one type of colonies, one colony was transferred into liquid medium with triethylamine as carbon source. The fastest growing strain was selected for further studies and was named RA1. The same steps were applied on a sample originating from the bio-scrubber “Biowäscher” of the same company and the fastest growing strain was selected for further studies. This strain was named RA2.

3.1.2 Identification of the triethylamine degrading bacteria RA1 and RA2

The growth of the two strains, RA1 and RA2, obtained in the enrichment procedure was tested. With 0.5 mM triethylamine, the growth rates at 25°C were 0.046 h^{-1} and 0.045 h^{-1}, respectively. The morphology and the physiological parameters of the strains were determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig. Strain RA1 was a motile gram negative rod of width 0.5 – 0.7 µm and length 1.8 – 3.0 µm. Strain RA2 was a non-motile, non-spore forming gram negative rod

of width 1.0 µm and length 2.0 µm. The properties of the two strains are summarized in Table 3.1. Based on these results, strain RA1 was classified as *Pseudomonas citronellolis*, and strain RA2 was identified as *Mycobacterium diernhoferi* (Figure 3.1 and Figure 3.2).

The colonies of RA1 were smooth and pale yellowish on LB medium; whereas the colonies of RA2 were chromium yellow. The strain RA1 was oxidase and catalase positive; while RA2 was not tested. The growth of RA1 at 41 °C was positive (Table 3.1). Neither growth of RA1 nor of RA2 was observed under anaerobic conditions.

The strain RA1 grew on a variety of substrates. Growth was observed on yeast extract, glucose, adipate, malate, phenylacetate, citrate, caprate, gluconate, azelate, geraniol and citronellol. No growth was observed on arabinose, mannitol, maltose, n-acetylglucosamine, acetamide, trehalose, sorbitol and citraconate.

Table 3.1: Morphological and physiological properties of Pseudomonas citronellolis, RA1 and Mycobacterium diernhoferi, RA2.

Properties of the strain	*Pseudomonas citronellolis*, RA1	*Mycobacterium diernhoferi*, RA2
Shape of cells:	Rods	Rods
width µm:	0.5 – 0.7	1
length µm:	1.8 – 3.0	2
Motility	+	-
Gram reaction	-	-
Lysis by 3% KOH	+	nd
Aminopeptidase (Cerny)	+	nd
Oxidase	+	nd
Catalase	+	nd
ADH	+	nd
Nitrate reduction	+	nd
Denitrification	+	nd
Urease	-	nd
Hydrolysis of gelatine	-	nd
Utilization of:		
glucose	+	nd
arabinose	-	nd
adipate	+	nd
malate	+	nd
mannitol	-	nd
phenylacetate	+	nd
citrate	+	nd
caprate	+	nd
gluconate	+	nd
maltose	-	nd
n-acetylglucosamine	-	nd
azelate	+	nd
acetamide	-	nd
geraniol	+	nd
trehalose	-	nd
sorbitol	-	nd
citraconate	-	nd
citronellol	+	nd
Growth at 41 °C	+	nd
Levan from sucrose	-	nd
Lecithinase	-	nd
Fluorescent pigment	+	nd
Pyocyanin	-	nd

+ : growth - : no growth nd : not determined

Fatty acid analysis: The fatty acid methyl ester (FAME) composition of the strain RA2 is shown in Table 3.2. The FAME profile displays only those fatty acids comprising ≥ 0.1% of the total. Straight chain saturated FAMEs were 34.3% total, terminally branched saturated FAMEs were 1.74% total and monounsaturated FAMEs were 56.86% total. 16:00 straight chain saturated FAME (25.82%), 17:1 ω6c monosaturated FAME (18.67%) and 11 methyl 18:1 ω7c (32.14%) were the most abundant FAMEs found.

Table 3.2: Fatty acid composition (%) of strain RA2.

Fatty acids	**[%]**
Straight-chain fatty acids	
12:00	-
14:00	3.21
15:00	-
16:00	25.82
17:00	-
18:00	2.92
20:00	2.35
Sum	**34.3**
Terminally branched saturated fatty acids	
11:0-iso	-
12:0-iso	-
15:0-iso 3OH	0.42
16:0-iso	-
TBSA 10Me18:0	1.32
Sum	**1.74**
Monounsaturated fatty acids	
14:1 ω5c	-
15:1 ω6c	-
15:1 ω8c	-
16:1 ω5c	2.68
16:1 ω9c	-
17:1 ω6c	18.67
17:1 ω7c	-
17:1 ω8c	3.37
11 methyl 18:1 ω7c	32.14
18:1 ω9c	-
Sum	**56.86**

Figure 3.1 shows an electron microscopic picture of strain RA1 and Figure 3.2 of strain RA2.

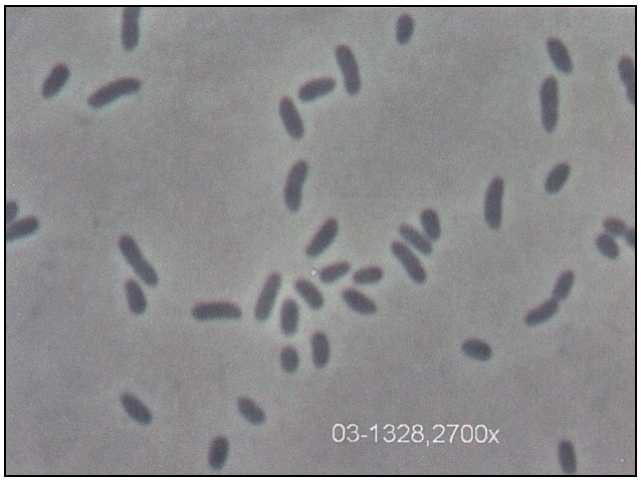

Figure 3.1: Pseudomonas citronellolis, RA1 (DSM ID 03-1328); cell form: rod; width 0.5-0.7µm; length 1.8-3.0µm.

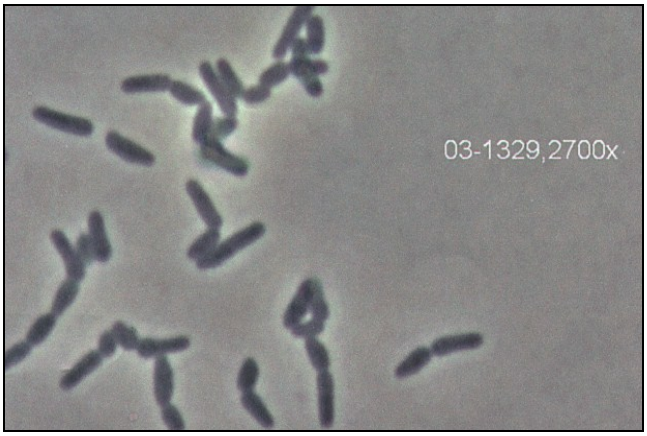

Figure 3.2: Mycobacterium diernhoferi, RA2 (DSM ID 03-1329); cell form: rod; width 1µm; length 2µm.

3.1.3 Determination of growth characteristics of *Pseudomonas citronellolis* RA1 and *Mycobacterium diernhoferi* RA2

The two bacterial strains RA1 and RA2 were cultivated and enriched at room temperature in a shaking culture containing mineral medium 1.10 M (-YE) supplemented with 0.5 mM triethylamine as the sole carbon and energy source. Neither addition of yeast extract nor vitamins was essential for growth. Both strains RA1 and RA2 utilized all the triethylamine within 4 days. After inoculation of either one of the strains a rapid growth was found immediately. Growth of RA1 and RA2 reached the stationary phase after 1 and 2 days of

cultivation, respectively. In the degradation experiments the substrate concentration was quantified by gas chromatography. 0.5 mM of the substrate was degraded within the first 4 days of inoculation (Figure 3.3 and Figure 3.4).

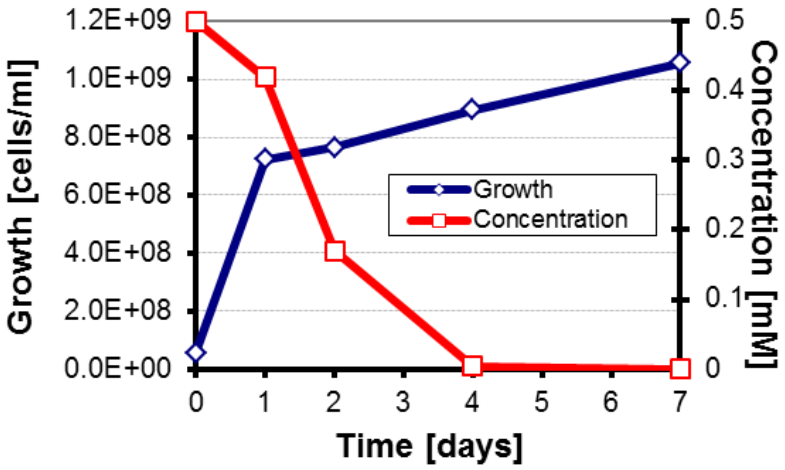

Figure 3.3: Degradation of triethylamine by RA1.

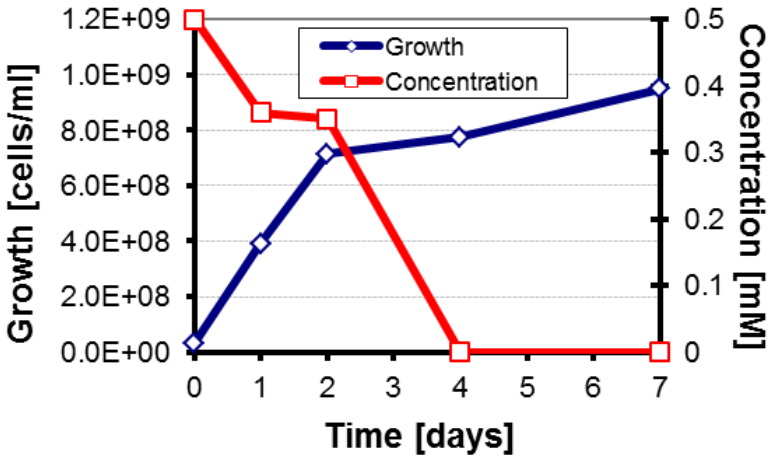

Figure 3.4: Degradation of triethylamine by RA2.

Both strains RA1 and RA2 were separately tested for their potential to degrade higher concentrations of triethylamine. Measurements for degradation of triethylamine on the GC were carried out for 22 days. Figure 3.5 shows that, concentrations 0.5 to 3.0 mM were completely degraded by RA1 in the first 7 days and that concentrations 4.0 to 9.0 mM were completely degraded in less than 16 days. Furthermore, the higher concentration of 10.0 mM was only degraded to 78% after 22 days. The same applied to the strain RA2 considering the concentrations 0.5 to 9.0 mM; however concentration 10.0 mM was only degraded to 84% after 22 days (Figure 3.7).

Similar measurements were also carried out for further higher concentrations of triethylamine (11.0 to 20.0 mM). After 14 days, and as shown by Figure 3.6, only the concentration of 12.0 mM of triethylamine was completely degraded by RA1. Concentrations 11.0, 13.0, 15.0, 17.0, 18.0, 19.0 and 20.0 mM were only degraded to 47, 43, 40, 37, 39, 43, 24% respectively; while no value of degradation was detected for both concentrations 14.0 and 16.0 mM (Figure 3.6). In addition, and as shown by Figure 3.8, after 14 days strain RA2 was found to degrade concentrations 11.0, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0, 18.0, 19.0, and 20.0 mM of triethylamine to 56, 60, 46, 58, 48, 48, 46, 14, 39 and 16% respectively.

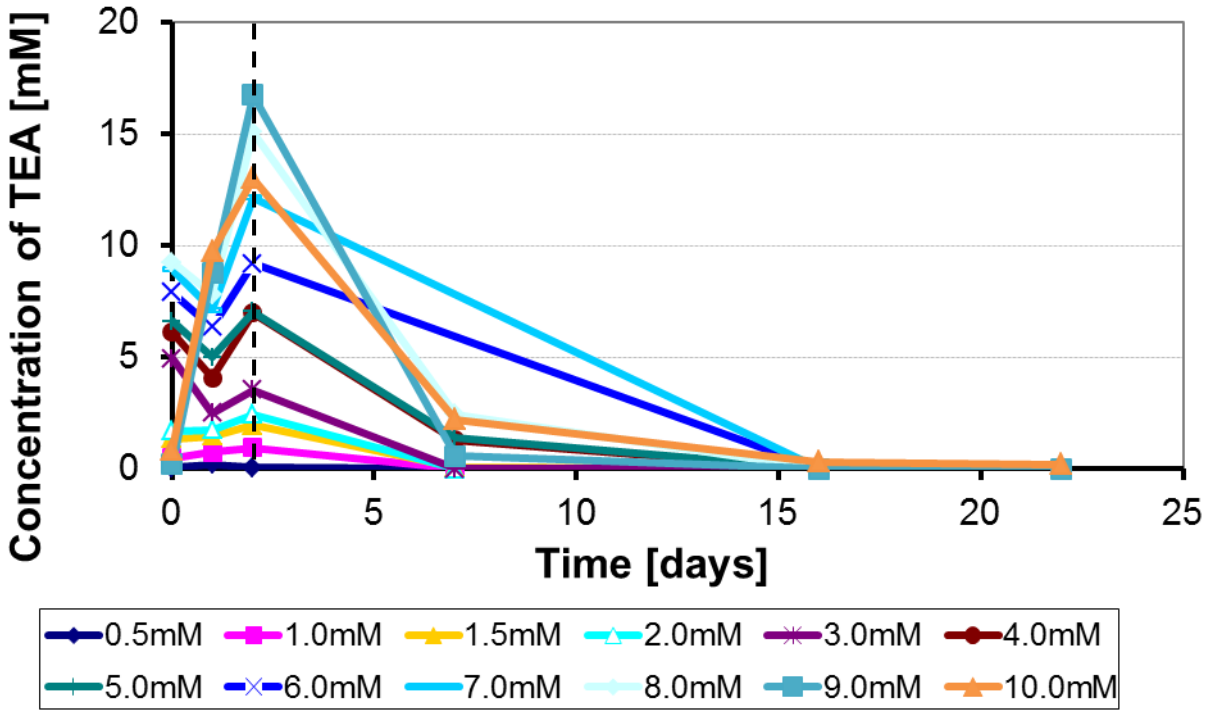

Figure 3.5: Degradation of higher concentration of triethylamine by RA1 (0.5mM – 10.0mM).

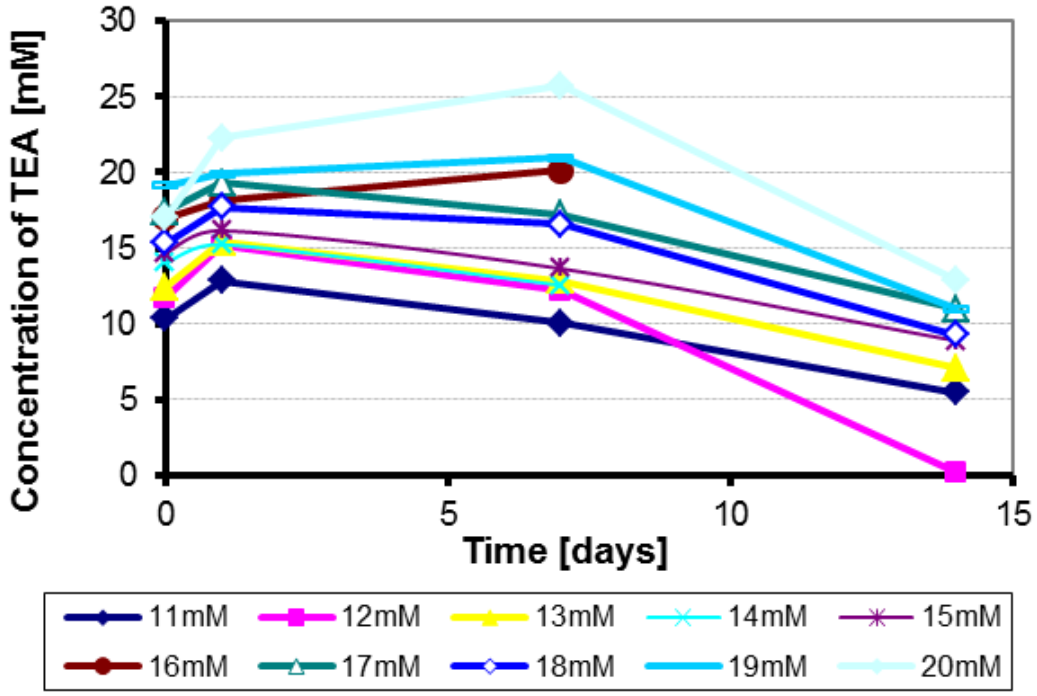

Figure 3.6: Degradation of higher concentration of triethylamine by RA1 (11.0mM – 20.0mM).

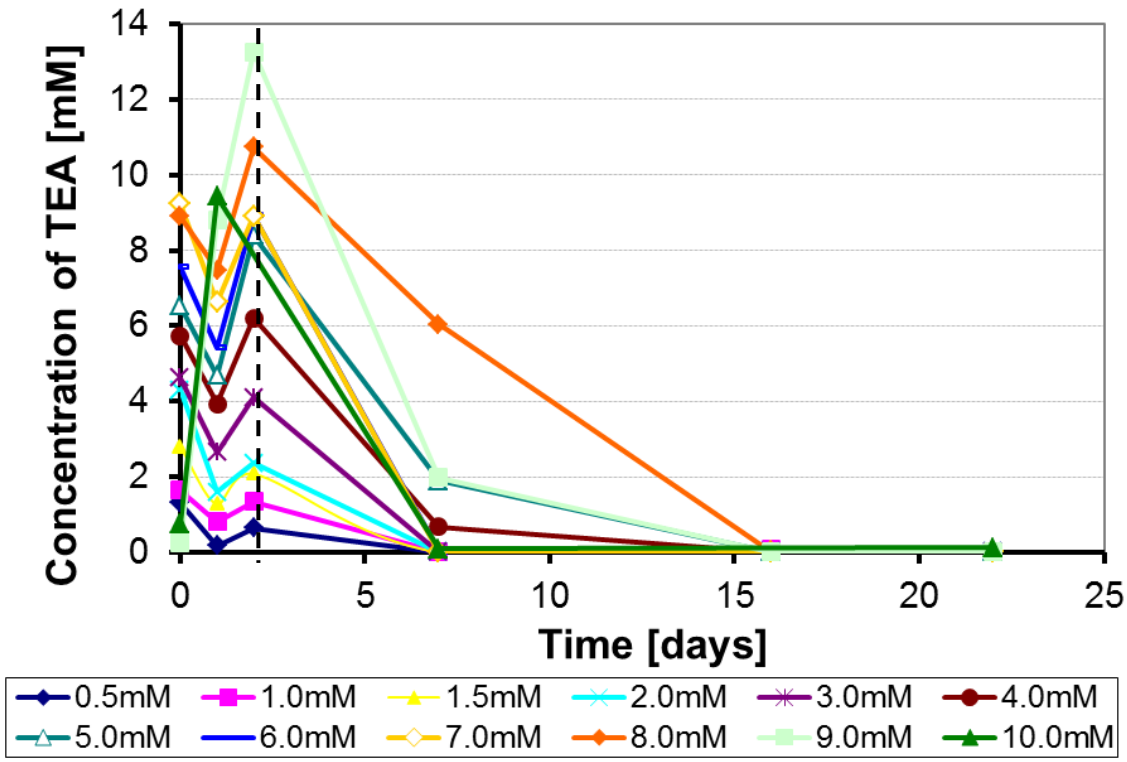

Figure 3.7: Degradation of higher concentration of triethylamine by RA2 (0.5mM – 10.0mM).

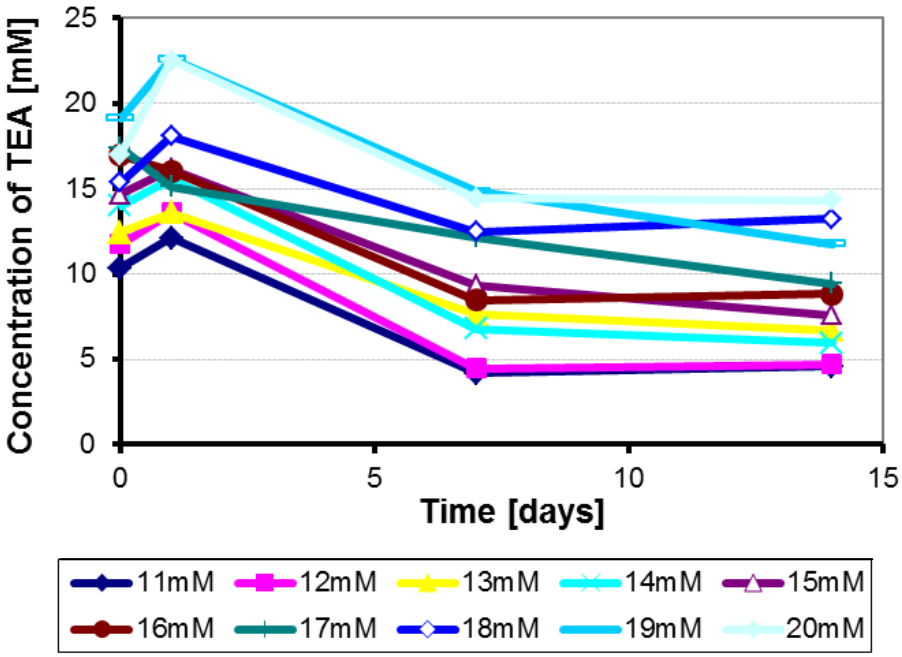

Figure 3.8: Degradation of higher concentration of triethylamine by RA2 (11.0mM – 20.0mM).

A study of the potential of RA1 and RA2 to degrade triethylamine under anaerobic conditions was performed (Section 2.3.2) and no significant growth was detected, which confirms that both strains can only degrade the substrate under aerobic conditions. A parallel study of the effect of Na_2S on the growth of RA1 and RA2 was carried out and no effect was determined, which rules out the possibility for either RA1 or RA2 to have been poisoned or inhibited by the presence of Na_2S.

3.1.4 Substrate spectra of *Pseudomonas citronellolis* RA1 and *Mycobacterium diernhoferi* RA2

Other odorous compounds in a concentration of 0.5 mM, besides triethylamine, were separately tested as a sole carbon and energy source to find out the growth capability and substrate spectrum for RA1 and RA2 compared to the trimethylamine-degrading strain, *Aminobacter aminovorans* A. Amin (Table 3.3). After 11 days, all three strains, RA1, RA2 and A. Amin had the potential to completely degrade furfurylmercaptan (FM) and only A. Amin was able to degrade trimethylamine (TMA) completely and Dimethyltrisulfide (DMTS) to 63%. Strain RA1 was a better degrader of dimethyldisulfide (DMDS) and trimethylamine (TME) but not of ethyl acrylate (ET), where strain RA2 showed a higher degradability

(87.5%). In addition, RA1 was the only strain to show degradability of pentanethiol (PT) and 2.5-dimethylpyrazine (DP) (53.8 and 33% respectively).

Table 3.3: Growth of amine degrading bacteria in the presence of other odour compounds.

Substrate / Bacteria	DMDS	DMTS	FM	PT	DM	DP	IP	TEA	TMA	ET
A. Amin* (TMA)	-	+ (63.0%)	+	-	-	-	-	-	+	-
RA1 (TEA)**	+ (44.1%)	-	+	+ (53.8%)	-	+ (33%)	-	+	+ (61.6%)	+ (32.7%)
RA2* (TEA)**	+ (24.1%)	-	+	-	-	-	-	+	+ (9.5%)	+ (87.5%)

+: total degradation -: no degradation (Measurements after 11 days)

* *Aminobacter aminovorans*
** *Pseudomonas citronellolis*
*** *Mycobacterium diernhoferi*

The growth capability and substrate spectrum for RA1, RA2 and a mixture of both RA1 and RA2 was studied in a mixture of 10 odorous compounds each at a concentration of 0.5 mM (Table 3.4). RA1, RA2 and RA1+RA2 degraded only furfurylmercaptan (FM) completely. Surprisingly, RA1 and RA1+RA2 were not able to degrade triethylamine (TEA) and RA2 only to 9.3%. All three compounds, 2,3-diethyl-5-methylpyrazine (DM), 2,5-dimethylpyrazine (DP) and 2-isobutyl-3-methoxypyrazine (IP) were not degraded by neither RA1 nor RA2 and also not by RA1+RA2. Pentanethiol (PT) was found to be the most degraded compound in the mixture by all RA1, RA2 and RA1+RA2 (82.9, 90.8, and 29.7% respectively). This was followed by ethyl acrylate (ET) (73.4, 88.4, and 84.1% respectively). Furthermore, dimethyldisulfide (DMDS) was not degraded by RA1 but by RA2 and RA1+RA2 (13.4 and 13.2% respectively); whereas dimethyltrisulfide (DMTS) was degraded by RA1, RA2 and RA1+RA2 (22.3, 63.9 and 39.6% respectively). In addition, trimethylamine (TME) was neither degraded by RA1 nor RA1+RA2 but to a 3.1% by RA2 (Table 3.4).

By comparing the different values of degradation in Table 3.4, it was noticed that mixing RA1 and RA2 strains did not have any influence on the degradation of dimethyldisulfide (DMDS) (13.2%), but it did decrease the degradation of pentanethiol (PT) to a value of 29.7%. In case of dimethyltrisulfide (DMTS), the highest degradation was accomplished by RA2 alone

(63.9%) and no significant effect was measured in case of degradation of ethyl acrylate (ET) (Table 3.4).

Table 3.4: Growth of RA1 and RA2 in the presence of other odour compounds.

Substrate / Bacteria	DMDS	DMTS	FM	PT	DM	DP	IP	TEA	TMA	ET
RA1*	-	+ (22.3%)	+	+ (82.9%)	-	-	-	-	-	+ (73.4%)
RA2**	+ (13.4%)	+ (63.9%)	+	+ (90.8%)	-	-	-	+ (9.3%)	+ (3.1%)	+ (88.4%)
RA1*+RA2**	+ (13.2%)	+ (39.6%)	+	+ (29.7%)	-	-	-	-	-	+ (84.1%)

+: total degradation -: no degradation (Measurements after 6 days)

* *Pseudomonas citronellolis*
** *Mycobacterium diernhoferi*

A further comparison of Table 3.3 and Table 3.4, lead to the conclusion that RA1 and RA2 better degraded triethylamine (TEA) in the case of single substrates (Table 3.3) than in the substrate mixture (Table 3.4). The same applied to the degradation of dimethyldisulfide (DMDS), 2,5-dimethylpyrazine (DP) and trimethylamine (TME) but not to the degradation of dimethyltrisulfide (DMTS), pentanethiol (PT) and ethyl acrylate (EA) were the degradation was enhanced by mixing the different odorous substrates. No effect was noticed in the case of degradation of furfurylmercaptan (FM), 2,3-diethyl-5-methylpyrazine (DM) and 2-isobutyl-3-methoxypyrazine (IP) (Table 3.3 and Table 3.4).

A number of aliphatic and aromatic amines were also separately tested as a sole carbon and energy source to find out the growth capability and substrate spectrum for RA1 and RA2 (Table 3.5). It was clear that both RA1 and RA2 showed similarities in utilizing and assimilating different compounds except in the case of 1-amino-2-naphthol hydrochloride where RA1 showed a positive growth in comparison to RA2.

Table 3.5: Amine degradation by RA1 and RA2.

Amine	**Growth of RA1***	**Growth of RA2****
Piperidine	+	+
Cyclohexylamine	+	+
Aniline	+	+
S(-)-Phenylethylamine	+	+
R(+)-Phenylethylamine	+	+
Ethanolamine	-	-
Diethylamine	-	-
Triethylamine	+	+
3-Amino-2,4,6,-Triiodobenzoic acid	+	+
6-Aminohexanoic acid	+	+
4-Aminobenzoic acid	+	+
6-Aminocaproic acid	+	+
4-Aminoantipyrene	+	+
Adenine	-	-
Ampicillin	+	+
1-Amino-2-naphthol hydrochloride	+	-
2-Amino-3-naphthol	-	-
4-Amino-1-naphthol hydrochloride	+	+
5-Amino-1-naphthol	+	+
D-α-Aminophenylacetic Acid	-	-
Ethylenediamine	+	+
3-Amino-1-propanol	-	-

+: growth -:no growth

**Pseudomonas citronellolis*
***Mycobacterium diernhoferi*
(measurements after 7 days)

3.1.5 Determination of the degradation pathway for triethylamine

The aerobic degradation of triethylamine by RA1 and RA2 strains was studied separately. The growth of RA1 and RA2 was individually examined on the following substrates supplied as the sole sources of carbon and energy: Triethylamine (TEA), diethylamine (DEA), triethanolamine, diethanolamine, ethanolamine, acetaldehyde and sodium acetate. The growth experiments were performed in a nitrogen-free mineral medium 1.10 M (-YE-N) and again in a mineral medium containing an ammonium source 1.10 M (-YE+N). Cell growth was determined by measuring optical density (OD) of cultures at 600 nm in a spectrophotometer.

3.1.5.1 Growth of RA1 on the different substrates in a nitrogen-free mineral medium 1.10 M (YE-N).

Strain RA1 was found to grow on 1.0 mM triethylamine as the sole carbon and energy source in a mineral medium without ammonium sulphate ($(NH_4)_2SO_4$) (Figure 3.9).

In Figure 3.9, the growth curves of RA1 on various substrates are presented. Growth was observed for triethylamine (A), diethylamine (B), ethanolamine (E) and sodium acetate (G). These all increased rapidly to a plateau within 50 hours, apart from triethylamine, for which growth was more gradual (within 100 hours). No growth was observed for triethanolamine (C), diethanolamine (D) or acetaldehyde (F). Despite a more gradual growth for triethylamine (A), this substrate achieved the greatest overall growth of any substrate, followed by ethanolamine (E), diethylamine (B) and sodium acetate (G).

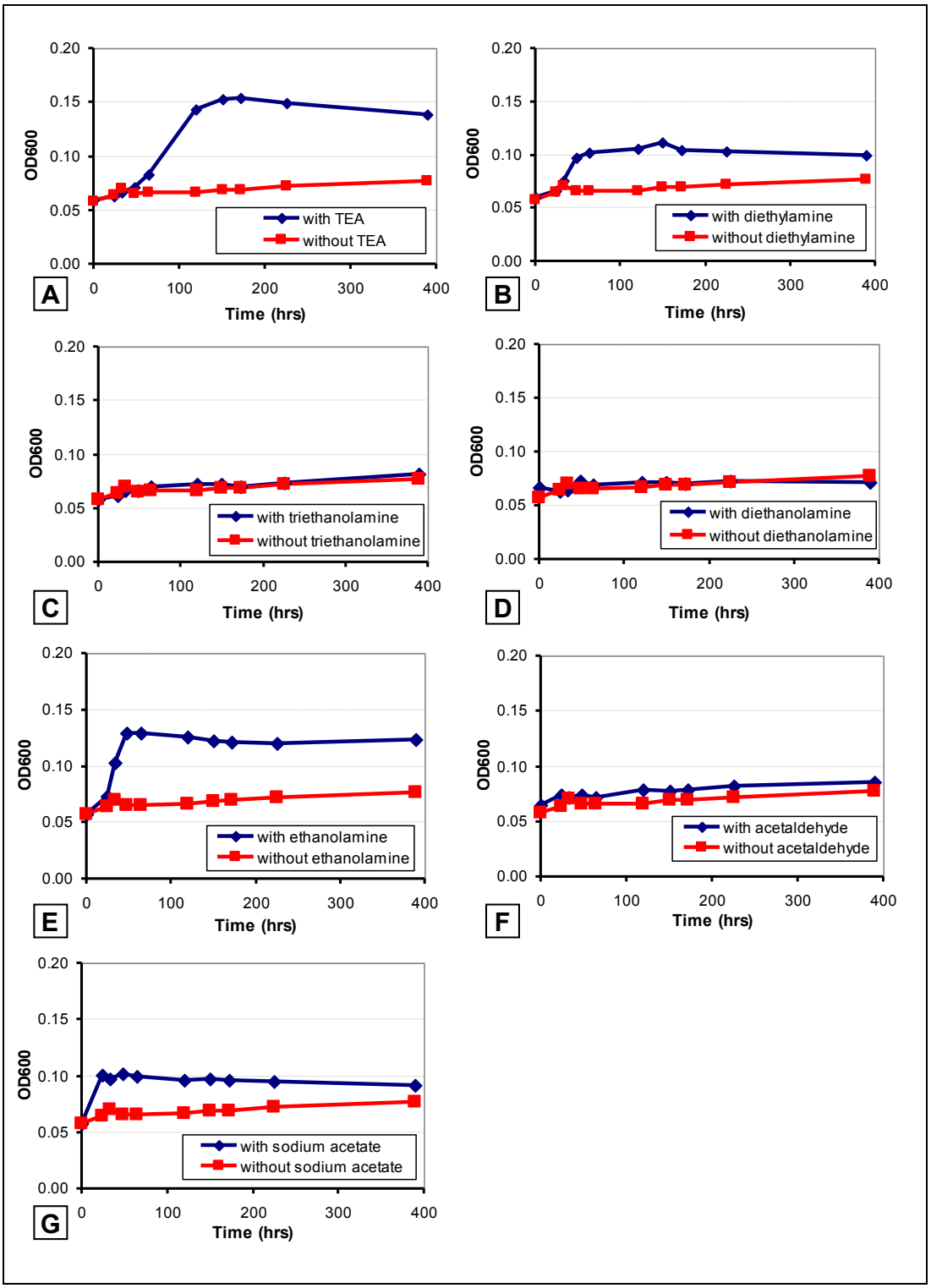

Figure 3.9: Growth of RA1 on 1.10 M (-YE-N) with/without (A) 1.0mM triethylamine; (B) 1.0mM diethylamine; (C) 1.0mM triethanolamine; (D) 1.0mM diethanolamine; (E) 1.0mM ethanolamine; (F) 1.0mM acetalhyde; (G) 1.0mM sodium acetate.

3.1.5.2 Growth of RA2 on the different substrates in a nitrogen-free mineral medium 1.10 M (YE-N).

In Figure 3.10, the growth curves of RA2 on the same substrates as for RA1 (Figure 3.9) are shown. Very similar trends were observed for the two strains. Thus, growth was measured for triethylamine (A), diethylamine (B), ethanolamine (E) and sodium acetate (G) and not for triethanolamine (C), diethanolamine (D) or acetaldehyde (F). However, despite similar growth rates, the growth peaks were higher for RA2 than for RA1 in each case, and therefore occurred later (200 hours for triethylamine (A), 150 hours for ethanolamine (E) and 50 hours for diethylamine (B) and sodium acetate (G)).

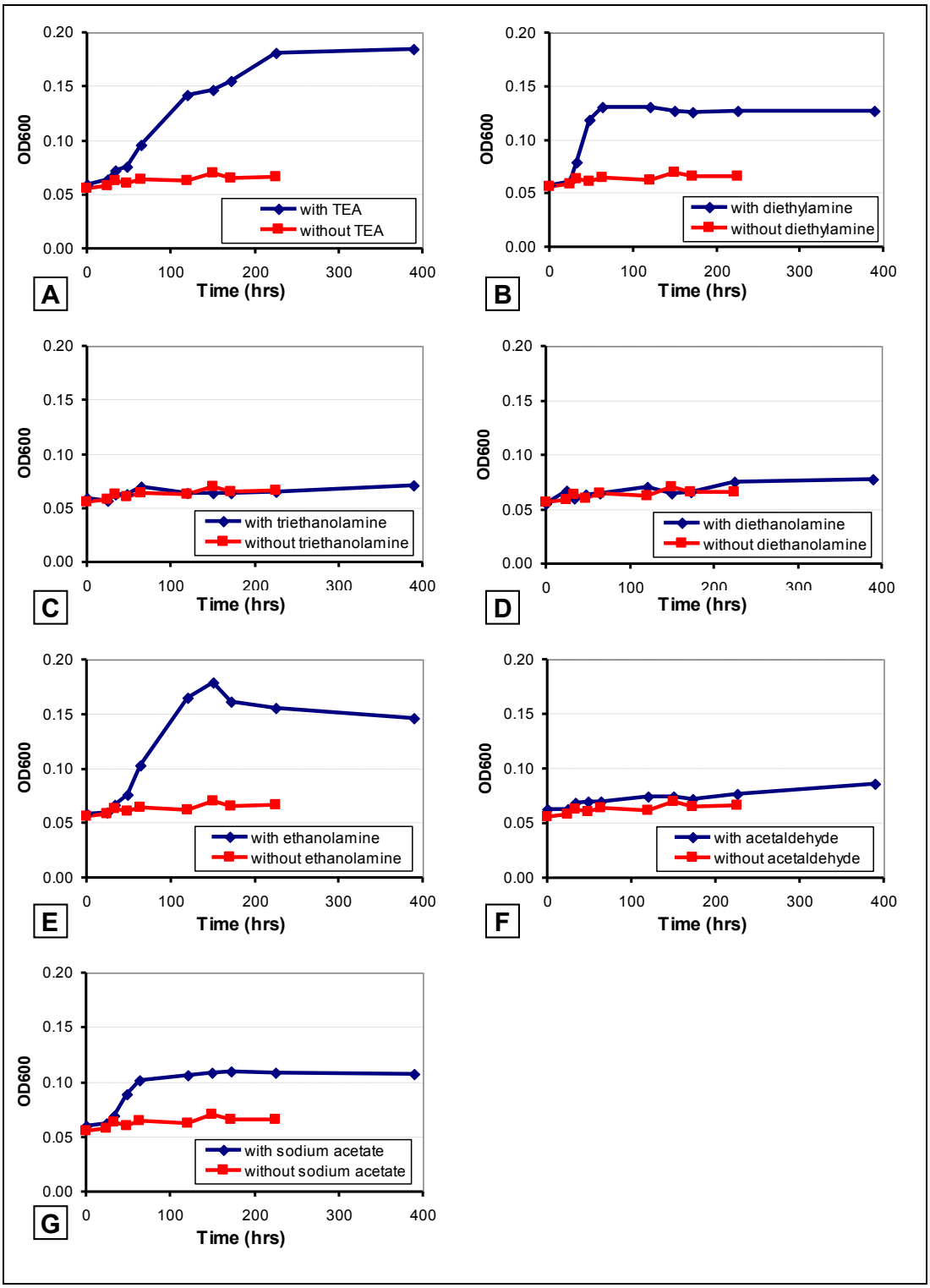

Figure 3.10: Growth of RA2 on 1.10 M (-YE-N) with/without (A) 1.0mM triethylamine; (B) 1.0mM diethylamine; (C) 1.0mM triethanolamine; (D) 1.0mM diethanolamine; (E) 1.0mM ethanolamine; (F) 1.0mM acetaldehyde; (G) 1.0 mM sodium acetate.

3.1.5.3 Growth of RA1 on the different substrates in minimal mineral medium with an ammonium source (1.10 M (-YE+N)).

Figure 3.11 shows the growth curves of RA1 on the various substrates shown in Figure 3.9, but with the addition of an ammonium source ($(NH_4)_2SO_4$). The growth trends and magnitudes are very similar, indicating that the ammonium source has no clear influence on the RA1 strain.

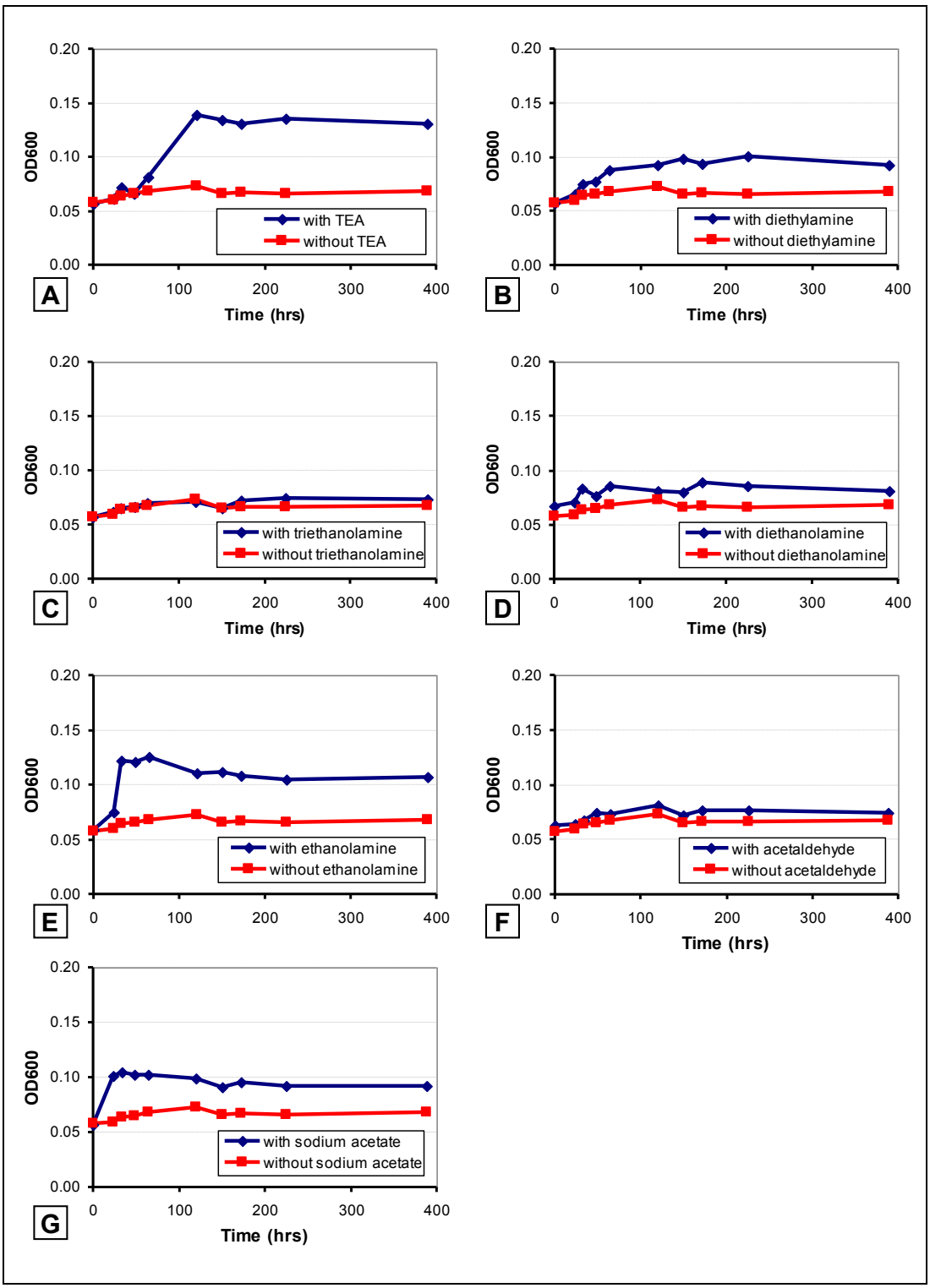

Figure 3.11: Growth of RA1 on 1.10 M (-YE+N) with/without (A) 1.0mM triethylamine; (B) 1.0mM diethylamine; (C) 1.0mM triethanolamine; (D) 1.0mM diethanolamine; (E) 1.0mM ethanolamine; (F) 1.0mM acetaldehyde; (G) 1.0mM sodium acetate.

3.1.5.4 Growth of RA2 on the different substrates in minimal mineral medium with an ammonium source (1.10 M (-YE+N)).

In Figure 3.12, the growth curves are presented for the RA2 strain, with the addition of an ammonium source ($(NH_4)_2SO_4$). These can be compared with the curves for the same strain with no ammonium source, shown in Figure 3.10. Similar trends are observed, but the presence of the ammonium source decreases the growth of RA2 on each of the four substrates on which it normally grows, (triethylamine (A), diethylamine (B), ethanolamine (E) and sodium acetate (G)).

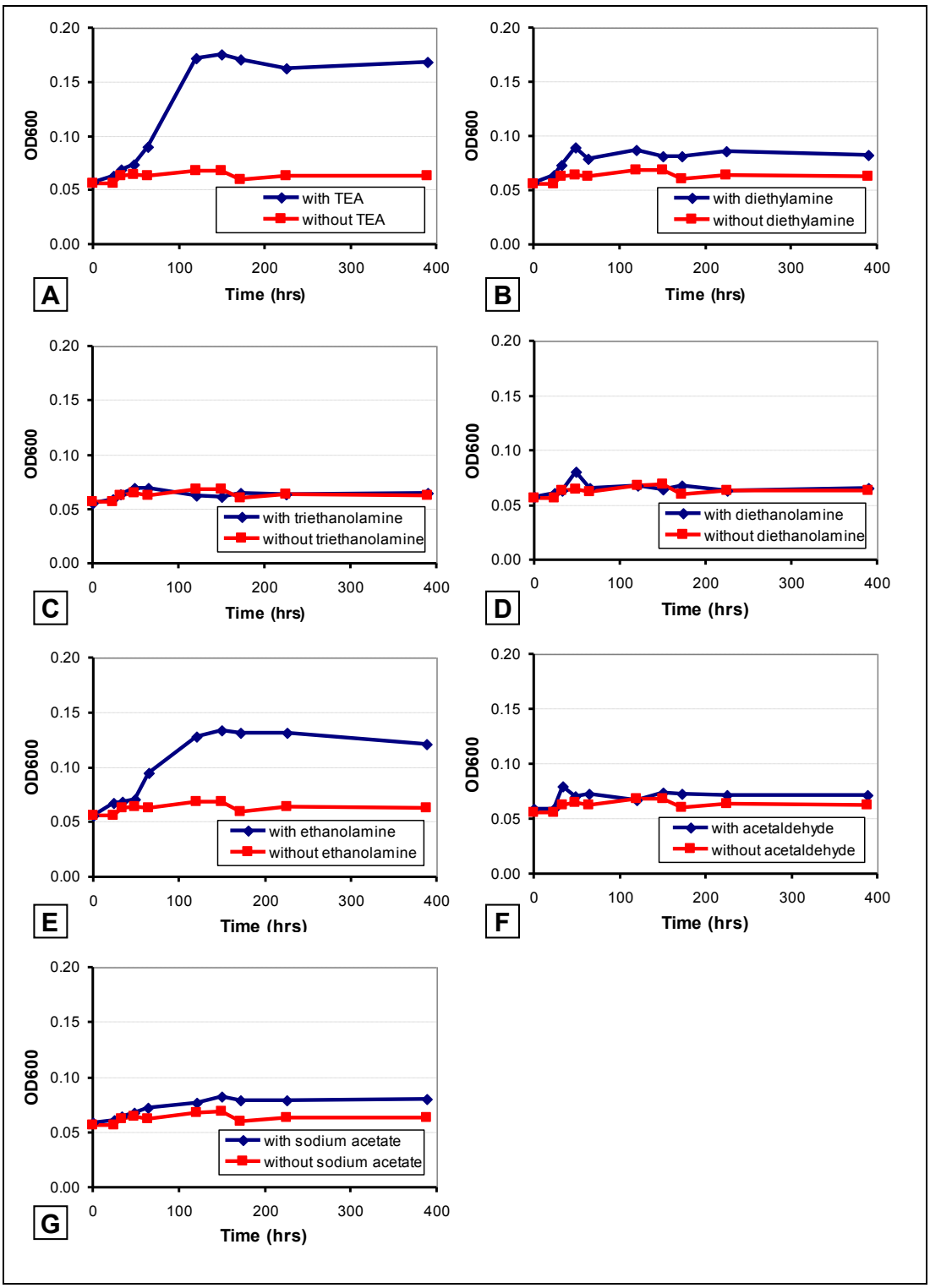

Figure 3.12: Growth of RA2 on 1.10 M (-YE+N) with/without (A) 1.0mM triethylamine; (B) 1.0mM diethylamine; (C) 1.0mM triethanolamine; (D) 1.0mM diethanolamine; (E) 1.0mM ethanolamine; (F) 1.0mM acetaldehyde; (G) 1.0mM sodium acetate.

By comparing Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12 it is observed that both RA1 and RA2 are able to grow on triethylamine (A), diethylamine (B), ethanolamine (E) and sodium acetate (G) but not on triethanolamine (C) nor diethanolamine (D). Also the growth of the two strains on acetaldehyde (F) is not very significant, due to the fact that acetaldehyde might be poisonous to the bacteria.

The ammonium concentration was measured during the growth of both RA1 and RA2 on the different substrates (A, B, C, D, E, F and G) in the absence of an ammonium source (Figure 3.9 and Figure 3.10). This was done using a test kit (Spectroquant, Merck, Germany) (Figure 3.13 and Figure 3.14).

3.1.5.5 Growth of RA1 on the different substrates in minimal mineral medium without an ammonium source (1.10 M (-YE-N)).

In Figure 3.13, the accumulation of ammonium during the growth of RA1 on various substrates is presented. An increase in the ammonium concentration was observed for triethylamine (A), diethylamine (B), ethanolamine (E) and sodium acetate (G). These all increased rapidly to a plateau within 50 hours, apart from triethylamine, for which increase was more gradual (within 100 hours). No increase of ammonium was observed for triethanolamine (C), diethanolamine (D) or acetaldehyde (F). Despite a more gradual increase of ammonium for triethylamine (A), this substrate achieved the greatest overall increase of any substrate, followed by ethanolamine (E), diethylamine (B) and sodium acetate (G).

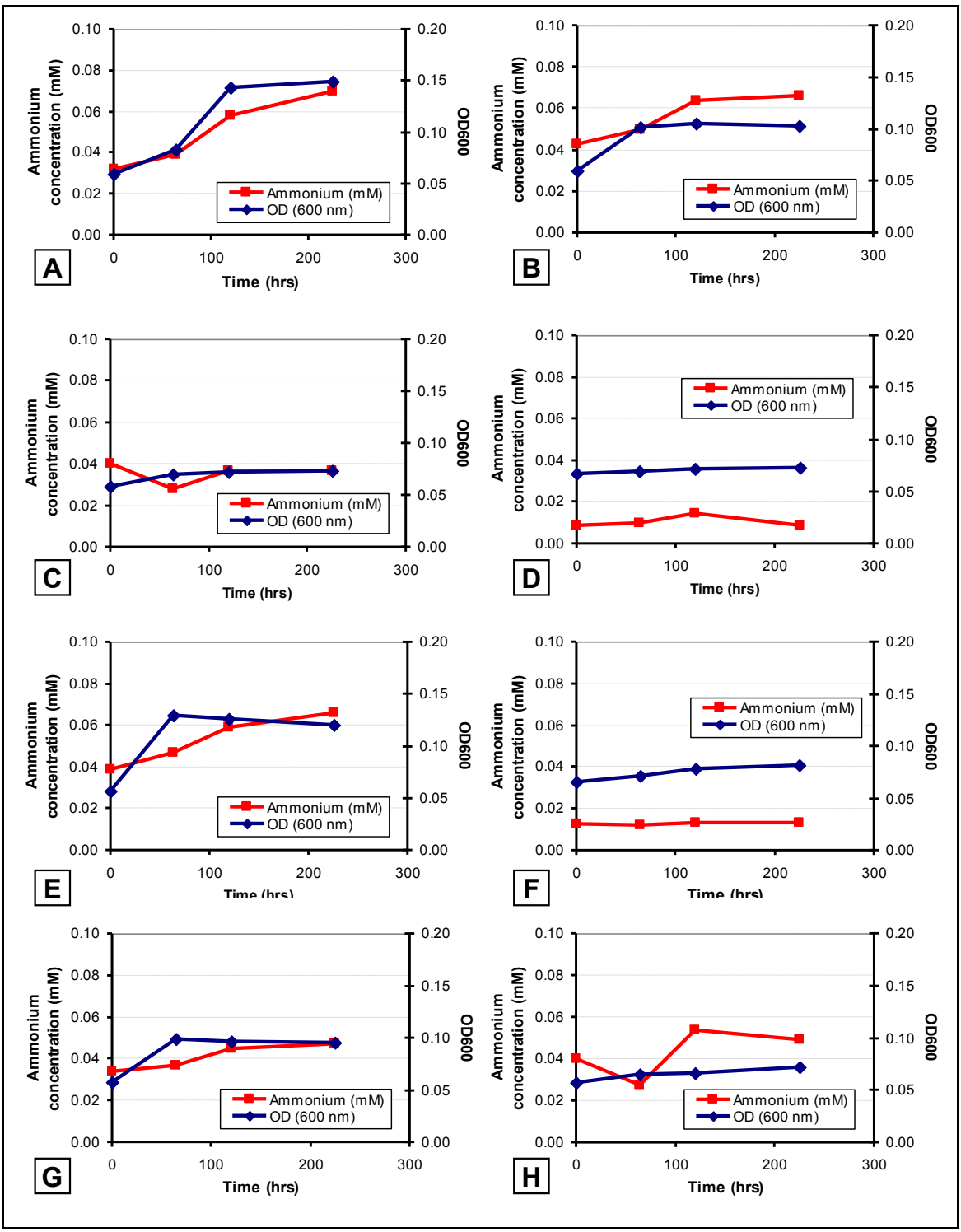

Figure 3.13: Accumulation of ammonium during growth of RA1 with (A) 1.0mM triethylamine; (B) 1.0mM diethylamine; (C) 1.0mM triethanolamine; (D) 1.0mM diethanolamine; (E) 1.0mM ethanolamine; (F) 1.0mM acetaldehyde; (G) 1.0mM sodium acetate; (H) on minimal mineral medium 1.10 M (-YE-N).

3.1.5.6 Growth of RA2 on the different substrates in minimal mineral medium without an ammonium source (1.10 M (-YE-N)).

In Figure 3.14, the accumulation of ammonium during the growth of RA2 on various substrates is presented as for RA1 (Figure 3.13). Very similar trends were observed for the two strains. An increase of ammonium was measured for triethylamine (A), diethylamine (B), ethanolamine (E) and sodium acetate (G) and not for triethanolamine (C), diethanolamine (D) or acetaldehyde (F). However, despite similar increase rates, the amount of ammonium produced was higher for RA2 than for RA1 in each case.

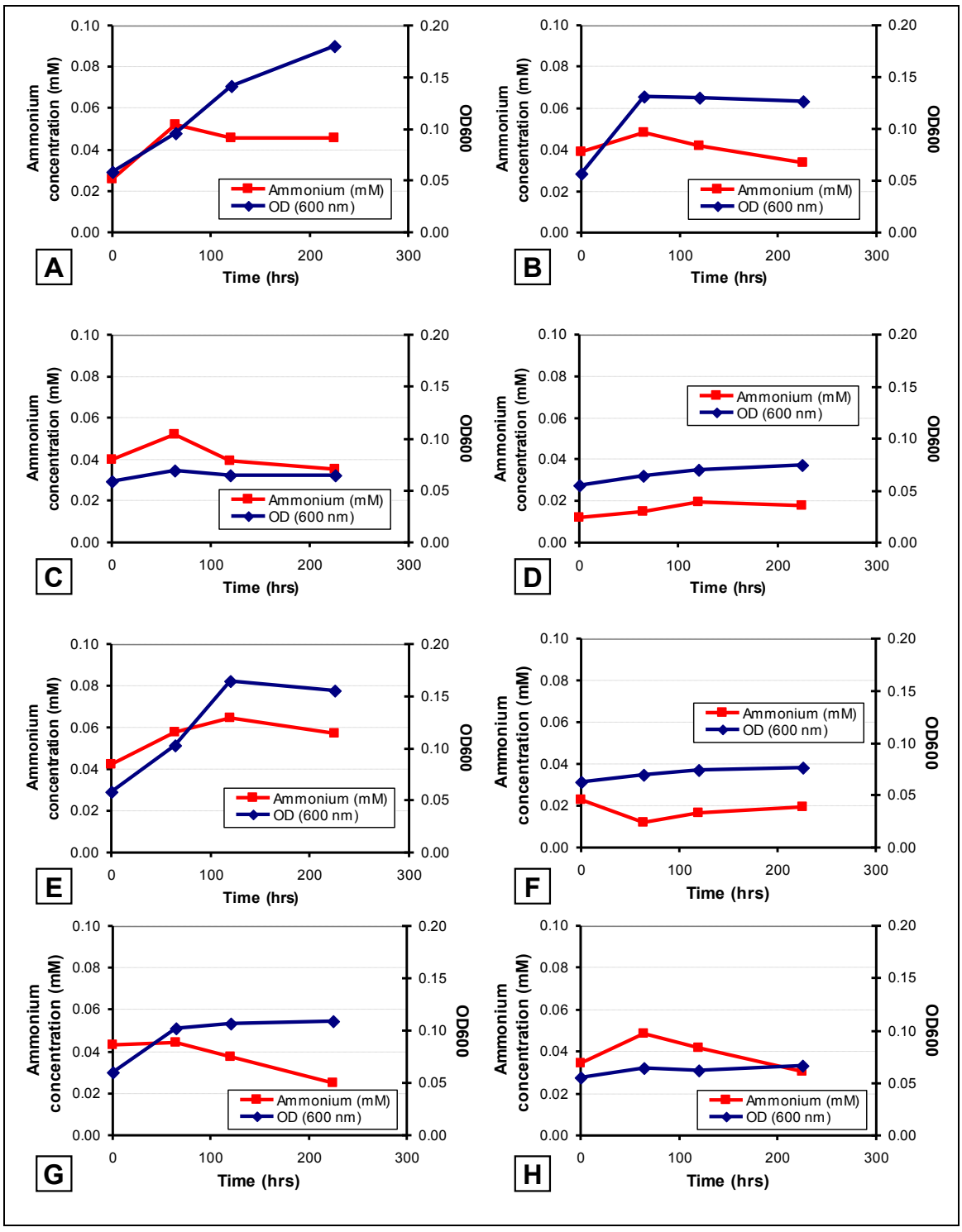

Figure 3.14: Accumulation of ammonium during growth of RA2 with (A) 1.0mM triethylamine; (B) 1.0mM diethylamine; (C) 1.0mM triethanolamine; (D) 1.0mM diethanolamine; (E) 1.0mM ethanolamine; (F) 1.0mM acetaldehyde; (G) 1.0mM sodium acetate; (H) on minimal mineral medium 1.10 M (-YE-N).

3.2 Bacteria that degrade ethyl acrylate

3.2.1 Isolation procedure

In order to isolate bacteria with the potential to degrade ethyl acrylate, 2 g of soil taken from Aussenmühlen Park in Harburg, Germany, were suspended in 20 ml of medium 1.10 M (+YE) with 2.0 mM ethyl acrylate as a carbon and energy source. After 10 - 14 hrs, the ethyl acrylate had disappeared and 2 ml were transferred into 20 ml of fresh medium. This procedure was repeated. After ~10 transfers, 50 µl were spread onto LB-medium plates and incubated at 25 °C. The strain was purified by consequent colony picking, different colonies were transferred onto fresh plates. After ~10 transfers, the bacteria obtained were checked for purity on nutrient broth agar plates. From the plates showing only one type of colonies, one colony was transferred into liquid medium with ethyl acrylate as carbon source. The two fastest growing strains were selected for further studies and were named 2C and 2Cbei.

3.2.2 Identification of the ethyl acrylate degrading bacteria 2C and 2Cbei

The growth of the two strains, 2C and 2Cbei, obtained in the enrichment procedure was tested. With 2.0 mM ethyl acrylate, the growth rates at 25 °C were 0.104 h^{-1} and 0.130 h^{-1}, respectively. The morphology and the physiological parameters of the strains were determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig. Both strains, 2C and 2Cbei, were gram negative with rod cell form of width 0.5 – 0.7 µm and length 1.0 – 2.5 µm. The properties of the two strains are summarized in Table 3.6. Based on these results, both strains 2C and 2Cbei were classified as *Defluvibacter lusatiensis* (Figure 3.15) (Fritsche *et al*., 1999a).

The colonies of 2C and 2Cbei were smooth and pale yellowish on LB medium. Both strains were oxidase and catalase positive (Table 3.6). No growth of 2C or 2Cbei was observed under anaerobic conditions.

The strains 2C and 2Cbei grew on a variety of substrates. Growth was observed on yeast extract, glucose, malate, mannose, n-acetylglucosamine, fructose and xylose. No growth was observed on arabinose, maltose, phenylacetate, citrate, adipate, β-hydroxybutyrate, methanol, trimethylamine, norvaline, malonate, rhamnose, raffinose, and ethanol. Additionaly, both strains grew on McConkey medium, L-tryptophan and glutarate (Table 3.6).

The partial sequencing of 16 s rDNA from both strains, 2C and 2Cbei, resulted in 100% similarity among each other. The similarity to the strain *Defluvibacter lusatiensis* was found to

be 100%. In addition, the profile of cellular fatty acid was found to be typical of the Group α2-Proteobacteria.

Figure 3.15 shows an electron microscopic picture of strains 2C and 2Cbei.

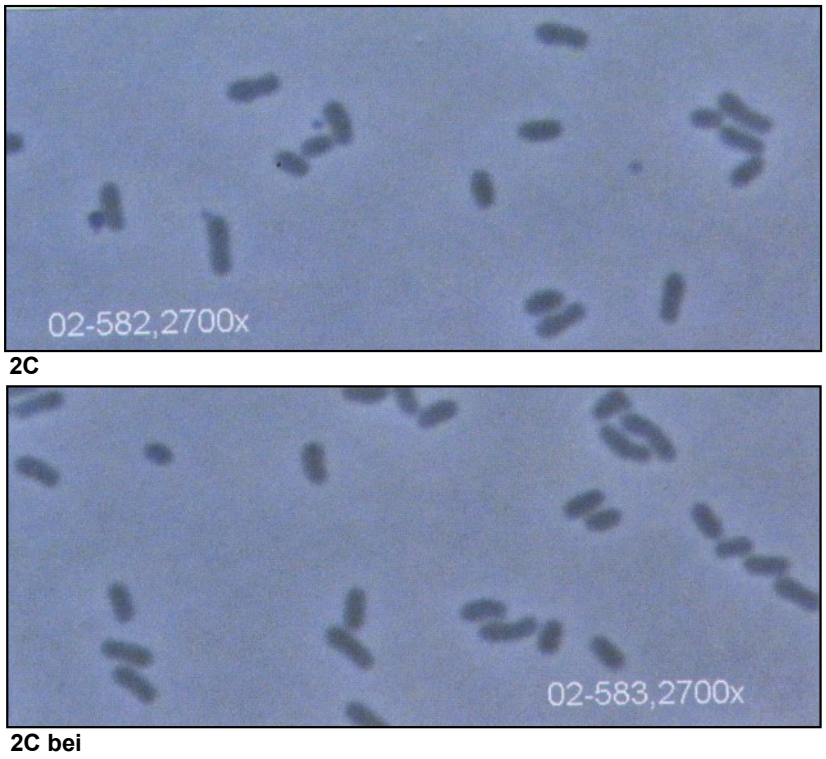

Figure 3.15: Defluvibacter lusatiensis, 2C (ID 02-582) and 2Cbei (ID 02-583).

Table 3.6: Morphological and physiological properties of Defluvibacter lusatiensis, 2C and 2Cbei.

Properties of the strain	Defluvibacter lusatiensis, 2C	Defluvibacter lusatiensis, 2Cbei
Shape of cells:	Rods	Rods
width µm:	0.5 – 0.7	0.5 – 0.7
length µm:	1.0 – 2.5	1.0 – 2.5
Gram reaction	-	-
Lysis by 3% KOH	+	+
Aminopeptidase (Cerny)	+	+
Oxidase	+	+
Catalase	+	+
ADH	-	-
Urease	-	-
Nitrate reduction	-	-
Hydrolysis of gelatine	-	-
Hydrolysis of Esculin	-	-
Hydrolysis of Tween 80	-	-
Hydrolysis of DNA	-	-
Hydrolysis of Starch	-	-
Indol reaction	-	-
Utilization of:		
glucose	+	+
arabinose	-	-
maltose	-	-
malate	+	+
mannose	+	+
phenylacetate	-	-
citrate	-	-
adipate	-	-
n-acetylglucosamine	+	+
β-hydroxybutyrate	-	-
methanol	-	-
trimethylamine	-	-
norvaline	-	-
malonate	-	-
L-tryptophan	w	w
Glutarate	w	w
Acid from:		
glucose	+	+
fructose	+	+
xylose	+	+
rhamnose	-	-
arabinose	-	-
raffinose	-	-
ethanol	-	-
Growth on McConkey	w	W

+ : growth - : no growth w : little growth

3.2.3 Determination of growth characteristics of *Defluvibacter lusatiensis 2C and 2Cbei*

The two bacterial strains 2C and 2Cbei were cultivated and enriched at room temperature in a shaking culture containing mineral medium 1.10 M (+YE) supplemented with 2.0 mM ethyl acrylate as a carbon and energy source. No addition of vitamins was essential for the growth.

The effect of yeast extract on the growth of both 2C and 2Cbei and on their degradability of 2.0 mM ethyl acrylate was studied separately. It was found that the strain 2C was faster in the degradation of 2.0 mM ethyl acrylate in the presence of 0.1% yeast extract. A complete degradation of the substrate was measured after 14 hours; while 21 hours were needed in the absence of 0.1% yeast extract (Figure 3.16). In addition, the growth of 2C on a mineral medium containing 0.1% yeast extract was higher than the growth of 2C on a mineral medium containing 0.1% yeast extract and 2.0 mM ethyl acrylate (1.10 M (+YE)). Strain 2C was found to grow slowest on a mineral medium containing 2.0 mM ethyl acrylate without 0.1% yeast extract (1.10 M (-YE)) (Figure 3.16).

While strain 2C was found to be highly affected by the presence of 0.1% yeast extract (Figure 3.16), strain 2Cbei was almost not affected (Figure *3.20*). The same time of 10 to 11 hours was needed by 2Cbei for the degradation of 2.0 mM ethyl acrylate both in presence and absence of 0.1% yeast extract (Figure *3.20*). Also, the growth of 2Cbei was not noticeably affected by the presence of 0.1% yeast extract. A similar pattern of growth was observed as shown in Figure *3.20*.

By comparing the two strains 2C and 2Cbei (Figure 3.16 and Figure 3.20), it is obvious that strain 2Cbei was faster in degrading 2.0 mM ethyl acrylate. 2Cbei required 10 to 11 hours both in presence and absence of 0.1% yeast extract; whereas 2C required 14 hours in the presence of 0.1% yeast extract and 21 hours in the absence of 0.1% yeast extract. The growth rate of strain 2C was calculated to be 0.160 h^{-1} in the fermenter.

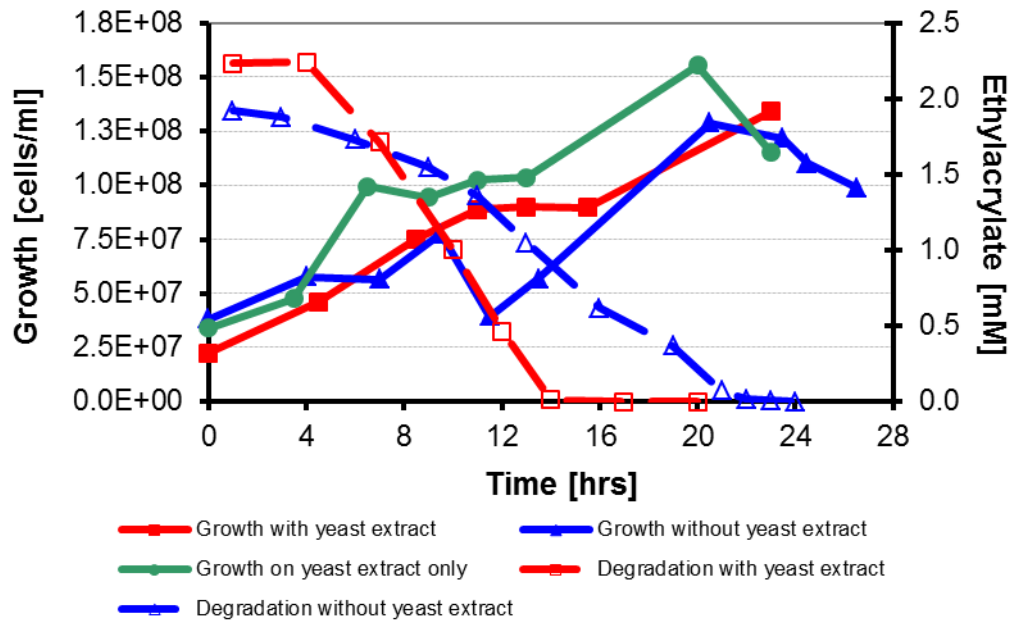

Figure 3.16: Effect of yeast extract on the degradation of ethyl acrylate by 2C.

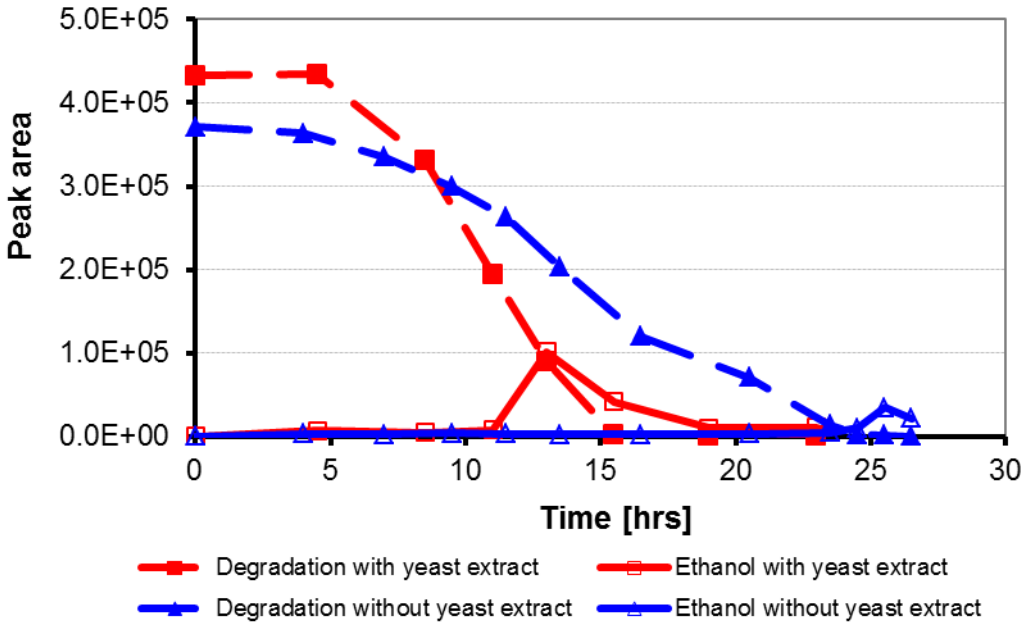

Figure 3.17: Effect of yeast extract on the degradation and product formation of ethyl acrylate by 2C.

Figure 3.17 shows that during the process of degradation of ethyl acrylate by the 2C strain, ethanol was measured in the reaction mixture as a by-product. In the case of presence of 0.1% yeast extract, the amount of ethanol produced was higher (Figure 3.17 and Figure 3.18).

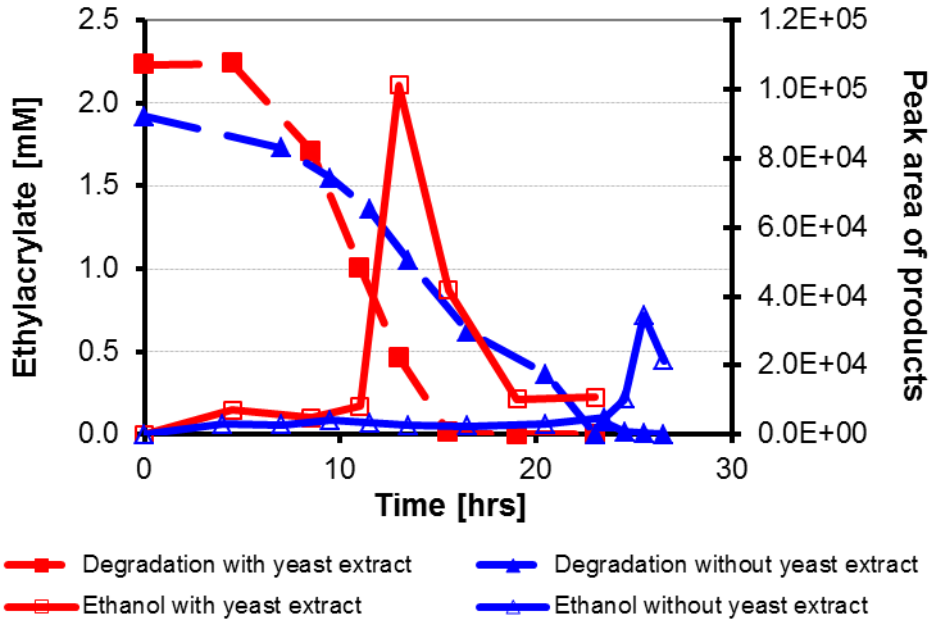

Figure 3.18: Effect of yeast extract on the degradation and product formation of ethyl acrylate by 2C

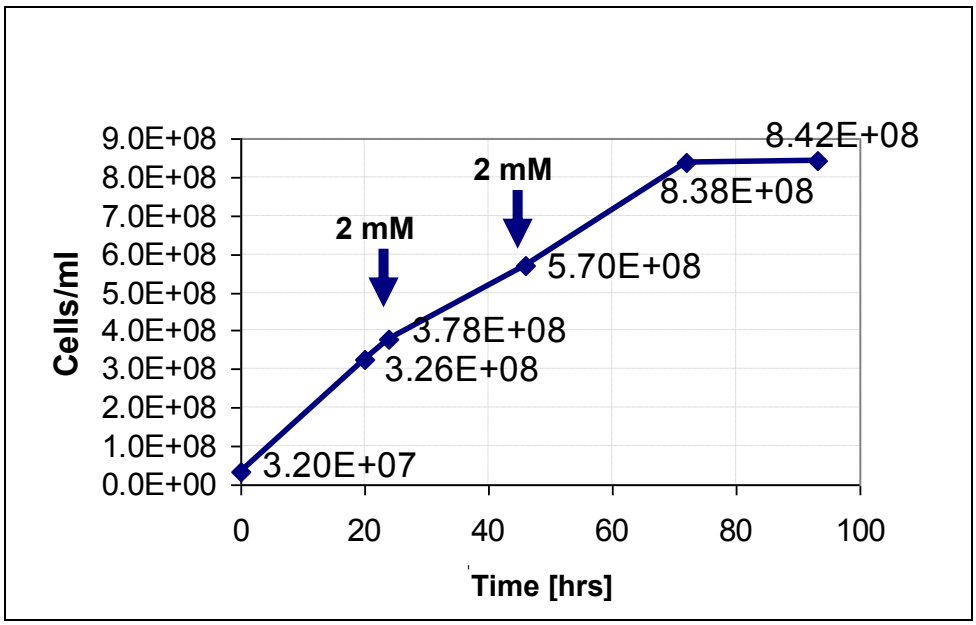

Figure 3.19: Growth of 2C Strain in a 30 Litre Fermenter.

The growth behaviour of the 2C strain was monitored in a 30 litre fermenter (Figure 3.19). An amount of 20 litre of 1.10 M (+YE) medium was inoculated with a 1 litre three-day-old inoculum of 2C strain. The growth was monitored for ~ 4 days (93 hours) and a refeed of a 2 mmol of ethyl acrylate was performed twice; once after 24 hours and again after 46 hours of incubation. The growth rate of strain 2Cbei was calculated to be 0.215 h^{-1} in the fermenter.

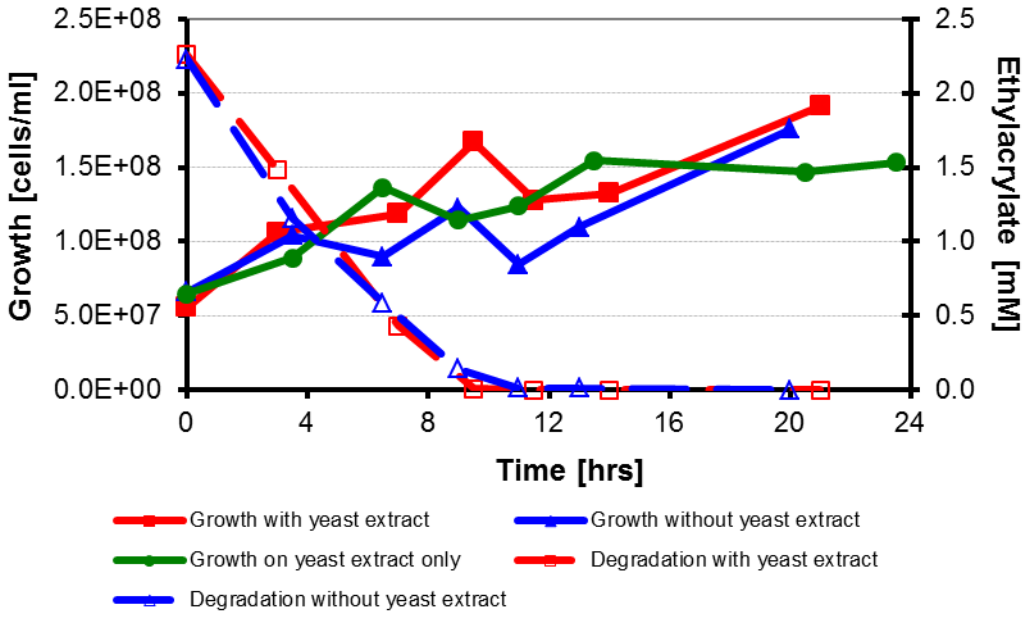

Figure 3.20: Effect of yeast extract on the degradation of ethyl acrylate by 2Cbei.

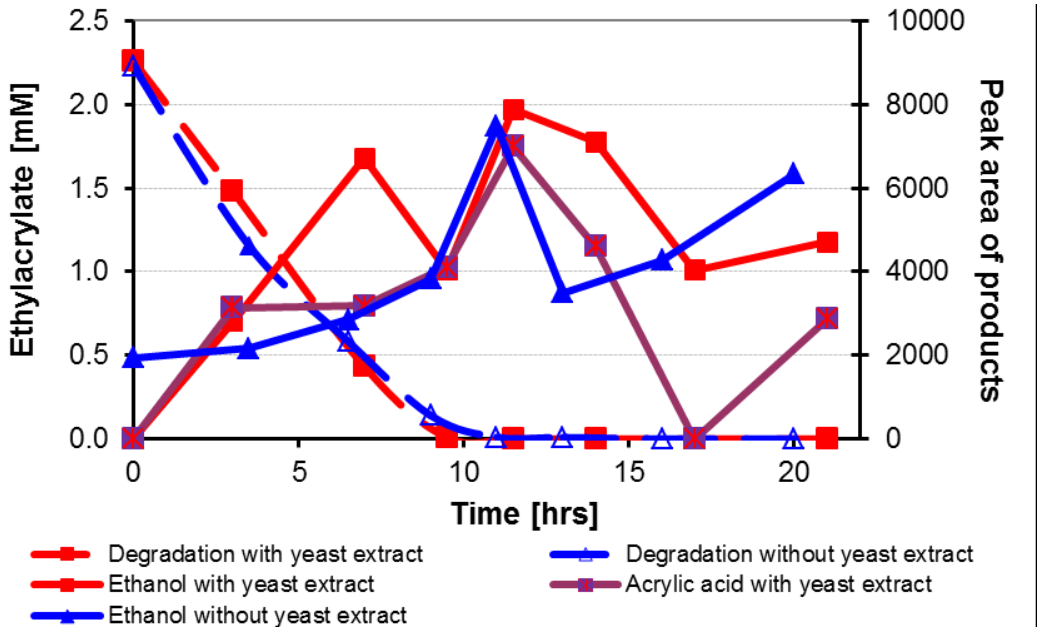

Figure 3.21: Effect of yeast extract on the degradation and product formation of ethyl acrylate by 2Cbei.

Figure 3.21 and Figure 3.22 show that both ethanol and propenoic acid were detected in the reaction mixture as by-products during the process of degradation of 2 mmol ethyl acrylate by the strain 2Cbei.

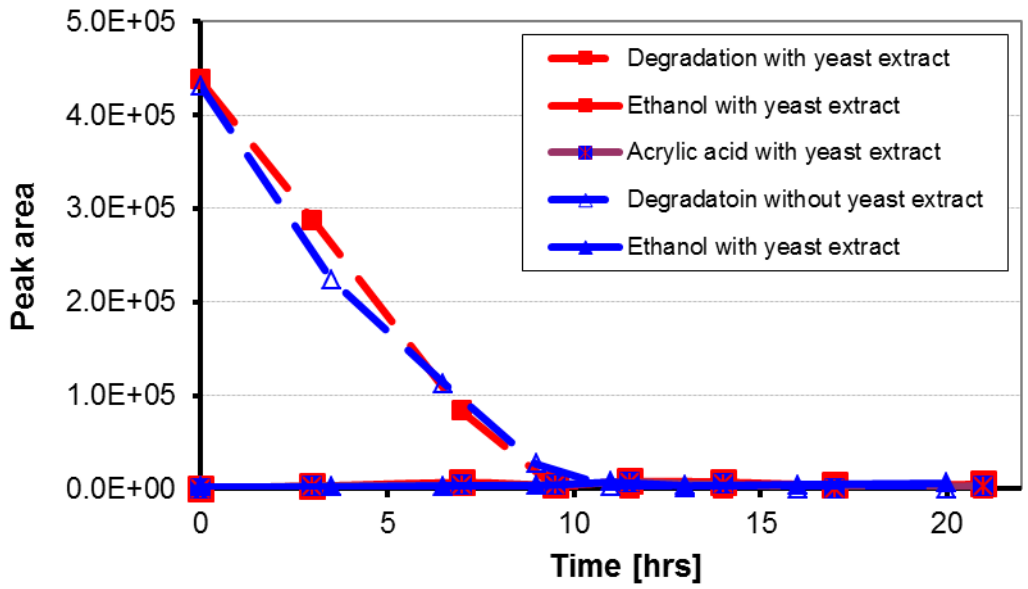

Figure 3.22: Effect of yeast extract on the degradation and product formation of ethyl acrylate by 2Cbei.

Both strains 2C and 2Cbei were separately tested for their potential to degrade higher concentrations of ethyl acrylate in 1.10 M (+YE) medium. Measurements for degradation of ethyl acrylate on the GC were carried out for 16 days. Figure 3.23 shows that, concentrations 0.5 to 3.0 mM were completely degraded by 2C after day one, and that concentrations 4.0, 5.0 and 6.0 were completely degraded after day five. Furthermore, 7.0 mM was completely degraded after sixteen days and concentrations 8.0, 9.0 and 10.0 mM were degraded to 74, 52 and 36 % respectively.

Similarly, Figure 3.24 shows that, concentrations 0.5 to 3.0 mM of ethyl acrylate were completely degraded by 2Cbei after day one, and that 4.0 mM was totally degraded after day two. Furthermore, a complete degradation of 5.0, 6.0 and 7.0 mM was measured after day five. And finally, after sixteen days, concentrations 8.0, 9.0 and 10.0 were degraded to 68, 54 and 34% respectively.

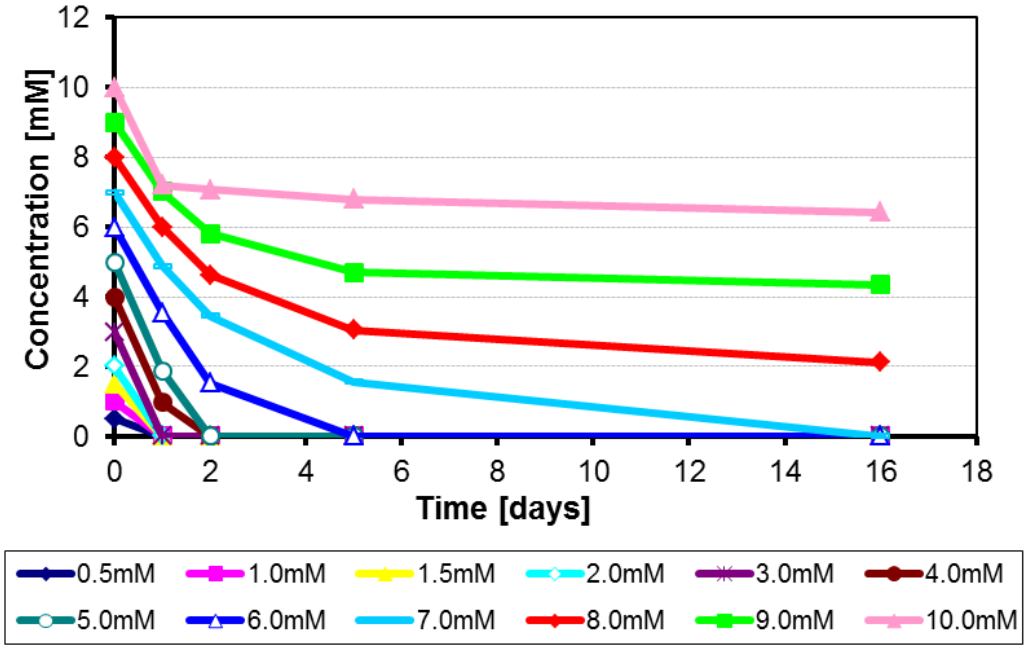

Figure 3.23: Degradation of higher concentrations of ethyl acrylate by 2C.

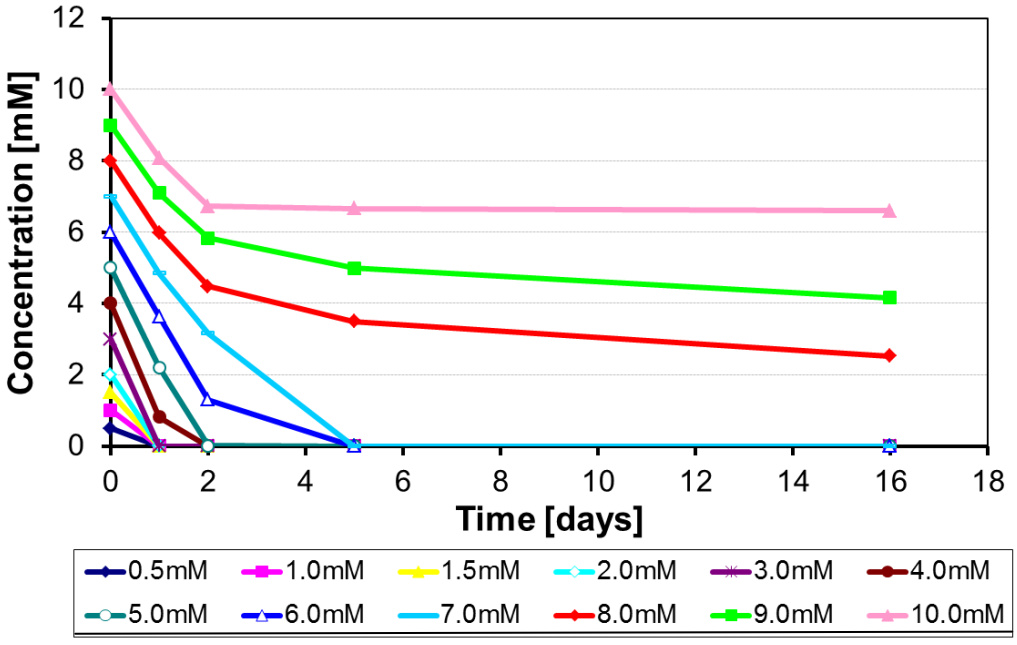

Figure 3.24: Degradation of higher concentrations of ethyl acrylate by 2Cbei.

A study of the potential of 2C and 2Cbei to degrade ethyl acrylate under anaerobic conditions was performed (Section 2.4.2) and no significant growth was detected. This confirms that

both strains can only degrade the substrate under aerobic conditions. A parallel study of the effect of Na_2S on the growth of 2C and 2Cbei was carried out and no effect was determined. This excludes the possibility of either 2C or 2Cbei having been poisoned or inhibited by the presence of Na_2S. It is also worth mentioning that after 4 to 5 hours of incubation, the pink colour reappeared in the incubation bottles, indicating the generation of oxygen in the cultures. This might be due to the formation of the intermediate (propenoic acid) in the first step of the proposed pathway of degradation as elucidated in Figure 4.3.

3.2.4 Determination of the degradation pathway for ethyl acrylate

The aerobic degradation of ethyl acrylate by 2C and 2Cbei strains was studied separately. Thus, the growth of 2C and 2Cbei was individually examined on the following substrates supplied as sources of carbon and energy: Acrylic acid (propenoic acid), ethanol, L(+)-Lactic acid, D(-)-Lactic acid, malonic acid and ethyl acrylate. The growth experiments were performed in a mineral medium containing 0.1% yeast extract (1.10 M (+YE)). Cell growth was determined by optical density (OD) of cultures at 600 nm in a spectrophotometer.

3.2.4.1 Growth of 2C on the different substrates in a mineral medium containing 0.1% yeast extract (1.10 M (+YE)).

Strain 2C was found to grow on 2.0 mM ethyl acrylate as a source for carbon and energy in a mineral medium that contains 0.1% yeast extract (1.10 M (+YE)) (Figure 3.25).

Figure 3.25 shows the growth curves for strain 2C on various substrates in 1.10 M (+YE) medium. Growth was observed for L(+) Lactic acid (C), D(-) Lactic acid (D) and ethyl acrylate (F) and no growth was observed for acrylic acid (A), ethanol (B) or malonic acid (E). The initial growth curves were similar for L(+) Lactic acid (C) and D(-) Lactic acid (D) but the former attained roughly double the growth of the latter and was the highest of any substrate. Growth on Ethyl acrylate (F) was less rapid than for the other substrates but attained the same maximum cell number as with D(-) Lactic acid (D).

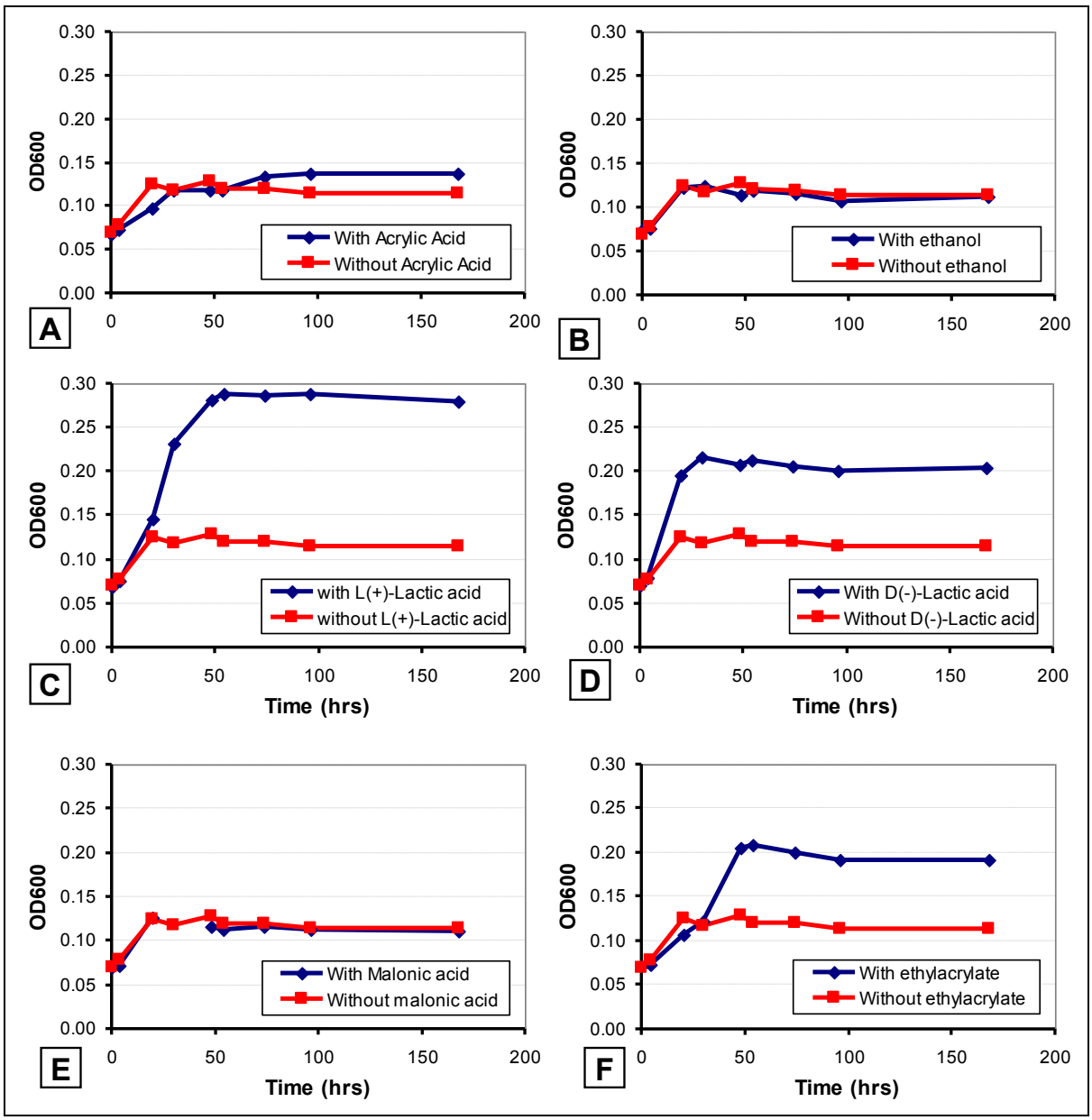

Figure 3.25: Growth of 2C on 1.10 M (+YE) with/without (A) acrylic acid, (B) ethanol, (C) L(+)-Lactic acid, (D) D-(-)-Lactic acid, (E) malonic acid, (F) ethyl acrylate

3.2.4.2 Growth of 2Cbei on the different substrates in a mineral medium containing 0.1% yeast extract (1.10 M (+YE)).

In Figure 3.26, the growth of 2Cbei is shown on the same substrates as for 2C in Figure 3.25. Similarly to growth for 2C no growth was observed on ethanol (B) or malonic acid (E) for 2Cbei. Furthermore, growth was similar on D(-)-Lactic acid (D) and ethyl acrylate (F) for

2Cbei and 2C. However, some clear differences were observed: In contrast to 2C, for which no growth was observed on acrylic acid, clear growth was observed for 2Cbei on this substrate (A). Also, the growth of 2Cbei on L(+)-Lactic acid (Figure 3.26C) was more rapid than that for 2C on the same substrate (Figure 3.25C) (~25 hours versus ~60 hours, respectively) but a slightly lower growth was attained for 2Cbei, which then decreased, while that for 2C maintained a maximum plateau.

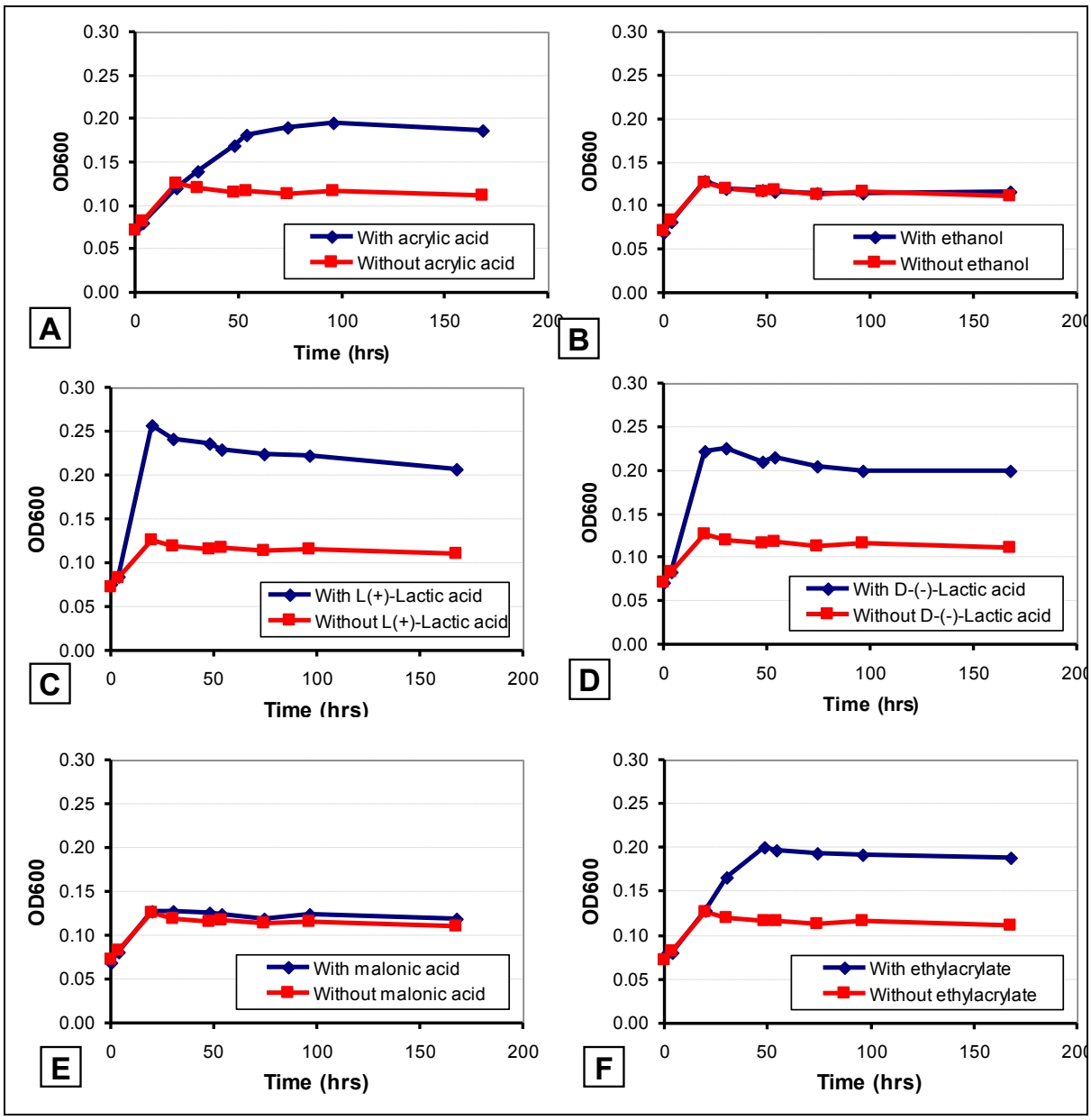

Figure 3.26: Growth of 2Cbei on 1.10 M (+YE) with/without (A) acrylic acid, (B) ethanol, (C) L(+)-Lactic acid, (D) D-(-)-Lactic acid, (E) malonic acid, (F) ethyl acrylate

3.2.5 Detection and characterization of an esterase from *Defluvibacter lusatiensis* 2Cbei

Using p-nitrophenyl ester as substrate, an esterase could be detected in crude extracts prepared from cells of 2Cbei grown with ethyl acrylate. In 20 mM phosphate buffer of pH 7 at 25 °C an activity of 0.28 U/mg was observed.

3.2.5.1 Substrate spectrum

The conversion of the following substrates by the esterase from 2Cbei was tested. The enzyme converted preferably esters of short chain aliphatic acids (Figure 3.27).

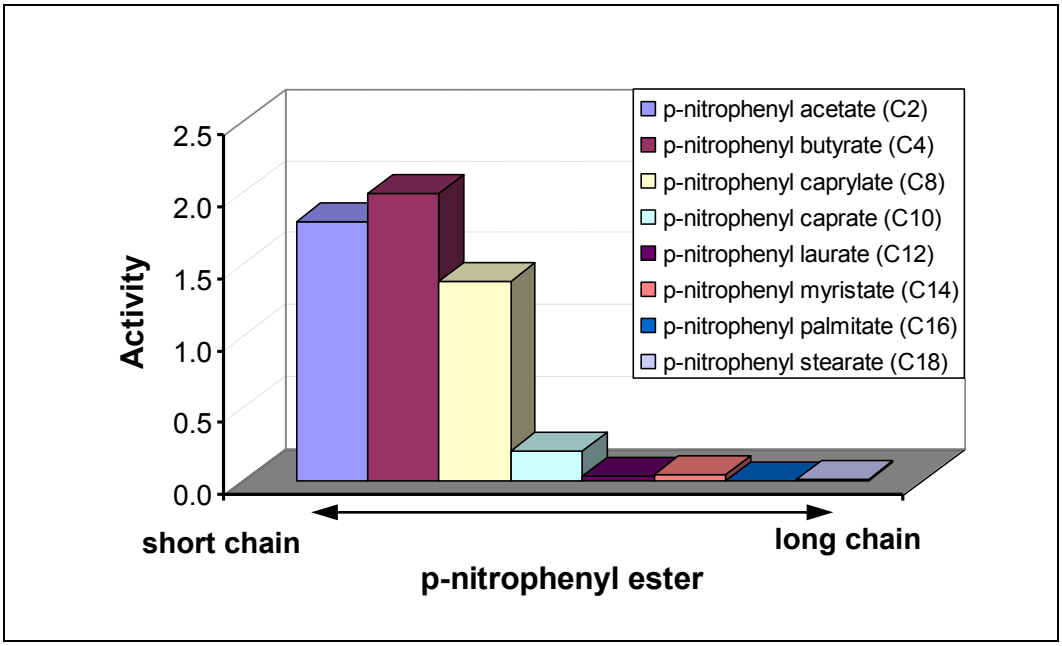

Figure 3.27: Substrate specificity of the 2Cbei enzyme

3.2.5.2 Inhibitors, detergents, solvents and metal ions

The effect of inhibitors, detergents, organic solvents and metal ions on *Defluvibacter lusatiensis* esterase is shown in Figure 3.28, Figure 3.29, Figure 3.30 and Figure 3.31. The effect of various inhibitors was investigated (Figure 3.28) and at concentrations of 10 mM, guanidine hydrochloride, urea, sodium 2-iodoacetate (cysteine protease), pefabloc (serine protease), EDTA (ethylenediamine tetraacetic acid) and disulfide bond reducing reagents such as β-mercaptoethanol and DTT showed no or mild inhibitory effect on the activity of the esterase. The effect of typical serine modifying reagents was tested. PCMB reduced the activity down to 65% whereas phenylmethylsulfonyl fluoride (PMSF) reduced the activity down to 25%.

The activity of the esterase was tested after incubation for 1 hour at 20 ˚C in the presence of different detergents at a concentration of 10% w/v (Figure 3.29). Polyvinyl alcohol and Triton X-100 had no effect on the activity of the esterase. CHAPS (3-[(3-Chol-amido-propyl) dimethylammonio]-1-propanesulfonic acid) had a mild effect on the esterase activity. Incubation with Tween-80, EDTA, Tween-20 and SDS decreased the activity down to 70, 60, 50 and 20%.

The effect of organic solvents is shown in Figure 3.30. The best solvents that did not have any effect on the esterase activity were 50% methanol, N,N-dimethyl formamide, 99% methanol, n-hexadecane, n-heptane, and isooctane. Solvents that had a mild effect on the esterase activity were 50% ethanol, acetone, DMSO, pyridine and 99% DMSO, pyridine, toluene, benzene, n-hexane and n-decyl alcohol. The esterase showed up to 40% of the original activity in 50% tert-butanol and 99% acetonitrile and was mostly deactivated by 99% tert-butanol, isopropanol and n-amyl alcohol.

Divalent and monovalent cations affected the activity of the enzyme as follows: the 2Cbei esterase was inhibited by 10 mM of Ag^{+1} (100% inhibition), Hg^{+2} (100% inhibition) and Mn^{+2} (35% inhibition); whereas Ni^{+2}, Cu^{+2}, Rb^{+1}, Zn^{+2}, Mg^{+2}, Fe^{+2}, Fe^{+3}, Ca^{+2}, K^{+1}, Cr^{+3} and Al^{+3} had no or limited effect. Na^{+1} and Sr^{+2} increased the activity by ~ 10% (Figure 3.31).

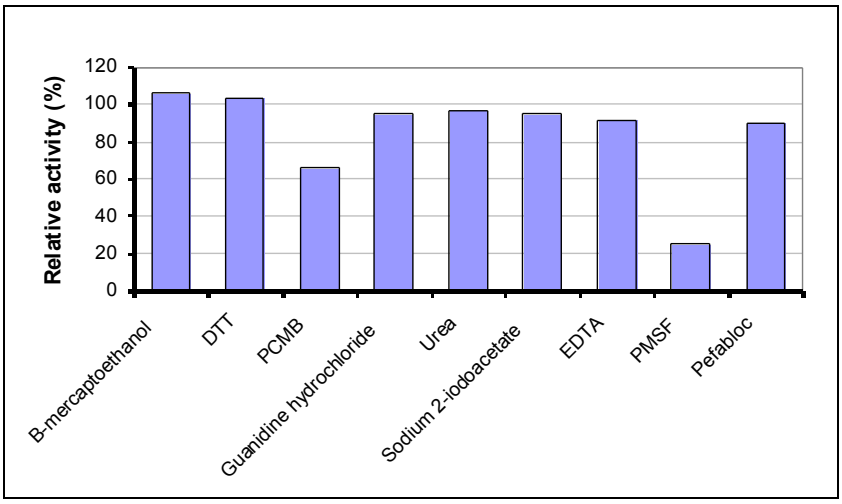

Figure 3.28: Effect of inhibitors on activity of 2Cbei enzyme (40%+60%) measured after 1 hour of incubation at 30 degrees.

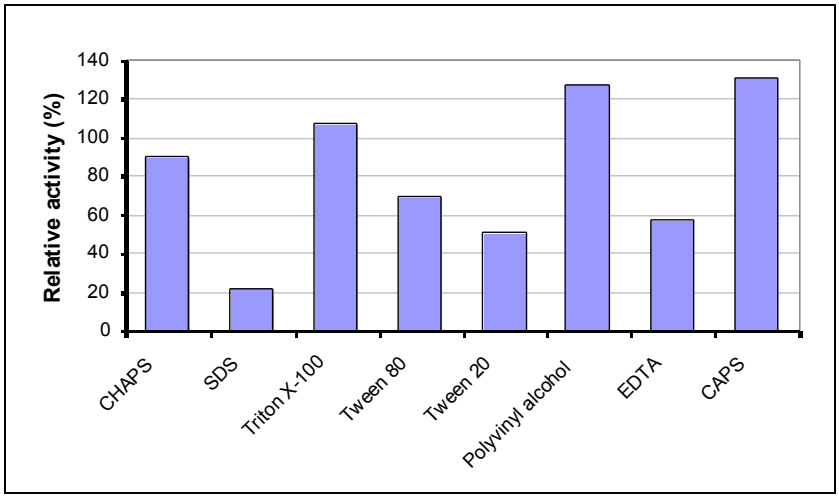

Figure 3.29: Effect of different detergents on the activity of the 2Cbei (40%+60%) enzyme.

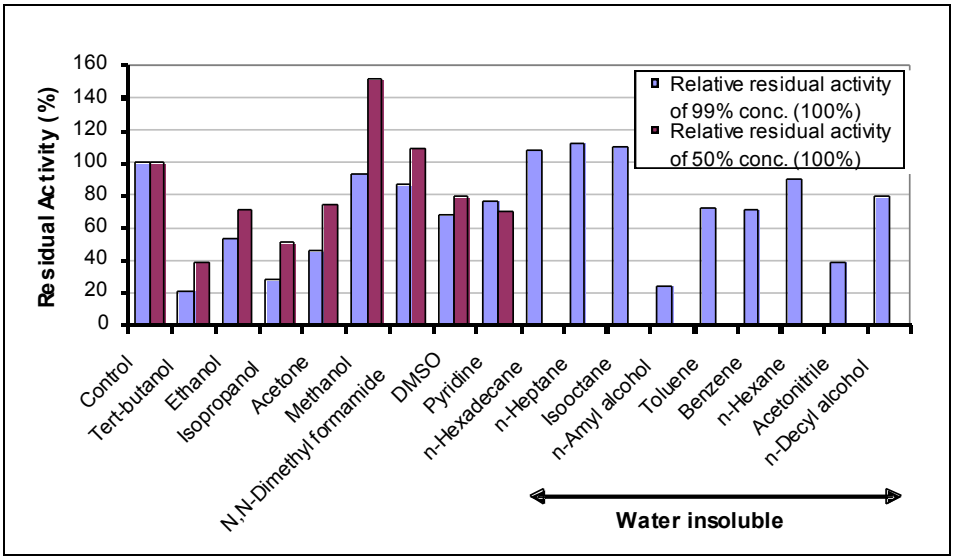

Figure 3.30: Effect of various organic solvents on the activity of 2Cbei enzyme (40%+60%).

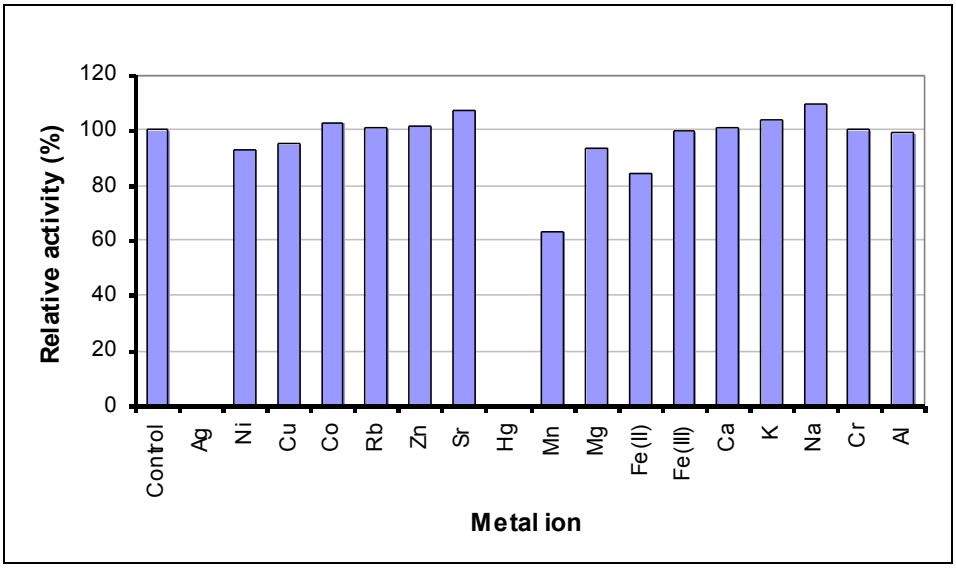

Figure 3.31: Effect of different metal ions on the activity of 2Cbei enzyme.

3.2.5.3 pH (optimum)

The 2Cbei esterase showed activity over a pH range of 6.0 – 8.5, with an optimum pH at pH 8.0 (Figure 3.32).

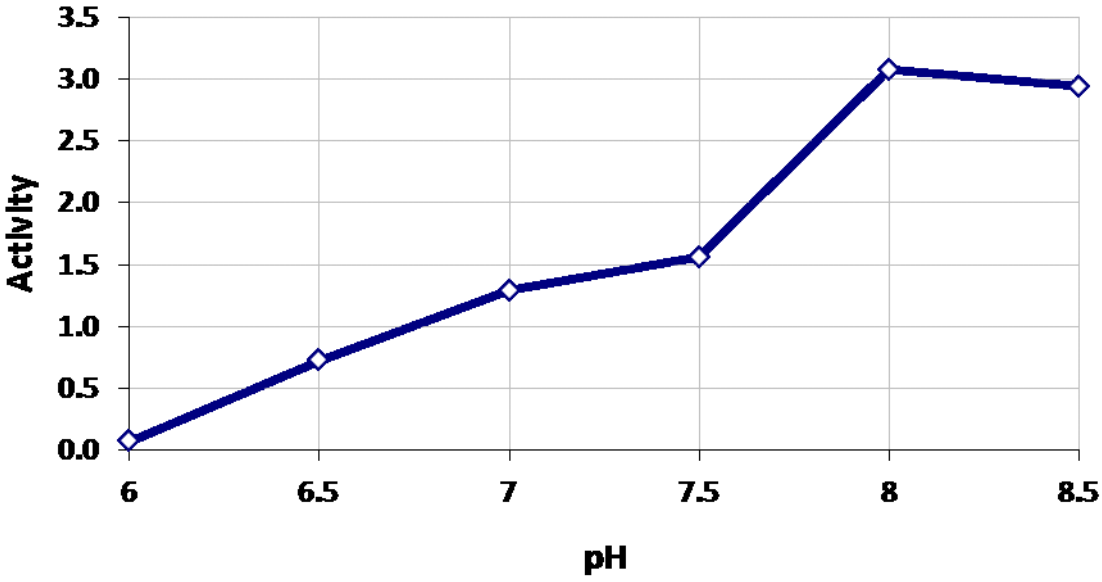

Figure 3.32: Optimum pH of the 2Cbei enzyme measured at the optimum temperature T 56°C.

3.2.5.4 Temperature (optimum)

The 2Cbei esterase was active between 25 and 60 ˚C. The temperature optimum was 56 ˚C and a rapid decrease in esterase activity was observed above 60 ˚C. 50% activity was retained at 50 ˚C (Figure 3.33).

The influence of temperature on the stability of the esterase was examined by measuring the enzymatic activity after incubation at temperatures ranging from 5 up to 85 ˚C (Figure 3.34). The enzyme is stable at temperatures of 5, 25, 35 and 45 ˚C. It lost around 80% of activity after 30 min of incubation at 55 and 60 ˚C. At higher temperatures 65, 70, 75 and 85 ˚C the enzyme lost 100% of activity after 30 min of incubation.

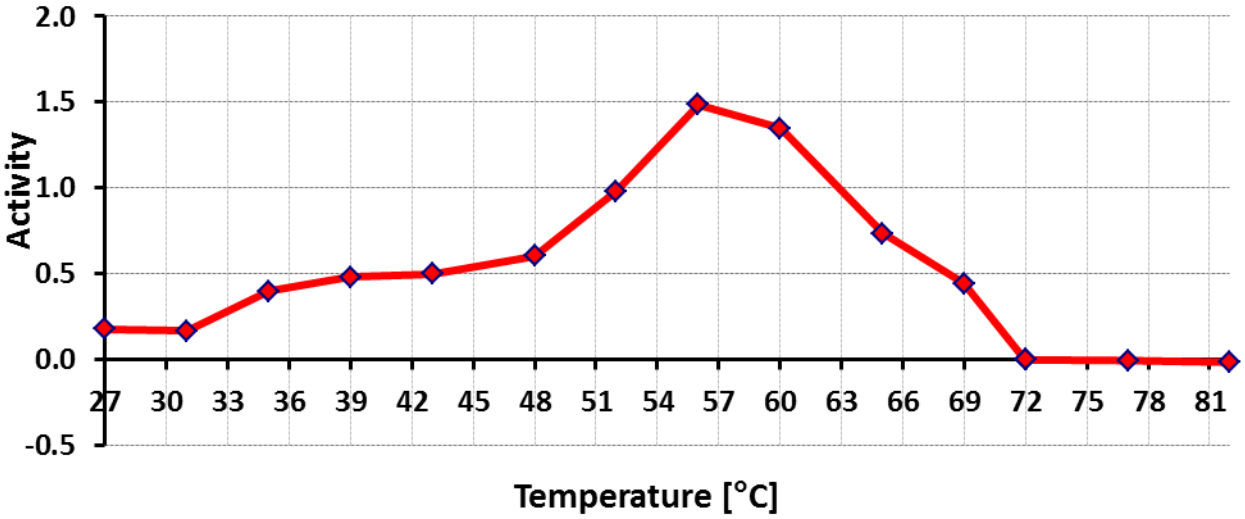

Figure 3.33: Optimum temperature of 2Cbei enzyme.

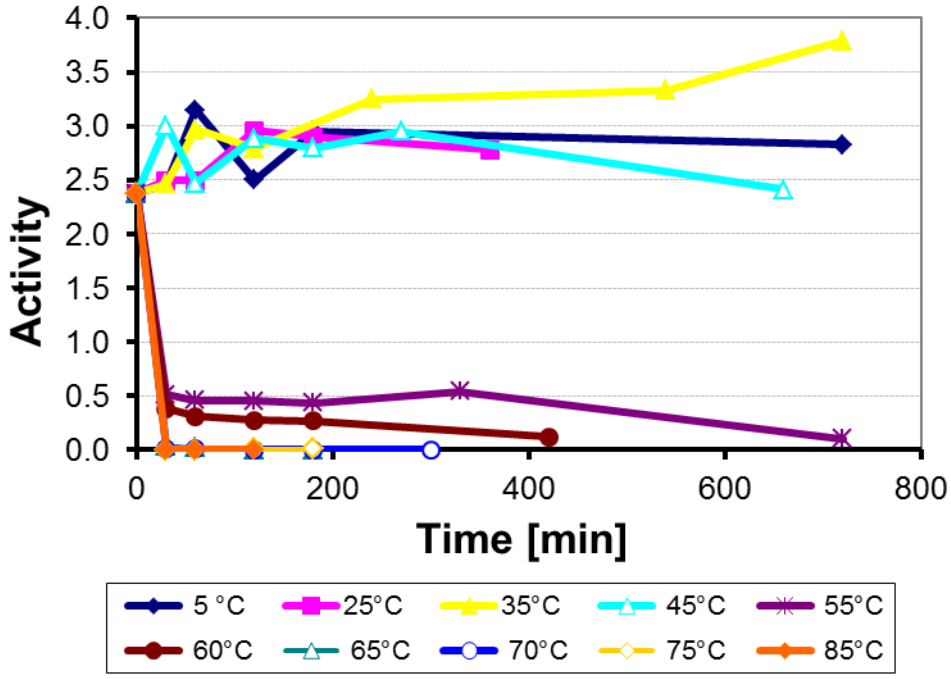

Figure 3.34: Thermal stability of the 2Cbei enzyme.

3.2.5.5 Purification

Table 3.7 summarizes the purification of the 2Cbei esterase by a three-step procedure as outlined in Materials and Methods (2.7.2). The specific activity of the esterase in the cell crude extract was 0.28 U/mg. Purification of the esterase after ammonium sulfate fractionation and Q-Sepharose chromatography resulted in a 4.6 fold increase in specific activity (1.29 U/mg) as shown in Table 3.7. Purification after the gel filtration step on Superdex G200 resulted in a 0.6 fold decrease in specific activity (0.18 U/mg). All purification steps were carried out at room temperature. The activity obtained after the ammonium sulfate fractionation step was enough to perform characterization of the native 2Cbei enzyme.

Table 3.7: Purification of the esterase of Defluvibacter lusatiensis 2Cbei.

Purification step	Vol [ml]	Protein conc. [mg/ml]	Total protein [mg]	Activity / ml [U/ml]	Total activity [U]	Spec. activity [U/mg]	Enrichment [fold]	Yield [%]
Crude extract	**100**	**8.172**	**817.2**	**2.3000**	**230.0**	**0.28**	**1.00**	**100.0**
Ammonium Sulfate	**96**	**5.227**	**501.8**	**1.5800**	**151.7**	**0.30**	**1.10**	**66.0**
Q-Sepharose	**288**	**0.034**	**9.9**	**0.0440**	**12.7**	**1.29**	**4.60**	**5.5**
Superdex G 200	**96**	**0.063**	**6.1**	**0.0113**	**1.1**	**0.18**	**0.60**	**0.5**

* One unit of esterase is defined as the amount of enzyme that releases 1 µmol of p-nitrophenol per min under essay conditions specified.

Chapter 4

DISCUSSION

The aim of this study was to isolate bacteria that have the potential to degrade triethylamine and ethyl acrylate. These two common industrial waste products are considered to be extremely annoying due to their exceedingly bad odour. Numerous environmental samples were collected from various sources for the screening process. A total of sixty one pure strains were isolated and tested for their ability to degrade triethylamine, and forty were isolated and tested for their ability to degrade ethyl acrylate. The two most effective strains were chosen for each substrate: RA1 and RA2 for triethylamine; 2C and 2Cbei for ethyl acrylate. All four strains were found to completely degrade the target compounds and are ready to be used in industrial purification processes. Furthermore, the mechanisms of degradation followed by these strains were determined.

4.1 Bacteria that degrade triethylamine

4.1.1 Isolation, characterization and identification of new triethylamine degrading bacteria, RA1 and RA2

The process of screening for bacteria that have the potential to degrade triethylamine was not very successful at first. Numerous samples were collected from different sources and locations that were expected to be contaminated with the target compound. After many attempts and much tedious work, several strains were isolated and tested for their degradation potential. The two best strains were selected for further studies and characterization: RA1 was isolated from a scrubber sample “Wäscher” taken from the fish-meal producing company See Löwe in Cuxhaven, Germany, and the strain RA2 was isolated from the same company but from a bio-scrubber “Biowäscher” sample. The company See Löwe was chosen due to the fact that fish odour contains some amine compounds, especially trimethylamine, as measured by Ranau and Steinhart (2004). It is also worth

mentioning that various samples taken from the bio-filter of the same company were successful, but to a lesser extent than those taken from the scrubber and bio-scrubber.

The characterization and identification of the strains were performed by DSMZ using the cell morphology, cell wall fatty acids analysis and 16S rDNA sequence. Strain RA1 was identified as *Pseudomonas citronellolis* and strain RA2 as *Mycobacterium diernhoferi*.

4.1.1.1 The species, *Pseudomonas citronellolis*, desription:

The new species of the genus *Pseudomonas* was first isolated from soil collected under pine trees in Northern Virginia. The isolation was carried out by the use of the enrichment culture technique with citronellol as the sole carbon source. After the morphological and nutritional characteristics of the organism were described, the name *Pseudomonas citronellolis* n. sp. was proposed. The bacterium was characterized as citronellol- and farnesol-oxidizing and after cultures were aerated with sterile air at 30 °C, the odour of citronellol had disappeared completely within 3 to 4 days (Seubert, 1959). This measured time is comparable to the 4 days needed by the isolated strain RA1 in order for it to completely degrade triethylamine which was also used as the sole carbon and energy source during the enrichment process followed in this study. Furthermore, it was stated that harvesting at an earlier stage usually resulted in inactivation of the cells caused by toxicity of the excess citronellol concentrated with the cells during centrifugation (Seubert, 1959); a case that was not comparable to RA1, where no signs of toxicity were to be detected.

Pseudomonas citronellolis is a gram-negative bacterium that is used to study the mechanisms of pyruvate carboxylase (Seubert and Remberger, 1961). The organism, measuring 0.5 by 1.0 to 1.5 µm, is rod-shaped, which usually occurs singly but occasionally in pairs. It possesses a single polar flagellum. Surface colonies observed on the basal medium containing 2 per cent agar are transparent, pin-point, raised circular colonies with slightly wrinkled margins (Seubert, 1959). All this morphological description of the organism may also be related to that of the isolated strain RA1.

Based on 16S rRNA analysis, *Pseudomonas citronellolis* has been placed in the *Pseudomonas aeruginosa* group (Anzai *et al*., 2000). It has also been found capable of the biosynthesis of polyhydroxyal-kanoates from “linear mono- and dicarboxylic acids”, a type of bacteria-synthesized polyester (Choi and Yoon, 1994).

Growth of the organism was found to occur anaerobically only in the presence of nitrate. It was measured to be vigorous over the range of 25 to 37 °C, with optimal growth at 31 °C (Seubert, 1959). For the isolated strain RA1 there was no anaerobic growth detected.

It is important to note that, excepting RA1, none of the strains described so far was shown to degrade triethylamine.

4.1.1.2 The species, *Mycobacterium diernhoferi*, description:

Mycobacterium diernhoferi (diern ho´ fer i. of Diernhof, who originally isolated the organisms) (Tsukamura *et al*., 1983). The name *Mycobacterium diernhoferi* has been revived for the organism originally described by Bönicke and Juhasz in 1965 (Bönicke and Juhasz, 1965). In previous articles it was already noted that in the vicinity of humans and animals there are bacteria of the genus *Mycobacterium*, the characteristic properties of which are different to the previously known *Mycobacterium* species (Bönicke and Juhasz, 1964). The immediate and broader environment of the cow was searched very carefully and systematically for Mycobacteria. The majority of the strains were isolated by Karl Diernhofer; thus the proposed name *Mycobacterium diernhoferi* for the new species. The bacterial cells are very consistent and are short and plump, 1-3 µm long and 0.5-0.8 µm wide. Their ends sometimes appeared to be thickened and rounded (Bönicke and Juhasz, 1965). All this morphological description of the organism may also be related to that of the isolated strain RA2.

Mycobacterium diernhoferi can be stained according to the Ziehl-Neelsen procedure. Its acid strength is equivalent to that of other fast-growing *Mycobacterium* species, such as *Mycobact. vaccae*, *Mycobact. phlei*, *Mycobact. smegmatis*, *Mycobact. borstelense* or *Mycobact. fortuitum* (Bönicke and Juhasz, 1965).

In the Löwenstein-Jensen, glycerin-broth agar or glycerol broth agar growth media, abundant growth of *Mycobacterium diernhoferi* was observed after an incubation time of 3 days at temperatures of 22-37 °C. At 17 °C growth was inhibited, even more at 40 °C. At 42 °C and higher temperatures, growth was completely absent (Bönicke and Juhasz, 1965). The growth behaviour of the isolated strain RA2 was found to be very similar when compared at room temperature where the cultures where grown.

Mycobacterium dienhoferi is not heat resistant. After 4 hours of heating at 60 °C, the growth was completely inhibited (Bönicke and Juhasz, 1965). No similar heat resistnace studies were performed on the isolated strain RA2; thus no comparison may be made here. This is due to the importance of degradation at normal temperatures and not at elevated ones.

Previously, *Mycobacterium diernhoferi* was regarded as a synonym for *Mycobacterium parafortuitum* (Liston *et al*., 1963). However, in a study by Tsukamura *et al*. (1983), it was established that organisms in this group could be differentiated from *Mycobacterium parafortuitum*. Kusunose *et al*. (1979) found that *Mycobacterium diernhoferi* (Source: Soil

obtained from a cattle field, Germany) contains 68- to 76-carbon mycolic acids; whereas *mycobacterium parafortuitum* contains 54- to 60-carbon mycolic acids (Tsukamura *et al*., 1983).

4.1.2 Determination of growth characteristics of *Pseudomonas citronellolis* RA1 and *Mycobacterium diernhoferi* RA2

The potential of RA1 and RA2 to degrade triethylamine was initially determined for a 0.5 mM concentration of the substrate. This low concentration was chosen to resemble the situation in exhaust contaminated air (Matz *et al*., 2004; Schlegelmilch *et al*., 2005) and is also easily detected using gas chromatography. The observation of growth of RA1 to saturation (stationary phase) within the first day, measured by the number of cells counted, confirms that this strain consumes triethylamine. Measurements showed that RA1 has the capacity to completely degrade this target compound within 4 days. RA2 was similarly found to completely degrade triethylamine within 4 days, but with less rapid growth, requiring twice as long to reach the stationary phase (see chapter 3).

Having proved the degradation potential of RA1 and RA2 towards a low concentration of triethylamine (0.5 mM), their effect under higher concentrations was investigated, to determine the maximum possible concentration without inhibiting their function. Increasing concentration corresponded to an increase in the time taken for complete degradation, but was found to be possible for RA1 within 16 days for substrate concentrations below 12 mM. For higher concentrations of up to 20 mM, degradation was found to be progressive at 14 days, and by extrapolation of the slopes of the degradation curves, degradation could therefore be expected to be complete within a few weeks. Since measured environmental concentrations of this substrate are roughly an order of magnitude lower than the maximum tested (Kleeberg *et al.*, 2005; Matz *et al.*, 2005), RA1 could be employed in industrial air purification applications with reasonable time scales.

RA2 demonstrated similar potential for degradation to that of RA1, for substrate concentrations below 10 mM. However, at higher concentrations (11-20 mM) a plateau was observed between 7 and 14 days, which demonstrates the diminished potency of RA2 in comparison with RA1. Despite this difference, the two strains demonstrate similar function for the lower concentrations expected in industry.

4.1.3 Substrate spectra of *Pseudomonas citronellolis* RA1 and *Mycobacterium diernhoferi* RA2

Having tested RA1 and RA2 for triethylamine degradation in isolation, they were then tested for their efficacy on other model substrates observed in exhaust gases, both individually, and as mixtures of compounds.

In addition, a bacterial strain, *Aminobacter aminovorans* (Lobo *et* al., 1997), which has been isolated for the degradation of trimethylamine (TMA) was compared with RA1 and RA2 (Table 3.3). RA1 and RA2 have broader spectra in degrading the model substrates than *Aminobacter aminovorans*. As well as degrading triethylamine, RA1 and RA2 were also found to at least partially degrade a number of other isolated substrates. Both strains partially degraded dimethyl disulfide (DMDS), furfuryl mercaptane (FM), trimethyamine (TMA) and ethyl acrylate (ET), where RA1 was more effective than RA2 for all of these except the ethyl acrylate (ET). RA1 was also found to partially degrade pentanethiol (PT) and 2,5-dimethylpyrazine (DP), which RA2 did not. Interestingly, this trend for single substrates was not reflected for the mixture of all substrates simultaneously (Table 3.4). In the case of combined substrates, RA2 was equal to, or more effective than RA1 at degrading the various substrates. This contrast suggests that an “a priori” knowledge of the industrial environment will dictate which strain to use: RA1 for isolated compounds, and RA2 for mixtures. There was no advantage observed for the use of combined RA1 and RA2.

Another novel denitrifying bacterial strain that degrades trimethylamine via two different pathways was isolated from an enrichment culture with trimethylamine inoculated with activated sludge (Kim *et al*., 2001). The isolate, strain T231, was gram-negative and consisted of non-motile cocci. On the basis of 16S rRNA gene sequence analysis, the strain was affiliated with the genus *Paracoccus aminovorans* with 98% sequence similarity. Aerobically, strain T231 degraded 9.1 mM trimethylamine in 18 h, 8.7 mM dimethylamine in 13 h and 8.9 mM methylamine in 18 h. Under anaerobic conditions, strain T231 completely degraded 10 mM trimethylamine in 22 h. During this anaerobic process of degradation, dimethylamine and methylamine appeared at concentrations up to 8.3 mM and 1.2 mM, respectively, and subsequently disappeared. Strains RA1 and RA2, isolated by this study, degraded 0.5 mM triethylamine aerobically within 4 days. No anaerobic growth of either strain was detected. By comparing the times of degradation needed by RA1 and RA2 with those for T231, it can be concluded that it is reasonable to apply RA1 and RA2 in industrial applications and odour minimization plants like scrubbers, bioscrubbers and biofilters.

It is also worth mentioning that in other studies, isolates identified as *Hyphomicrobium* were capable of degrading trimethylamine both aerobically and under denitrifying condition (Attwood and Harder, 1972). However, no strains have been reported so far that have the potential to degrade triethylamine. This is the first research that presents the isolation and characterization of bacterial strains that can utilize triethylamine (TEA) as a sole carbon and energy source.

Another recent study performed by Wang *et al*. (2006) aimed to remove triethylamine by using a biological method, as well as to understand the ability of mixed bacteria cultures to treat a triethylamine compound from synthetic wastewater. It was found that the mixed bacteria cultures could not remove triethylamine, whether the activated sludge came from an acrylonitrile-butadiene-styrene resin manufactured wastewater treatment system or a waterborne polyurethane resin manufactured wastewater treatment system. On the other hand, it was found that when the mixed bacteria cultures were acclimated to triethylamine, they could utilize 650 mg/l triethylamine for growth and when the initial triethylamine concentration was below 200 mg/l, the triethylamine removal efficiency could reach 100%. Hence, the results of this study indicate that triethylamine removal using the biological method is practicable but no strains were isolated during this study which again confirms that we were the first to isolate strains that can degrade triethylamine.

4.1.4 Determination of the degradation pathway for triethylamine

The degradation pathway for triethylamine was determined by examining the aerobic growth of RA1 and RA2 in isolation on the following substrates supplied as the sole sources of carbon and energy: Triethylamine (TEA), diethylamine (DEA), triethanolamine, diethanolamine, ethanolamine, acetaldehyde and sodium acetate. The growth experiments were performed once in a nitrogen-free mineral medium 1.10 M (-YE-N) and again in a mineral medium containing an ammonium source 1.10 M (-YE+N).

Based on the growth behaviours shown in Section 3.1.5, the following degradation pathway followed by both RA1 and RA2 is suggested (Walles *et al.*, 2003; Sun *et al.*, 2004) (*Figure 4.1*):

Triethylamine was initially oxidized to diethylamine and acetaldehyde. We assume that this reaction is catalyzed by a monooxygenase which hydroxylates the α-carbon of the ethyl group resulting in a semiacetal-like structure which spontaneously decomposes into the diethylamine and acetaldehyde, although we cannot completely exclude an attack at the

nitrogen as suggested in the degradation of trimethylamine (see Figure 1.2). The same enzyme should remove all three ethyl groups step by step in the same way (Figure 4.2).

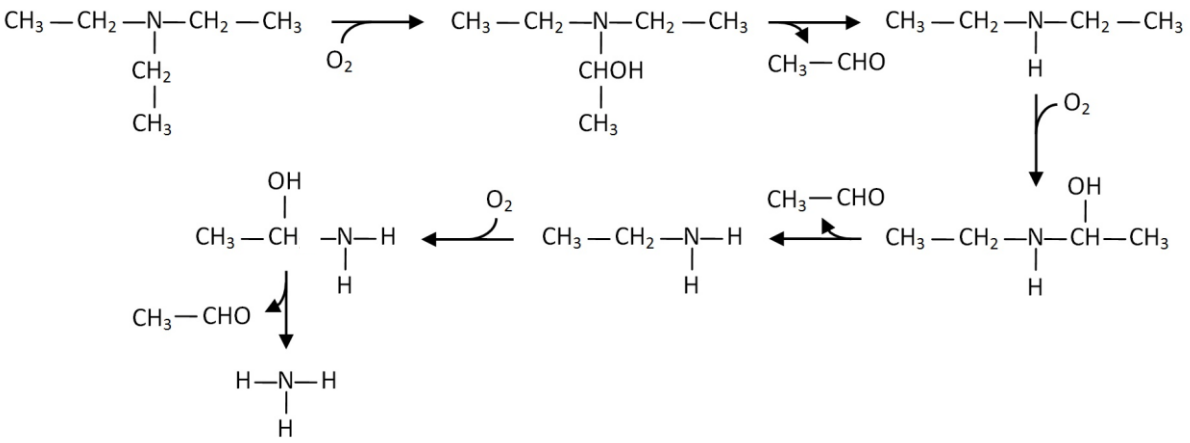

Figure 4.1: Proposed pathway of aerobic metabolism of triethylamine in strains RA1 and RA2.

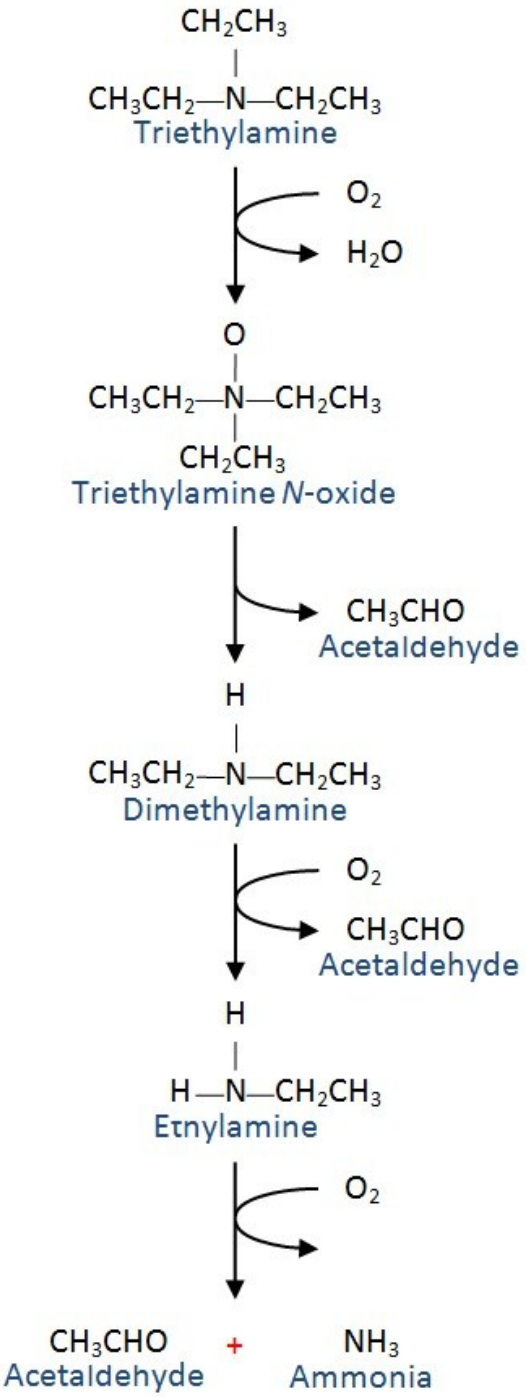

Figure 4.2: Proposed pathway of aerobic metabolism of triethylamine in strains RA1 and RA2.

4.2 Bacteria that degrade ethyl acrylate

4.2.1 Isolation, characterization and identification of new ethyl acrylate degrading bacteria, 2C and 2Cbei

The process of screening for bacteria that have the potential to degrade ethyl acrylate was carried out where a number of soil samples had to be collected from different sources and locations that were thought to be contaminated with the target compound. Several strains were isolated and tested for their degradation potential. The two best strains were selected for further studies and characterization: 2C and 2Cbei were isolated from a soil sample taken from the Aussenmühlen Park in Harburg, Germany. It is worth mentioning that many other soil samples from diverse locations also showed activity towards degrading ethyl acrylate. The screening process for ethyl acrylate was therefore not as time-consuming and arduous as for triethylamine, for which only three sources were found, and all at the same site. The sources for triethylamine were scrubbers, bio-scrubbers and bio-filter from a fish-meal producing company (Seelöwe, Cuxhafen).

The characterization and identification of the strains were carried out by DSMZ using the cell morphology, cell wall fatty acids analysis and 16S rDNA sequence. Both strains 2C and 2Cbei were found to belong to the species *Defluvibacter lusatiensis* (Fritsche *et al*., 1999a).

For the two strains 2C and 2Cbei of *Defluvibacter lusatiensis*, there were no significant differences in the colonies shape in case of growing on both LB and mineral salt media.

4.2.1.1 Description of the genus *Defluvibacter* gen. nov. :

The monospecific genus *Defluvibacter* was proposed by Fritsche *et al*. (1999a) (Kämpfer *et al*., 2009). *Defluvibacter* (De.flu.vi.bac´ter. M. L. n. *defluvium*, waste water; Gr. hyp. masc. n. *bacter*, rod; M. L. masc. n. *Defluvibacter*, referring to its origin from activated sludge of a waste water treatment plant) (Fritsche *et al.*, 1999a). Cells are Gram-negative short rods that occur as single cells. Cells are motile by means of a flagellum. Strictly aerobic. Oxidase and catalase positive. Nitrate is not reduced. Only a limited number of carbohydrates are utilized for growth, e.g. D-glucose, D-fructose, D-mannose, D-ribose, D-xylose and L-lyxose. Different amines and amino acids serve as carbon sources. Ubiquinone 10 is the major ubiquinone. The abundant fatty acid is octadecenoic acid (C18:1). The hydroxyl fatty acid 3-OH C12:0 is present in low quantities. Spermidine is the major polyamine. The genus can also be recognized by the 16S rDNA sequence. Phylogenetically, the genus belongs to the α-subgroup of the Proteobacteria with members of *Pseudaminobacter*, *Mesorhizobium* and

Phyllobacterium being the closest relatives. The type species is *Defluvibacter lusatiae*. (Fritsche *et al.*, 1999a).

Kämpfer *et al*. (2009) later proposed to reclassify *Defluvibacter lusatiensis* to the genus *Aquamicrobium* as *Aquamicrobium lusatiense* comb. nov. A novel species, *Aquamicrobium aerolatum* sp. nov., is described. This proposal was based on the remarkable congruence in phenotypic characters. (Kämpfer *et al*., 2009).

4.2.1.2 Description of the type species *Defluvibacter lusatiae* sp.nov.

Defluvibacter lusatiae (lu.sa.ti.ae. M. L. gen. n. *lusatiae*, referring to the German province of Lausitz [latin name *Lusatia*], where the organism was first isolated). The Gram-negative strain S1, isolated by Fritsche *et al*., exhibited a stable capability to degrade 2,4-dichlorophenol, 4-chloro-2-methylphenol, 4-chlorophenol and phenol. This capability to degrade chlorophenols and phenols was stable over 30 transfers on nutrient agar. The strain S1 was also described as type strain of a new species and assigned to a new genus with the proposed name *Defluvibacter lusatiae* (Fritsche *et. al*, 1999a). The name was then corrected to *Defluvibacter lusatiensis* on validation (Fritsche *et al*., 1999b; Kämpfer *et al*., 2009). Cells are short rods (0.6 to 0.8 µm in width and 1.5 to 3 µm in length), occur singly and are motile by means of a single polar flagellum. Spores are not formed. After 2 days of incubation on nutrient agar, colonies are white-greyish, circular with a diameter of 2 mm, which can extend to 4 mm after prolonged incubation, and appear slightly mucoid. Optimal growth is at 30-37 °C and pH 7.0-7.5. These properties were all found in our isolated strains. Nevertheless, none of the stains described so far degraded ethyl acrylate.

4.2.2 Determination of growth characteristics of *Defluvibacter lusatiensis*, 2C and 2Cbei

In order to determine the potential of 2C and 2Cbei to degrade ethyl acrylate, an initial concentration of 2 mM was tested. And because these strains were originally isolated using yeast extract as an additional carbon source to the mineral medium, degradation was tested both with and without yeast extract. The 2C strain was found to have the capacity to completely degrade the ethyl acrylate substrate without yeast extract, but its addition decreased the time to complete degradation from 22 to 14 hours. 2Cbei was also found to completely degrade ethyl acrylate both with and without yeast extract. However, the time required was somewhat lower than for the 2C strain and degradation was not affected by the presence of yeast extract. This suggests that 2Cbei is the more efficient strain in the degradation of ethyl acrylate, with no extra yeast extract resources necessary.

Subsequently, the concentration of the substrate was varied. This set of experiments was performed 9 months later than the original series discussed above. After this period the bacteria were found to be less potent and required yeast extract to completely degrade the substrate. Degradation took almost twice as long for the 2 mM concentration used in the original series. This phenomenon remains unexplained, but could be related to mutations of the bacterial strains over time. Contamination was ruled out, since multiple sources of the strains demonstrated the same trend. Complete degradation was still shown to occur for both strains for substrate concentrations of up to 7 mM. For higher concentrations, of up to 10 mM, degradation was observed to be progressive at 16 days, and could be therefore expected to eventually lead to complete degradation. Despite their diminished function, 2C and 2Cbei are clearly candidates for the degradation of ethyl acraylate in an applied industrial setting.

During the degradation of ethyl acrylate by 2C, a peak was noted in the GC measurements, referring to ethanol, which was therefore shown to be a by-product of ethyl acrylate degradation. The concentration of ethanol was found to be three times higher with the addition of yeast extract.

4.2.3 Determination of the degradation pathway for ethyl acrylate

The degradation pathway for ethyl acrylate was determined by monitoring the aerobic growth of 2C and 2Cbei in isolation on the following substrates supplied as sources of carbon and energy: Acrylic acid (propenoic acid), ethanol, L(+)-Lactic acid, D(-)-Lactic acid, malonic acid and ethyl acrylate.

From these data (Section 3.2.4) it was concluded that the ester bond in the ethyl acrylate is hydrolyzed first. The ethanol was used as a carbon source and to the double bond of the acrylate water was added and the resulting lactate was also used as carbon source.

These growth experiments (Figure 3.25 and Figure 3.26) confirm the suggested degradation pathway for ethyl acrylate, which is illustrated as follows (Figure 4.3):

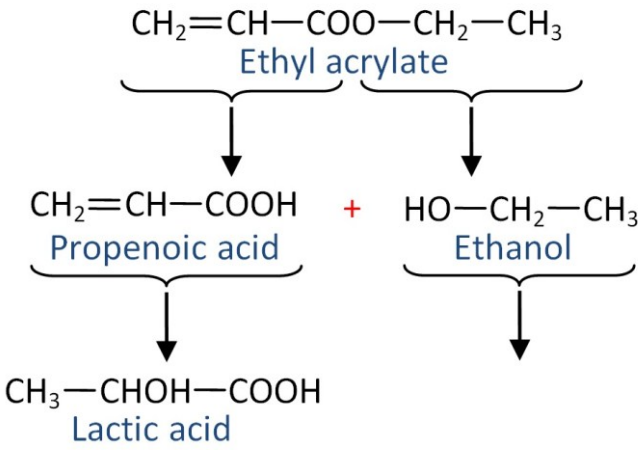

Figure 4.3: Proposed degradation pathway for ethyl acrylate.

4.2.4 Detection and characterization of an esterase from *Defluvibacter lusatiensis* 2Cbei

This work presents the enrichment and properties of an esterase from a novel isolate *Defluvibacter lusatiensis* 2Cbei.

The esterase was enriched by a three-step-procedure. Both specific activity and yield of the enzyme dramatically decreased during enrichment. The highest specific activity was found after ion exchange chromatography (1.29 U/mg). This value is rather low when compared to other known esterases. The reasons for this low value and for the loss of activity during enrichment are not clear. Therefore, the enzyme was characterized after ammonium sulfate fractionation.

The highest activity was found at 56°C and pH 8.0. Up to 50% of the activity is retained at 50°C. These values are similar to many esterases described (Al Khudary, 2006; Shin and Chen, 2007; Choi *et al.*, 2004).

The esterase was stable at temperatures of 5, 25, 35, and 45°C. It lost around 80% activity after 30 min incubation at 55 and 60°C. At higher temperatures 65, 70, 75 and 85°C the esterase lost 100% activity after 30min of incubation. Moreover, 2Cbei esterase was stable at acidic rather than at alkaline pH.

A hydrolase-catalyzed reaction in a conventional aqueous system thermodynamically favours hydrolysis. Because an esterase-catalyzed reaction is also such a case, a condensation reaction to produce an ester is carried out in a non-aqueous medium such as an organic

solvent, a solvent-free system, or an ionic liquid. To carry out a condensation reaction, the esterase should be stable in the organic solvent. The organic solvents recommended for 2Cbei esterase are: 50% methanol, N,N-dimethyl formamide, 99% methanol, n-hexadecane, n-heptane and isooctane where it showed 100% stability upon incubation for 60 min. Other organic solvents like 50% ethanol, acetone, DMSO, pyridine and 99% DMSO, pyridine, toluene, benzene, n-hexane and n-decyl alcohol decreased the activity of the esterase to 70, 75, 80, 75, 90, 80, 75, 75, 90, 80% repectively. The esterase showed up to 40% of the original activity in 50% tert-butanol and 99% acetonitrile and was mostly deactivated by 99% tert-butanol, isopropanol and n-amyl alcohol. No 100% inhibition of the 2Cbei esterase was caused by any of the organic solvents tested.

Generally, esterases are cofactor-independent enzymes. 2Cbei esterase was inhibited by 10 mM of Ag^{+1} (100% inhibition), Hg^{+2} (100% inhibition) and Mn^{+2} (35% inhibition); whereas Ni^{+2}, Cu^{+2}, Rb^{+1}, Zn^{+2}, Mg^{+2}, Fe^{+2}, Fe^{+3}, Ca^{+2}, K^{+1}, Cr^{+3} and Al^{+3} had no or limited effect. Na^{+1} and Sr^{+2} increased the activity by ~10%. Hg^{+2} and Ag^{+1} were reported to inhibit activity of the esterase from *Lactobacillus plantarum* 2739 (Gobetti *et al.*, 1996) and of the esterase from *Lactobacillus fermentum* DT41 (Gobetti *et al.*, 1997). Ca^{+2} is known to increase activity in some lipases and was reported to slightly increase the activity of PsyEst from *Psychrobacter sp.* Ant300 (Kulakova et al., 2004) whereas Ca^{+2} had no effect on the activity of 2Cbei esterase.

Various inhibitors were used to study the structure and catalytic mechanism of the esterase. There are two classes of enzyme inhibitors: Reversible and irreversible. The reversible inhibitors are classified further in two groups as non-specific and specific reversible inhibitors. Compounds that inhibit esterase activity by changing the conformation or interfacial properties, but do not act directly on the active site are defined as non-specific inhibitors such as surfactants. Specific inhibitors directly interact with the active site of the enzyme and can be either reversible or irreversible. Specific irreversible inhibitors inhibit the catalytic activity of enzymes by reacting with the amino acids at or near the active site. Serine inhibitors are potential esterase active-site irreversible inhibitors, because esterases belong to a class of serine hydrolases with the catalytic triad Ser-His-Asp. Phenylmethylsulfonyl fluoride (PMSF), phenylboronic acid, Pefablock and diethyl ρ-nitrophenyl phosphate belong to serine-specific inhibitors. PMSF reduced the activity of 2Cbei esterase down to 25%. Therefore, the enzyme belongs to the serine hydrolases.

PCMB reduced the activity of the 2Cbei esterase only slightly to 65%. The specific irreversible inhibitors such as 2-mercaptoethanol, iodoacetate and DTT, which react with sulphhydryl groups and thus modify the protein conformation, did not have an inhibitory effect

on the 2Cbei esterase activity. Therefore, the esterase of 2Cbei does not have a sulphhydryl group at the active site.

No inhibitory effect of the 2Cbei esterase by EDTA was observed suggesting that no divalent ions are required for activity.

The 2Cbei esterase had a broad substrate specificity considering the chain length of fatty acids. However; the enzyme exhibited higher activity towards water soluble substrates with short chains, such as ρ-nitrophenyl esters with fatty acid chain shorter than C10. Therefore the enzyme is a true esterase and not a lipase.

4.3 Concluding remarks and further prospects

This study had been successful in contributing more to the understanding of biological degradation of some key odorous compounds that needed to be improved. The necessary microorganisms, needed for the purpose of the degradation of both triethylamine and ethyl acrylate, were successfully isolated and characterized. The results and the information obtained by the research were important and will aid our understanding of biodegration in natural ecosystems and will assist the development of strategies for effective biological odour treatment technologies.

The research study determined: (1) the degradation ability of the isolated microorganisms and (2) the influence of the presence of other odorous compounds on the degradation of the target odorous compound. The latter was necessary since the waste gases always consist of a mixture of various odorous compounds.

It is also worth pointing out that this research has been the first that presents the isolation and characterization of bacterial strains that can utilize triethylamine as a sole carbon and energy source.

The biological degradation pathways of both triethylamine and ethyl acrylate were described. And finally, the esterase of the 2Cbei enzyme was purified and further characterized.

It was concluded that the goals of the study were achieved and new strains were isolated: RA1 and RA2 for the degradation of triethylamine; 2C and 2Cbei for the degradation of ethyl acrylate.

Last but not least, more detailed research is recommended concerning further investigations of the degradation pathways and an isolation of the different metabolites for both triethylamine and ethyl acrylate.

Chapter 5

SUMMARY

In this study, bacteria that have the potential to degrade triethylamine and ethyl acrylate were isolated. These two common industrial waste products are considered to be extremely annoying due to their exceedingly bad odour. A biological degradation of these compounds has not been previously described.

Numerous environmental samples were collected from a large number of sources that were believed to be contaminated with the target compounds. Sources included biofilter material, bio-scrubber "Biowäscher", scrubber "Wäscher" and soils. Several strains were isolated and the two bacterial strains demonstrating the most efficient degradation of each odorous compound were chosen for further analysis in this study: RA1 and RA2 for triethylamine; 2C and 2Cbei for ethyl acrylate.

RA1 was isolated from the scrubber "Wäscher" of the fish-meal producing company Seelöwe in Cuxhaven, Germany; whereas RA2 was isolated from a sample originating from the bio-scrubber "Biowäscher" of the same company. The growth of the two strains, RA1 and RA2, obtained in the enrichment procedure was tested. With 0.5 mM triethylamine, the growth rates at 25 °C were 0.046 h^{-1} and 0.045 h^{-1}, respectively. After 4 days, the 0.5 mM triethylamine had disappeared and was completely degraded by RA1 and RA2 separately.

Strain RA1 was found to be a motile gram negative rod of width 0.5 – 0.7 µm and length 1.8 – 3.0 µm. Strain RA2 was found to be a non-motile, non-spore forming gram negative rod of width 1.0 µm and length 2.0 µm. Based on these results and on the characterization of DSMZ, RA1 was classified as *Pseudomonas citronellolis* and RA2 as *Mycobacterium diernhoferi*.

Both 2C and 2Cbei were isolated from a soil sample taken from Aussenmühlen Park in Harburg, Germany. The growth of these two strains, obtained in the enrichment procedure, was tested. With 2.0 mM ethyl acrylate, the growth rates at 25 °C were 0.104 h^{-1} and

0.130 h^{-1}, respectively. By comparing the two strains, strain 2Cbei was faster in degrading 2.0 mM ethyl acrylate. 2Cbei required 10 to 11 hours both in the presence and absence of 0.1% yeast extract; whereas 2C required 14 hours in the presence of 0.1% yeast extract and 21 hours in the absence of 0.1% yeast extract.

2C and 2Cbei were found to be gram negative with rod cell form of width 0.5 – 0.7 µm and length 1.0 – 2.5 µm. The partial sequencing of 16 s rDNA from both strains resulted in 100% similarity between them. The similarity to the strain *Defluvibacter lusatiensis* was found to be 100%; thus both strains were classified as *Defluvibacter lusatiensis.*

A study of the potential of RA1 and RA2 to degrade triethylamine and of 2C and 2Cbei to degrade ethyl acrylate under anaerobic conditions was performed and no significant growth was detected. This confirms that all four strains can only degrade the target substrate strictly under aerobic conditions.

Optimum temperature, pH and substrate concentration were determined and the substrate-degradation potential of each bacterial strain was examined. The aerobic mechanisms of degradation by these strains were determined and degradation pathways were suggested for each compound.

Finally, from all four strains, *Defluvibacter lusatiensis* 2Cbei, the enzyme initiating the degradation of ethyl acrylate was extracted, purified and characterized. Using p-nitrophenyl ester as substrate, an esterase could be detected in crude extracts prepared from cells of 2Cbei grown with ethyl acrylate. In 20 mM phosphate buffer of pH 7 at 25 °C an activity of 0.28 U/mg was measured. The extracted esterase was purified by a three-step procedure: Purification of the esterase after ammonium sulfate fractionation and Q-Sepharose chromatography resulted in a 4.6 fold increase in specific activity (1.29 U/mg). Purification after the gel filtration step on Superdex G200 resulted in a 40% decrease in specific activity (0.18 U/mg). All purification steps were carried out at room temperature. The activity obtained after the ammonium sulfate fractionation step was sufficient to perform characterization of the native 2Cbei enzyme.

The 2Cbei esterase showed activity over a pH range of 6.0 – 8.5, with an optimum pH of 8.0. The enzyme was active between 25 and 60 °C. The optimum temperature was 56 °C and a rapid decrease in esterase activity was observed above 60 °C. 50% activity was retained at 50 °C. The influence of temperature on the stability of the esterase was examined by measuring the enzymatic activity after incubation at temperatures ranging from 5 to 85 °C. The enzyme was stable at temperatures of 5, 25, 35 and 45 °C. It lost around 80% of activity

after 30 min of incubation at 55 and 60 °C. At higher temperatures 65, 70, 75 and 85 °C the enzyme lost 100% of activity after 30 min of incubation.

The effect of inhibitors, detergents, organic solvents and metal ions on the esterase of *Defluvibacter lusatiensis* was studied and the enzyme converted preferentially esters of short chain aliphatic acids.

In this study all four strains studied were found to degrade the target compounds completely. In combination with the determination of optimum degradation conditions and discovery of the mechanism, the strains are now ready to be used in industrial air and waste water purification processes.

Chapter 6

ZUSAMMENFASSUNG

In dieser Studie wurden Bakterien, die Triethylamin und Ethylacrylat abbauen, isoliert. Diese zwei gängigen, industriellen Abfallprodukte sind extrem lästig aufgrund ihres äußerst schlechten Geruchs. Ein biologischer Abbau von diesen Gemischen wurde bislang noch nicht beschrieben.

Zahlreiche Boden- und Wasserproben wurden von verschiedenen Quellen entnommen, von denen angenommen wurde, dass sie mit Triethylamin und Ethylacrylat kontaminiert sind. Dabei handelte es sich um Biofiltermaterial, Biowäscher, Wäscher und Bodenproben. Mehrere Bakterienstämme wurden isoliert. Die zwei besten Bakterienstämme, die den jeweiligen Geruch von Triethylamin und Ethylacrylat am effizientesten abbauen konnten, wurden näher untersucht: RA1 und RA2 für Triethylamin; 2C und 2Cbei für Ethylacrylat.

RA1 wurde von einem „Wäscher“ der Fischmehl Fabrik, Seelöwe in Cuxhaven Deutschland, isoliert. RA2 wurde von einem „Biowäscher“ der selbigen Firma isoliert. Es wurde das Wachstum der beiden Bakterienstämme untersucht. Unter Zugabe von 0,5 mM Triethylamin waren die Wachstumsraten für RA1 und RA2 0.046 h^{-1} und 0.045 h^{-1} bei einer Temperatur von 25°C. Nach 4 Tagen wurde das Triethylamin von RA1 und ebenso von RA2 vollständig abgebaut.

RA1-Bakterien sind Gram-negativ, stäbchenförmig und beweglich. Die Abmaße sind: 0,5 - 0,7 µm Breite und 1,8 – 3,0 µm Länge. RA2-Bakterien sind Gram-negativ, nicht sporenbildend, stäbchenförmig und nicht beweglich mit einer Breite von 1,0 µm und einer Länge von 2,0 µm. Basierend auf diesen Ergebnissen und der Charakterisierung der DSMZ wurde RA1 als *Pseudomonas citronellolis* klassifiziert. RA2 wurde als *Mycobacterium diemhoferi* klassifiziert.

Beide Stämme 2C und 2Cbei wurden von einer Bodenprobe aus dem Außenmühlen Park, Hamburg-Harburg, Deutschland, isoliert. Es wurde das Wachstum beider Bakterienstämme

untersucht. Unter Zugabe von 2,0 mM Ethylacrylat betrugen die Wachstumsraten für 2C und 2Cbei 0.104 h^{-1} und 0.130 h^{-1} bei Temperatur 25°C; wobei 2Cbei schneller Ethylacrylat abbaute als 2C. 2Cbei benötigte dafür 10 bis 11 Stunden sowohl mit als auch ohne Zugabe von 0,1% Hefeextrakt. 2C hingegen benötigte für den Abbau 14 Stunden bei Zugabe von 0,1% Hefeextrakt und 21 Stunden ohne die Zugabe von 0,1% Hefeextrakt.

2C und 2Cbei sind Gram-negative und stäbchenförmige Bakterien. Sie weisen eine Breite von 0,5 – 0,7 µm und eine Länge von 1,0 – 2,5 µm auf. Die Teilsequenzen von 16 s rDNA der beiden Stämme hatten eine Ähnlichkeit von 100%. Folglich wurden beide Stämme als *Defluvibacter lusatiensis* klassifiziert.

Zusätzlich wurde das Potential von RA1 und RA2 Triethylamin bzw. von 2C und 2Cbei Ethylacrylat unter anaeroben Bedingungen abzubauen untersucht. Es zeigte sich kein signifikantes Wachstum der Bakterienstämme. Dies unterstreicht, dass alle vier Bakterienstämme ausschließlich unter aeroben Bedingungen die Substrate abbauen können.

Optimale Temperatur, pH-Wert und Substratkonzentration in Bezug auf das Abbauverhalten wurden für jeden Bakterienstamm ermittelt. Der aerobe Abbaumechanismus des Geruchs bzw. der Verstoffwechselungsweg dieser Bakterienkulturen wurde heraus gearbeitet.

Von den vier Bakterienstämmen wurde das Enzym von *Defluvibacter lusatiensis* 2Cbei, welches Ethylacrylat abbaut, extrahiert, gereinigt und charakterisiert. Unter Verwendung von p-Nitrophenylester als Substrat konnte eine Esterase im Rohextrakt, erzeugt durch 2Cbei unter Einfluss von Ethylacraylat, detektiert werden. In 20 mM Phosphat-Puffer bei einem pH-Wert von 7 und einer Temperatur von 25°C wurde eine Aktivität von 0,28 U/mg gemessen. Die extrahierte Esterase wurde in 3 Stufen gereinigt: Reinigung der Esterase nach Fraktionierung mit Ammoniumsulfat und die Q-Sepharose-Chromatographie ergaben eine 4,6-fache spezifische Aktivität (1,29 U/mg). Die Reingung nach der Gelfiltrationchromatographie mit Superdex G200 ergab 60% an spezifischer Aktivität (0,18 U/mg). Alle Reinigungsschritte wurden bei Raumtemperatur durchgeführt. Die spezifische Aktivität, die sich nach der ersten Reinigungsstufe, der Ammoniumsulfatfraktionierung, einstellte, war ausreichend zur Charakterisierung des nativen Enzyms 2Cbei.

2Cbei Esterase zeigte in einem pH-Bereich von 6,0 – 8,5 eine Aktivität, wobei der optimale pH-Wert bei 8,0 lag. Das Enzym war aktiv bei einer Temperatur zwischen 25 °C und 60 °C. Die optimale Temperatur war 56 °C; eine rasche Abnahme der Aktivität wurde über einer Temperatur von 60 °C beobachtet. 50% der Aktivität wurde bei einer Temperatur von 50 °C gemessen. Der Einfluss der Temperatur auf die Stabilität der Esterase wurde durch die

Messung der Enzymaktivität nach Inkubation der Proben bei einer Temperatur zwischen 5 °C und 85 °C untersucht. Das Enzym war bei den Temperaturen 5 °C, 25 °C, 35 °C und 45 °C stabil. Es verlor ~80% der Aktivität nach einer Inkubation von 30 Minuten bei einer Temperatur von 55 °C und 60 °C. Bei höheren Temperaturen 65 °C, 70 °C, 75 °C und 85 °C verlor das Enzym 100% der Aktivität nach einer Inkubation von 30 Minuten.

Der Einfluss von Hemmstoffen, Detergenzien, organischen Lösungsmitteln und Metallionen auf die Esterase von *Defluvibacter lusatiensis* wurde untersucht. Das Enzym wandelte vorzugsweise Ester von kurzkettigen, aliphatischen Säuren um.

Zusammengefasst zeigte sich, dass alle vier Bakterienkulturen die Geruchsstoffe Triethylamin und Ethylacrylat vollständig abbauen können. Durch die Bestimmung der optimalen Abbaubedingungen und der Entschlüsselung des Abbaumechanismus sind diese vier isolierten Bakterienkulturen einsatzbereit für industrielle Luft- und Wasserreinigungsanlagen.

Chapter 7

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