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DEHALOGENATION OF 3-CHLORO-1,2-PROPANEDIOL BY STRAINS OF *PSEUDOMONAS AERUGINOSA*

DEHALOGENIRANJE 3-KLORO-1,2-PROPANDIOLA S SEVI *PSEUDOMONAS AERUGINOSA*

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The research work was carried out at the Biomedical Research Center (CIBM) in Granada, Spain, under the supervision of my co-mentor prof. dr. Antonio Suárez García and home mentorship of prof. dr. Irena Mlinarič-Raščan.

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STATEMENT

I hereby declare that I have independently carried out work for my master thesis, under the mentorship of prof. dr. Irena Mlinarič-Raščan and co-mentorship of prof. dr. Antonio Suárez García.

Master degree commission

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Organske halogenske spojine so v našem okolju široko prisotne. 3-kloro-1,2-propandiol sodi v skupino halogeniranih alkoholov in ima s študijami dokazano splošno toksično, nevrotoksično, kancerogeno in *in vitro* genotoksično delovanje, povzroča pa tudi neplodnost pri podganjih samcih. Evropski znanstveni komite za hrano (The European Scientific Committee on Food of the European Union) ga je uvrstil v skupino negenotoksičnih in potencialno kancerogenih spojin. Nevarnost ljudem predstavlja njegova prisotnost v številnih živilih.

Znanih je nekaj raziskav o sposobnosti razgradnje 3-CPD in drugih halogeniranih alkoholov s talnimi sevi bakterij. Ključna je prisotnost encima haloalkohol dehalogenaze (Hhe, E.C.4.5.-.-), ki lahko prek poznanega mehanizma razgradi toksično spojino. Prisotnost Hhe je bila dokazana tudi za nekatere talne seve *Pseudomonas* spp., nismo pa zasledili raziskave o prisotnosti encima pri sevih bakterij iz človeškega črevesja. Naš namen je bil raziskati sposobnost razgradnje 3-CPD s sevi *Pseudomonas aeruginosa* izoliranimi iz človeškega prebavnega trakta. Dokaz zmožnosti razgradnje bi pripomogel k razumevanju vloge človeške mikroflore pri razgradnji toksičnega kontaminanta iz hrane.

Sevi *Pseudomonas aeruginosa* uporabljeni v tej študiji so dobro uspevali *in vitro* v prisotnosti 1,2-propandiola (PD), ki je lahko produkt razgradnje 3-CPD, ter po 7,5 h pri 0,6 OD dosegli maksimalno rast in hkrati tvorili 10^{12} CFU/ml. Bakterije so uspevale *in vitro* tudi v prisotnosti 3-CPD kot edinega vira ogljika, kar je nakazovalo na sposobnost, da ga (3-CPD) razgradijo. Morebitna prisotnost in aktivnost Hhe bi povzročila razpad 3-CPD; predvidevali smo, da bi takrat bilo v mediju moč zaznati naraščajočo koncentracijo Cl^- ionov. Za detekcijo Cl^- smo optimizirali spektofotometrično metodo. Preizkušali smo različne pogoje, potrebne za izražanje Hhe. Eksperiment z bakterijami pozne faze, gojenimi na LB-gojišču preko noči in nato prenesenimi v medij s 3-CPD, ni povzročil sproščanja kloridnih ionov. Postopek indukcije encima, ki je vključeval obogatitev gojišča z glukozo, pa se je izkazal za uspešnega. Po izpostavitvi kulture bakterij 3-CPD kot edinemu viru ogljika je bilo v gojišču moč zaznati naraščajoče koncentracije Cl^- ionov, ki so sovpadale s koncentracijami razpoložljivega 3-CPD. Po rezultatih sodeč je bil pri našem sevu *Pseudomonas aeruginosa* izražen encim Hhe in je uspešno degradiral onesnaževalca okolja, hrane in vode 3-CPD. Za potrditev hipoteze so potrebne nadaljnje raziskave.

ABSTRACT

Organohalogen compounds are abundantly present in our environment. Compound 3-chloro-1,2-propanediol (3-CPD), belongs to the group of haloalcohols. 3-CPD is known rodent anti-fertility agent, generally toxic, neurotoxic, carcinogenic and *in vitro* genotoxic. The European Scientific Committee on Food has classified it as a nongenotoxic, threshold carcinogen substance. As a free compound or in ester form it is present in numerous food and can cause harm to consumer's health.

One of the options for removal of organohalogens is microbiological degradation. Few soil bacteria strains are known for their ability to degrade 3-CPD or other haloalcohols, for such activity, the presence of haloalcohol dehalogenases (Hhe, E.C.4.5.-.-) is crucial. These enzymes are able, over well-known mechanisms, to perform degradation of toxic compounds. The presence of such enzymes is, among other bacteria, proven for some *Pseudomonas* spp. soil strains. To best of our knowledge, no study has been performed on capacities of gut bacteria for 3-CPD degradation. The aim of our study was to test the ability of human gut *Pseudomonas aeruginosa* strains to degrade 3-CPD. Such evidence would help us understand microbiota's role in removing the toxic contaminant from the body.

Pseudomonas aeruginosa gut strains grew well *in vitro* in the presence of possible 3-CPD degradation product 1,2-propanediol (PD), reaching maximal growth at 0,6 OD and forming 10^{12} CFU/ml. Bacteria also grew *in vitro* in the presence of 3-CPD as a sole carbon source indicating ability of bacteria to degrade it. We optimized spectrophotometric method to measure chloride ions release as a consequence of 3-CPD degradation. We also tested different conditions required for higher Hhe expression. The use of late phase bacteria, grown on LB media overnight and later inoculated in media with 3-CPD as a sole carbon source, did not result in increase of chloride ions release. The enzyme induction protocol, including growth media supplemented with glucose resulted in increased concentration of released chloride ions. The correlation between 3-CPD concentrations and chloride ions release was shown and could be a consequence of expression and activity of Hhe in *Pseudomonas aeruginosa* gut strains. Further experiments need to be performed to prove this hypothesis.

Keywords: 3-chloro-1,2-propanediol, *Pseudomonas aeruginosa*, haloalcohol dehalogenase, enzyme induction

RAZŠIRJENI POVZETEK

Organske halogenske spojine, naravnega ali sinteznega izvora so v našem okolju široko prisotne. Predstavniki sinteznih organskih halogenskih spojin se uporabljajo kot zdravila, fungicidi, insekticidi, topila, hladilna sredstva, potisni plini, spojine so lahko stranski produkti raznih industrijskih procesov ali intermediati pri laboratorijskih sintezah. Med naravno prisotnimi organskimi halogenskimi spojinami je po nekaterih podatkih znanih čez 4000 različnih spojin, ki so lahko zelo enostavne ali kompleksne molekule. Obe vrsti molekul lahko nastaneta kot produkt živih organizmov, molekule pa lahko nastanejo tudi abiotsko npr. pri raznih požarih v naravi, vulkanski ali drugi geotermalni aktivnosti ali pa so del raznih kamnin. Organske halogenske spojine, npr. antibiotik vankomicin, predstavljajo dodano vrednost, po drugi strani pa so npr. dioksini, škodljivi, zelo obstojni onesnaževalci okolja. Ne glede na naravni ali sintezni izvor so organske halogenske spojine lahko relativno nereaktivne, nekatere med njimi pa močno toksične.

Organski halogenid 3-kloro-1,2-propandiol (3-CPD) spada v skupino halogeniranih alkoholov. Prav tako kot nekateri drugi predstavniki iz te skupine ima s študijami dokazano splošno toksično, nevrotoksično, kancerogeno in *in vitro* genotoksično delovanje, povzroča pa tudi neplodnost pri podganjih samcih. Evropski znanstveni komite za hrano (The European Scientific Committee on Food of the European Union) ga je leta 2001 uvrstil v skupino negenotoksičnih in potencialno kancerogenih spojin. Najdemo ga v proizvodnji dinamita, barv, kot sterilizacijsko sredstvo za glodalce in tudi kot produkt hidrolize epiklorhidrina, ki je široko uporabljen v proizvodnji smol, tekstila in papirja. Največjo grožnjo ljudem predstavlja njegova prisotnost v hrani. V prosti obliki se lahko nahaja v živilih z dodanimi ojačevalci okusa iz vrste kislinsko hidroliziranih rastlinskih proteinov. Takšna živila so juhe iz vrečk, jušne kocke, na splošno pred-pripravljena hrana, kot so razne vnaprej pripravljene omake. Najdemo ga v sojini omaki, slanih prigrizkih, toplotno obdelanih izdelkih iz žit in drugih termično predelanih živilih. V hrani je lahko tudi v obliki estrov, iz katerih se enostavno sprosti s pomočjo hidrolize. Takšne 3-CPD estre lahko zasledimo tako v predelani kot nepredelani hrani. Njihovo prisotnost so dokazali v slanih krekerjih, vloženih slanikih, krofih, kruhu, temnem sladu, ocvrtem krompirčku, praženem ječmenu in rafiniranih jedilnih oljih. Zaradi uporabe epiklorhidrinskih kationskih izmenjevalcev pri čiščenju odpadnih voda se lahko 3-CPD nahaja v pitni vodi.

Poznamo različne fizikalne, kemične in mikrobiološke procese odstranjevanja organskih halogenidov iz narave, številne nove metode pa se še razvijajo. Posebna pozornost se posveča odstranjevanju onesnaževalcev s pomočjo živih organizmov. Bakterije so sposobne s pomočjo encimov oz. kemijskih reakcij, kot so oksidativna dehalogenacija, dehidrohalogenacija, substitutivna dehalogenacija, reduktivna dehalogenacija, razgraditi onesnaževalce v obliki organskih halogenskih spojin. Nekaj raziskav o sposobnosti razgradnje 3-CPD s pomočjo talnih sevov bakterij je bilo že narejenih. Izkazalo se je, da je skupna točka vsem bakterijam pri mikrobiološki razgradnji 3-CPD in drugih molekul iz skupine halogeniranih alkoholov prisotnost encima haloalkohol dehalogenaze (Hhe, E.C.4.5.-.-). Ta encim lahko prek poznanega mehanizma cepi vez med ogljikom in halogenom. Prisotnost encima Hhe je bila potrjena za nekatere talne seve *Pseudomonas* spp.

V človeškem prebavnem traktu živi čez 100 trilijonov mikroorganizmov. Z novimi metodami metagenomike in metodami 16S/18S rRNA so se odprle nove možnosti za raziskovanje tega področja. Pripeljale so nas do spoznanja, da je človeška mikroflora »organ«, ki ima pomembno vlogo pri zdravju posameznika. Mikroorganizmi imajo vlogo pri presnovi ogljikovih hidratov, porabi dušikovih spojin in bio-transformaciji žolčnih kislin. Pomembni so za normalno črevesno funkcijo, razgradnjo hranil, aktivacijo imunskega sistema, izgradnjo imunosti, regulacijo metabolizma, vplivajo na bio-razpoložljivost zdravil, razgradnjo toksinov, obnovo črevesnega epitelija in sintezo vitaminov. Njihova vloga je raznovrstna, spremembe v mikrobioti pa se lahko izrazijo v posameznikovem zdravju.

Naš namen je bil raziskati sposobnost razgradnje 3-CPD s sevi *Pseudomonas aeruginosa,* izoliranimi iz človeškega prebavnega trakta. Pri sevih bakterij iz človeškega črevesja nismo zasledili nobene raziskave o prisotnosti encima Hhe in s tem zmožnosti razgradnje 3-CPD. Dokaz prisotnosti encima Hhe bi pripomogel k razumevanju vloge mikroorganizmov prebavnega trakta pri razgradnji toksičnega kontaminanta iz hrane. Ker je metabolna kapaciteta mikroorganizmov prebavnega trakta ljudi zelo velika in za še ni povsem raziskana, je pot do boljšega javnega zdravja lahko skrita tudi v poznavanju sposobnosti prilagajanja črevesnih mikroorganizmov na razne vplive okolja. Iz slednjega lahko neprestano prek različnih poti v telo vnesemo različne organske halogenske spojine, ki nam lahko s svojim kopičenjem v telesu škodijo. Z našim delom smo poskušali odgovoriti na vprašanji kaj se zgodi s temi spojinami, ko enkrat vstopijo v naše telo, in ali so morda bakterije prebavnega trakta udeležene pri odstranjevanju teh spojin.

Sevi *Pseudomonas aeruginosa*, uporabljeni v tej študiji so dobro uspevali *in vitro* v prisotnosti 1,2-propandiola (PD), ki je lahko produkt degradacije 3-CPD, in po 7.5 h pri 0,6 optične gostote (OD) dosegli maksimalno rast in hkrati tvorili 10^{12} CFU/ml. Bakterije smo nato gojili *in vitro* v prisotnosti izključno 3-CPD kot vira ogljika. Morebitna prisotnost in aktivnost encima Hhe v našem sevu bakterij bi povzročila razpad molekule 3-CPD. Predvidevali smo, da bi ob razpadu 3-CPD v mediju bilo moč zaznati naraščajočo koncentracijo Cl^- ionov. Za merjenje prostih kloridnih ionov smo zato optimizirali spektofotometrično metodo. Kot smo predvidevali, so bili za izražanje encima potrebni posebni pogoji v gojišču. Rast bakterij *in vitro* v prisotnosti izključno 3-CPD kot vira ogljika, je bilo moč spektofotometrično zaznati, a je bila zelo počasna, istočasno pa signifikantno naraščajoče koncentracije Cl^- ionov ni bilo mogoče določiti. S spremembami koncentracije razpoložljivega 3-CPD nismo povzročili nobenih sprememb v merjeni koncentraciji Cl^- ionov. Prav tako eksperiment z bakterijami pozne faze rasti, gojenimi v s hranili bogatem mediju LB prek noči in nato prenešenimi v medij s 3-CPD kot edinim virom ogljika, ni povzročil sproščanja kloridnih ionov. Postopek indukcije encima pa se je izkazal za uspešnega. Med postopkom predpriprave bakterij so imele slednje kot dodaten vir ogljika na voljo glukozo. Kultura bakterij je bila nato izpostavljena izključno 3-CPD kot viru ogljika in zaznana je bila naraščajoča koncentracija kloridnih ionov v gojišču. Koncentracija kloridnih ionov je sovpadala s koncentracijo razpoložljivega 3-CPD. Možno je, da je glukoza preprečila inhibicijo encima s 3-CPD in podprla izražanje encima Hhe. Po naših rezultatih sodeč je bil encim Hhe izražen pri izoliranih sevih *Pseudomonas aeruginosa* in je uspešno razgradil onesnaževalca okolja, hrane in vode- 3-CPD. Za potrditev hipoteze so potrebne nadaljnje raziskave.

Ključne besede: 3-kloro-1,2-propandiol, *Pseudomonas aeruginosa*, haloalkohol dehalogenaza, indukcija encima

LIST OF ABBREVIATIONS

1,3-DCP	1,3-dichloro-2-propanol
2,3-DCP	2,3-dichloro-1-propanol
3-CPD	3-chloro-1,2-propanediol
3-MCPD	3-monochloropropane-1,2-diol
3α/20β-HSD	3-alpha (or 20-beta)-hydroxysteroid dehydrogenase
acid-HVP	Acid hydrolyzed vegetable protein
Arg	Arginine
CFC	Chlorofluorocarbon- Freon
CFU	Colony forming unit
DDT	Dichlorodiphenyltrichloroethane
GC	Gas chromatography
Hhe	Halohydrin hydrogen-halide lyases or haloalcohol dehalogenases
LB	Luria-Bertani media
Lys	Lysine
Mm	Molar mass (g/mol)
M9	M9 Minimal medium
MiM	Mineral medium
MM	Minimal medium
NAD	Nicotinamide adenine dinucleotide
NAD(P)+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NAD(P)+
OD	Optical density
PCB	Polychlorinated biphenyl
PD	1,2-propane-diol
PVC	Polyvinyl chloride
rpm	Revolutions per minute
SDRs	NAD(P)(H)-dependent short-chain dehydrogenases/reductases
Ser	Serine
TDI	Tolerable daily intake
TFE	Tetrafluoroethylene
Tyr	Tyrosine

1. INTRODUCTION

1.1 Organic halogen compounds

The term organic halogen compounds (organohalogens) describes a broad class of natural and synthetic chemicals. This chemicals are composed of one or more halogens (fluorine, chlorine, bromine or iodine) combined with carbon and other elements. Further we can divide them into alkyl, vinyl, aril and acyl halides. Alkyl- there are (all) four single bounds to the carbon that bears halogen, vinyl- carbon that bears halogen is double bounded to another carbon, aril- carbon that bears halogen is part of an aromatic structure and acyl halides have halogen-bearing carbon bonded to oxygen with double bond (Figure 1).

A B C D

Figure 1. Examples of organic halogen compounds (1). A; alkyl chloride: 1-Chloropropane, B; vinyl chloride: 1-Chloropropene, C; aryl chloride: Chlorobenzene and D; acyl chloride: Propanoyl chloride.

1.1.1 Physicochemical characteristics

When talking about halogens we have in mind the VII group of periodic table from which astatine is exempt, because it is radioactive. Halogens are all very reactive. They have 7 electrons on the outermost shell, it means, they need to get only one electron, with chemical reaction, to have perfect configuration and stability. Such characterization makes them life threatening for living organisms. Halogen atoms are more electronegative than carbon atoms, so the bond between them is polar (2). Between above described four types of oganohalogen compounds, aryl halides have the strongest carbon-halogen bond and the alkyl halides the weakest. The correlation between the strength and the rate of reaction it's inversely. The strongest the bond the slower the rate of reaction. However, the reactivity of

organohalogens can be very different. We can have very reactive and highly toxic compounds on one side and relatively inert compounds on the other (3).

1.1.2 Presence of organic halogen compounds

The organohalogens are widely present in nature. Among organohalogens most abundant halogen is chlorine. On basis of their origin, organohalogens can be classified as being biogenic (e.g. CH_3Cl), natural/geogenic (e.g. specific dioxins in clay), having anthropogenic non-halogenated precursors (e.g. halogenated phenols formed from phenol), having anthropogenic halogenated precursors (e.g. chlorophenols formed from chlorobenzenes, pentachlorophenyl methyl ether formed from pentachlorophenol), or anthropogenic (e.g. freons) (4).

From synthetic made halogen- containing compounds, especially alkyl halides are used as important laboratory synthesis intermediates (2). At industrial level, we can find halogenated organic compounds as pharmaceuticals, fungicides, insecticides, solvents, flame retardants etc. Many compounds are by-products during different chemical syntheses (5). Halogenated aliphatic compounds are constituents of industrial waste gases. Several of these compounds are produced in millions of tons annually (6). Beside the usefulness of organohalogens, they are persistent pollutants in our environment (7). The reasons for persistence is precisely the halogenation of organic molecules (8). Here are some examples of persistent organohalogens:

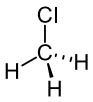
- insecticide; eg. dichlorodiphenyltrichloroethane (DDT)
- industrial processes by-products; eg. dioxins
- a component of dielectric and coolant fluids, heat transfer fluids, plasticizers, pesticide extenders, lubricating oils, hydraulic fluids; eg. polychlorinated biphenyl (PCB)
- refrigerant, propellant (in aerosol applications), and solvent; eg. chlorofluorocarbon (CFC, brand name Freon)

Halogenated aliphatic compounds are extended pollutants of groundwater. Important group of halogenated aliphatic are chlorinated aliphatic. Around 50 000 tons of these pollutants enter and pollutes the environment annually. In these concentrations they present a risk for

public and environment health (9). There are reports of presence of chlorinated ethers in industrial effluents, air samples and in river sediments (10, 11).

Beside man-made molecules, halogenated organic compounds can be produced by nature. These compounds can be very similar or even identical to some synthetically made halogenated compounds. They have identified over 2000 unique naturally occurring organochlorines, over 2000 organobromines, over 100 organoiodines and over 30 organofluorines. There are covered simple molecules such as methyl chloride and also complex molecules, such as vancomycin (7) (Figure 2). Most of naturally produced organohalogens are produced by living organisms. There are known marine and terrestrial plants, marine animals, bacteria, fungi, lichen, insects, and some mammals, that can produce organohalogens (3, 12). Some of halogenated metabolites are very important for humans because we used them as antibiotics. Such compounds are chloramphenicol, 7-chlorotetracyclin and vancomycin (13). Organic halogen compounds can also be formed abiotically. Sources of such production are forest fires, volcanic and other geothermal activities. Rocks also contains organohalogens, several natural fluorite minerals contains tetrafluoroethylene (TFE) the chemical precursor to Teflon (7).

A



B

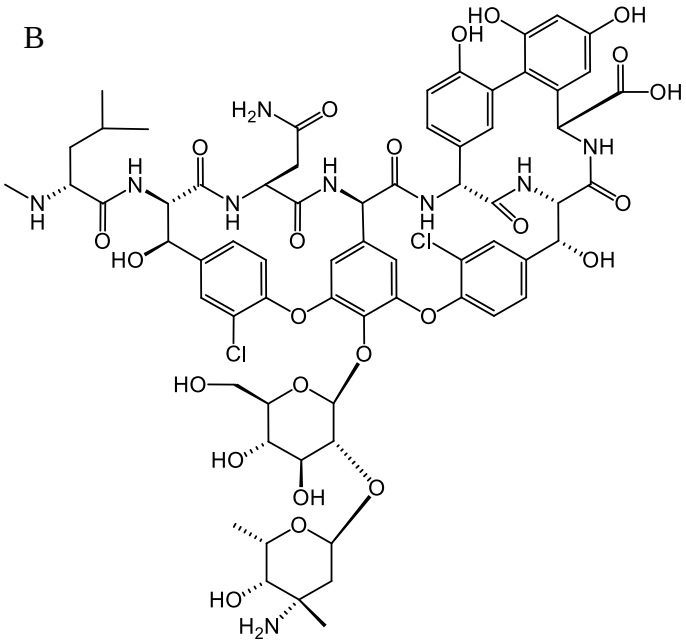


Figure 2. The example of naturally produced organohalogens. Nature can produce simple halogen- containing compounds like A; methyl chloride and also complex halogen-containing compounds like B; vancomycin.

1.1.4 Toxicity of organic halogen compounds

The part that definitely shouldn't be overlooked is the toxicity of organohalogens. Characteristics, such as physical properties and biological activity, of organic halogen molecule, depends on the number and position of halogen atoms. Therefor individual molecules can have different toxicity. Lipophilic, stable and persistent organohalogen molecules are slowly metabolized and eliminated, which leads to a net increase of such substances in organism over time (14). Description of all differences between individual organic halogen compounds could be another thesis or article. Here we are describing only some common toxicity characteristics. Sensitivity and response to organohalogens are although depended on the different species. There may be differences and these differences can be considerable. Exposure to high or low doses of organohalogens, can cause acute or chronic effects. The effects of exposure can be shown as effects on reproduction, development, cytochrome P450-dependent enzymes, porphyrins, the immune system, the adrenals, the thyroid gland, thyroid hormone levels, and vitamin A levels. Some organohalogens can also cause visible changes in the liver. Hypertrophy, lesions, and even, tumors may occur (14).

1.1.5 Degradation of organic halogen compounds

Different physical, chemical and microbiological processes, which are enabling the removal of organic halogenated compounds are known. One of them is volatilization from water in to atmosphere followed by destruction with photooxidation. In case of 2-chloroethylvinylether photooxidation, mechanism of decomposition involves electrophilic attack by hydroxyl radicals, ozone or other oxidants on the double bound (10). Other processes are e.g. dehalogenation processes under oxidative, alkaline, or irradiation conditions, pyrolysis, hydrolysis, photodegradation. Unfortunately, due to various reasons, these methods are very limited (15). Method with reactive, permeable barrier consisting of zerovalent iron is being investigated, and offers a new approach for removal of halogenated compounds. Iron offers electrons which are able to reduce organohalogens through reductive dehalogenation (15, 16). Beside physical and chemical methods microbiological degradation is also possible. Microorganisms are able, either under aerobic or anaerobic conditions, to transform and degrade organohalogens. Degradation under aerobic conditions is widespread,

because aerobic culture techniques are simpler, efficient and generally applicable. There is broad range of microorganisms that participate in biodegradation reactions (16). Bacteria trigger mechanisms that produce metabolites which are further used for cell material and production of energy. When talking about halogenated compounds, the first step of degradation under aerobic conditions is replacement of the halogen, triggered by enzymes called dehalogenases (18). There are, as mentioned, many different organohalogen molecules with different properties. Bacteria have also developed many different strategies for enzyme catalyzed dehalogenation and degradation of these molecules. Mechanisms of different strategies such as oxidative dehalogenation, dehydrohalogenation, substitutive dehalogenation, thiolytic dehalogenation, dehalogenation by methyl transfer and reductive dehalogenations are well known and described (19). More detailed presentation of, for our research relevant, enzymes and chemical reactions, will be present in following chapters.

1.2 Haloalcohols

Haloalcohols also called halohydrins are organic compounds, consisting of one carbon atom with halogen substituent and adjacent carbon atom with hydroxyl substituent. One of the chemical reactions in which haloalcohols are formed is electrophilic addition. Halogen is added to alkene in aqueous solution (2). In everyday use, we can find haloalcohols as solvents and reactants for pharmaceuticals, agrochemicals, polymers and resins (9, 20). Examples of haloalcohols are: 1,3-dichloro-2-propanol (1,3-DCP), 2,3-dichloro-1-propanol (2,3-DCP), and 3-chloro-1,2-propanediol (3-CPD). 1,3-DCP and 2,3-DCP are known as being carcinogenic, mutagenic and genotoxic (9, 20).

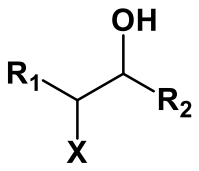


Figure 3. Halohydrin. General structure of haloydrins, containing halogen (X) and hydroxyl (OH) substituent.

1.2.1 3-chloro-1,2-propanediol (3-CPD)

3-chloro-1,2-propanediol (3-CPD), many times also named 3-monochloropropane-1,2-diol (3-MCPD) or α-chlorohydrin is widely used organic halogenated compound. We can find 3-CPD in production of dynamite, in manufacture of dye intermediates, as a rodent chemosterilant (21). After hydrolyses, 3-CPD is formed from epichlorohydrin, which is also used widely for resins manufacture, textiles and paper products (22). 3-CPD can also be found in food. Together with 1,3-DCP they are known food contaminants (23). Since now they were detected in many food products. One of the reasons for 3-CPD occurrence in food is acid hydrolyzed vegetable protein (acid-HVP). 3-CPD is formed as reaction product of hydrochloric acid with triacylglycerols, phospholipids and glycerol during production of acid-HVP. This protein is used as flavor enhancer, ingredient of savoury food such as soups, prepared meals, savoury snacks and gravy mix (24). 3-CPD was also found in soy sauce, heat-treated cereal products, and wide range of other thermally processed food (22-24). In acid-HVP, 3-CPD occurs as a free compound. Beside free compound form, they have also found esters of 3-CPD in unprocessed and processed food. With hydrolysis reaction, 3-CPD can be easy released from these molecules. In high levels 3-CPD esters were found in salty crackers, pickled herrings, doughnuts, crisp bread, dark malt, French fries, roasted barley, refined edible oils (25). They (3-CPD esters) occurs there as contaminants, being formed, during manufacturing or cooking, from lipids and sodium chloride, which were (lipids and sodium chloride) naturally present or added to the food (24). Because sometimes epichlorohydrin-linked cationic polymer resins are used for purification of waste water, 3-CPD can also occur in drinking water (22).

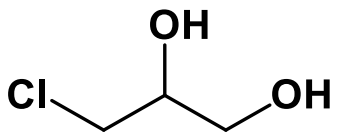


Figure 4. 3-chloro-1,2-propanediol (3-CPD). Skeletal formula of 3-CPD.

1.2.1.1 Toxicity of 3-chloro-1,2-propanediol (3-CPD)

As we can see above, humans can consume 3-CPD in many different food products. This intake is worrying, because excessive amount of 3-CPD can cause harm to consumers health. Usually 3-CPD is present as racemic mixture of R and S enantiomers (25). R enantiomer supposed to have an effect on the kidneys and S enantiomer is a male anti-fertility agent (22). Due to the ability of crossing the blood-testis and blood-brain barrier and distribution in the body fluids, 3-CPD is general toxic, neurotoxic, carcinogenic and genotoxic (26). Animal studies has shown that 3-CPD, after chronic oral exposure, can cause nephropathy and tubular hyperplasia and adenomas, it also induce infertility and suppression of the immune function in rats. It is genotoxic *in vitro*, but in *in vivo* study this was not confirmed. There are also some evidence about carcinogenic effect on male and female rats (27). If dally intake is not too high, 3-CPD is not acute toxic, but the long term accumulated toxicity is harmful to humans (26). The European Scientific Committee on Food of the European Union has classified 3-CPD in 2001 as a nongenotoxic, threshold carcinogen with a tolerable daily intake (TDI) of 2 μg/kg body weight, per day (28).

1.2.2 Degradation of haloalcohols

For the elimination of toxic contaminants, different methods are being developed. For instance, hydrolysis and photolysis are methods that can efficiently remove 3-CPD from water (22). Biological methods include the utilization of microorganisms that are able to degrade haloalcohols, are also being developed. Many bacteria are being tested on their ability to perform degradation of haloalcohols. *Agrobacterium sp*. A1, *Agrobacterium radiobacter* AD1, *Pseudomonas sp*. AD1, *Pseudomonas putida* DSM 437, *Pseudomonas sp*. OS-K-29, *Flavobacterium sp., Arthrobacter sp*. AD2, are all being proved to degrade or convert haloalcohols (6, 9, 13, 29, 30).

For degradation or conversion of halogenated compounds the crucial step is precisely the removal of halogen substituent. After removal of halogen substituent, during metabolism generated, toxic intermediates are less likely to occur. Compound can lose its toxic and xenobiotic character (30). Common to all above mentioned bacteria is presence and activity of required enzymes that are able to perform dehalogenation (6, 9, 13, 29, 30).

1.2.2.1 Halohydrin hydrogen-halide lyase (haloalcohol dehalogenase, Hhe)

Halohydrin hydrogen-halide lyases or haloalcohol dehalogenases (Hhe, E.C.4.5.-.-) are enzymes that are able to perform the reaction of dehalogenation. They are specific for vicinal haloalcohols and haloketones (19). These enzymes can cleave carbon halogen bond. Reports about isolation of 6 different Hhe are known. Hhe can be, on basis of their amino acid sequence similarities, classified in 3 different subtypes labeled A, B and C. Evolutionarily these enzymes belong to the large family of NAD(P)(H)-dependent short-chain dehydrogenases/reductases (SDRs), whit similar serine-tyrosine-lysine/arginine (Ser-Tyr-Lys/Arg) catalytic triad (31, 32). There are few differences between SDRs and Hhe. Hhe do not require $NAD(P)^+$ as a coenzyme and in active site Lys residue of SDRs is replaced by Arg residue (5) (Figure 5 A, B). The chemical reaction carried out by Hhe is intramolecular nucleophilic substitution (19, 31). In, by Hhe generated, reaction of dehalogenation, haloalcohols (3-CPD, 1,3-DCP) are dehalogenated and formatted to epoxide glycidol. Glycidol may be converted by epoxide hydrolase (EC 3.3.2.3) to glycerol (6, 19) (Figure 5, 1-5). Mechanism of reaction is probably started with removal of proton from vicinal hydroxyl group. Deprotonated Tyr residue along with adjacent Arg residue carry out this step. Following step is formation of an epoxide and elimination of halide ion. In this step alkoxide attacks adjacent carbon, which has a lack of electrons. The function of Ser is to stabilize the reaction intermediate by forming a hydrogen bond to the hydroxyl group (31) (Figure 6).

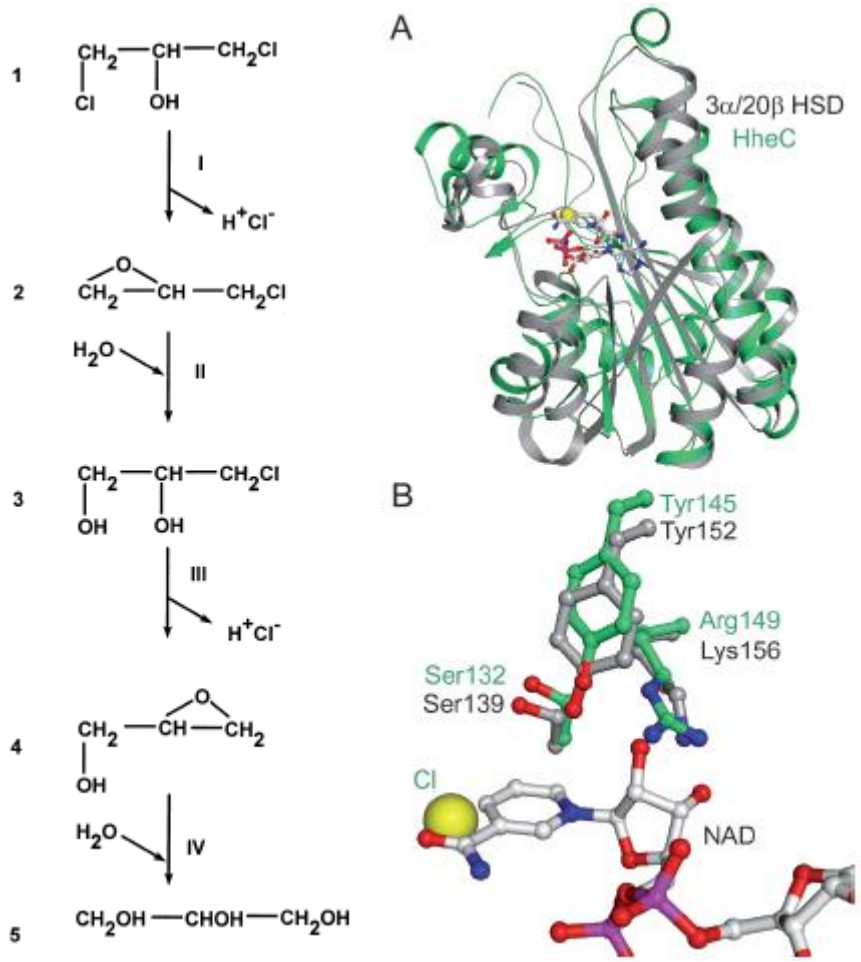


Figure 5. Haloalcohol dehalogenase structure and reaction of dehalogenation. On the left side; dehalogenation of 1,3-DCP→1 and 3-CPD→3 with Hhe→I, III and formation of epoxide, glycidol→4 and conversation of epichlorohydrin→2 and glycidol→4 by epoxide hydrolase→II, IV to glycerol→5. On the right side; A→ similarity of α/β fold architecture, when comparing classical SDR, 3-alpha (or 20-beta)-hydroxysteroid dehydrogenase (3α/20β-HSD) (grey), and Hhe (green). B→ comparison of 3α/20β-HSD (grey) and Hhe (green) active sites. We can also see the NAD molecule from 3α/20β-HSD and chloride bound to Hhe (19, 31).

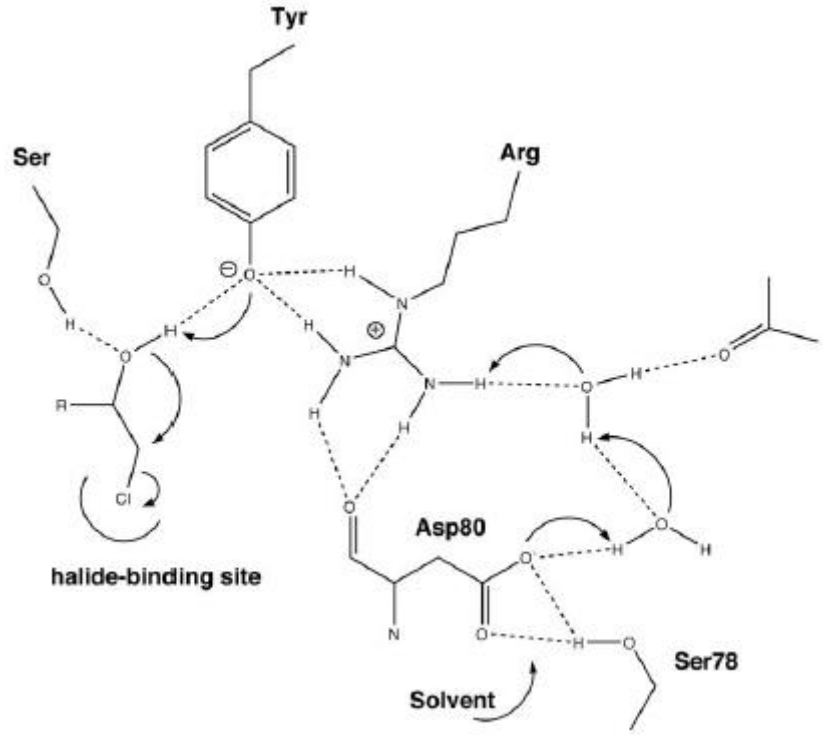


Figure 6. Catalytic mechanism of haloalcohol dehalogenase. Vicinal hydroxyl group is bounded by Ser and Tyr. Tyr, as catalytic base, activates OH group for nucleophilic attack on the halogen-bearing carbon atom. Arginine, water molecules and Aspartic acid (Asp) at the surface of Hhe, are helping to release the abstracted proton to the solvent (32).

1.3 Gut microbiota

In human gut there is more than 100 trillion microorganisms, consisting of a complex community of microorganism species. Bacteria dominate, but Archaea and Eukarya are also represented (33). In monogastric species, which includes humans, gut bacteria most widely inhabit the distal part of intestine (34, 35). Variations on the genomic, transcriptomic, proteomic and metabolomic methods, from which metagenomic and 16S/18S rRNA gene-based methods are the most common, are used for determination of functions in the microbiome and presence of species. New knowledge, optimization and improvement of mentioned methods, especially in years after 1990, are one of the reasons, for recognition that microbiota is an important element of host's health and one of the reasons for numerous research with hypothesis in which microbiota are having a key role for health and diseases (36). Every human has its own complex community of microorganism species, but it is known that most of microorganisms (over 90% in average) belongs to two main phyla -

Bacteroidetes and Firmicutes. There are also Actinobacteria phylum, Proteobacteria phylum and Verrucomicrobia phylum as minority representatives (37) (Figure 7). There is broader range of bacteria when talking about species (a few hundred) or strains (a few thousand) (36).

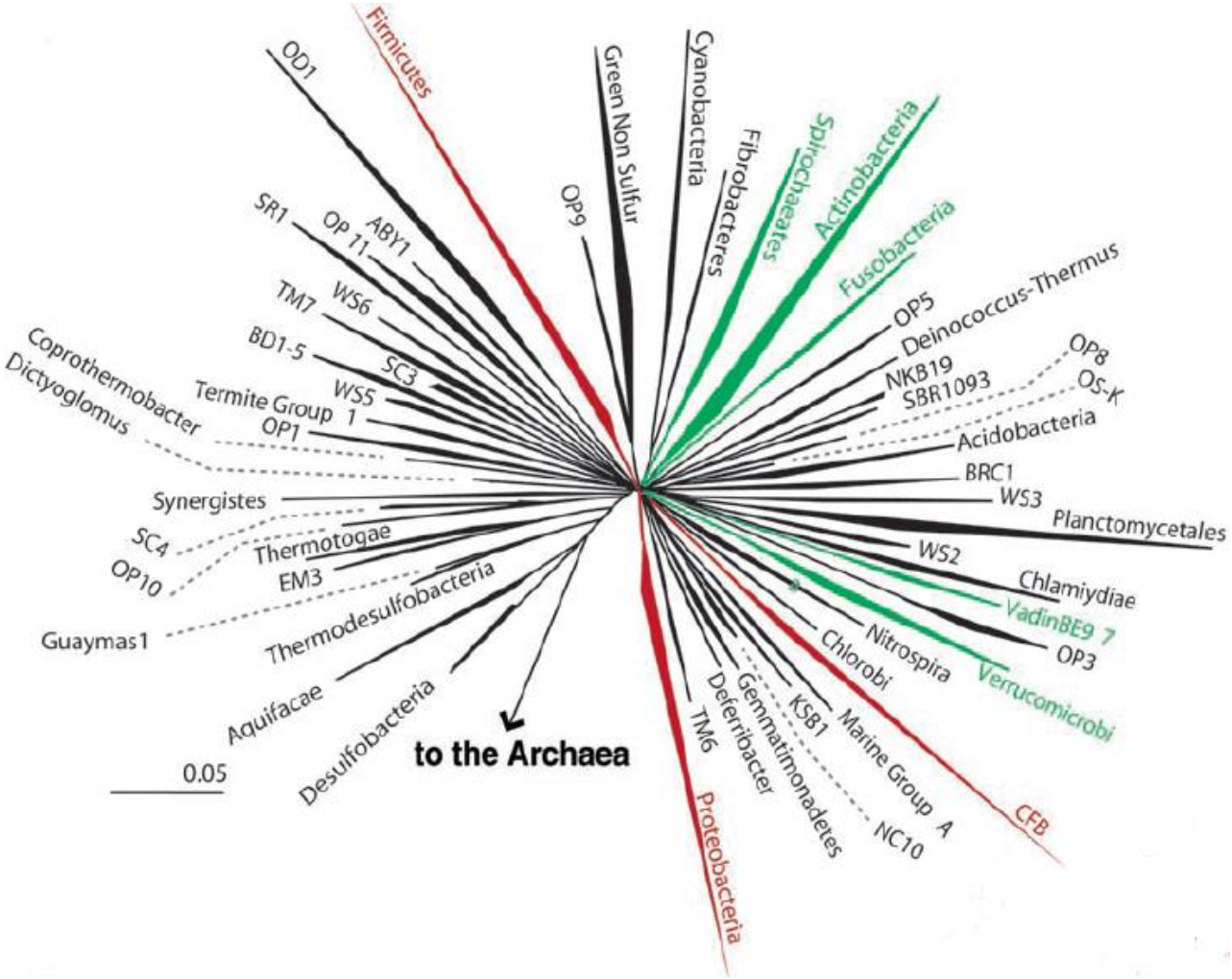


Figure 7. Phylogenetic tree of human gut bacteria. Tree was made on basis of 8903 representative 16S rDNA gene sequences. Wedges represent phylum. Wedge length represents evolutionary distance from the common ancestor. Red color→ bacteria present in large number, green→ bacteria present in small number, black→ bacteria that are not being detected (35).

1.3.1. Metabolic activity of gut microbiota

Gut microbiota can be imagined as an organ because of all their metabolic activities. It has a capacity to communicate with each other and with host, to consume, store and redistribute energy, it also mediates important physiological, chemical transformations and can maintain and repair itself with ability of self-replication (35). Metabolic activities are very diverse. Carbohydrate, lipid and amino acid metabolisms, production of vitamins and existence of trans-genomic, trans-mural co-metabolism of multiple substrates, including those involved in host metabolic regulation, are all known and described (37). Bacteroidetes representatives are involved in carbohydrate fermentation, nitrogen-substances consumption and bile acids biotransformation. They have moderate saccharolityc activity with ability to hydrolyze complex plants polysaccharides such as cellulose, pectin and xylan. Firmicutes representatives are also having important role in fermentation of polysaccharides. Although there is only a small part of them, Archaea are also helping to increase the energy yield during food fermentation (34). It seems that gut microbiota is very important for normal intestinal function, for adoption and degradation of nutrients. Its role is in installation and activation of host immune system and immunity development. Other functions are; regulation of host metabolism, bioavailability of drugs, degradation of dietary toxins, gut epithelial cells renewal and vitamin synthesis. Microbiota have an impact on physiology modulation, impact on host's behavior and also impact on etiology of diseases, like inflammatory bowel disease, colorectal cancer, heart diseases, diabetes and metabolic syndrome (38, 39). Incidence of diseases in relation to gut microbiota can be associated with bacterial metabolites in the human gut. These most commonly metabolites are short-chain fatty acids, butyrate, acetate, lactate and propionate. Butyrate, produced mainly by *Faecalibacterium prausnitzii* and *Eubacterium rectale*/*Roseburia intestinalis* groups, has an impact on controlling apoptosis, cytokine production, energy for colonocytes and mucus synthesis (36). Gut microbiota is however very dynamic system. Few metagenomic studies are describing the difference between lean and obese microbiota genes that encode enzymes involved in indigestible dietary polysaccharides degradation. These genes are being enriched in human gut microbiome of obese people when comparing with lean control. Such a condition can lead to greater utilization of energy from the diet (37). We can see that there is a great probability that any changes in gut microbiota can result in changes of different host functions and physiology or generally- in changes of host's health.

1.3.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a rod-shaped gram negative, bacteria that belongs to phylum Proteobacteria and class of gama Proteobacteria. It has a wide distribution in environment such as water, soil, sewage, plants, hospitals, animals and humans. It is an opportunistic human pathogen and can causes infection in immunocompromised patients (40).

If growing bacteria in laboratory under certain conditions, besides possible other colors, green-yellow color occurs as a result of one of the metabolites of *Pseudomonas aeruginosa*, pyoverdine (fluorescein). This green-yellow pigment, because of its florescence, can clearly be visible under UV light (41). A wide range of metabolic activities, probably as result of the large number of enzymes, give this bacteria an opportunity for biotechnological applications in areas of bioremediation, biocatalysis, function of biocontrol agent in plant protection and for bioplastic production (42).

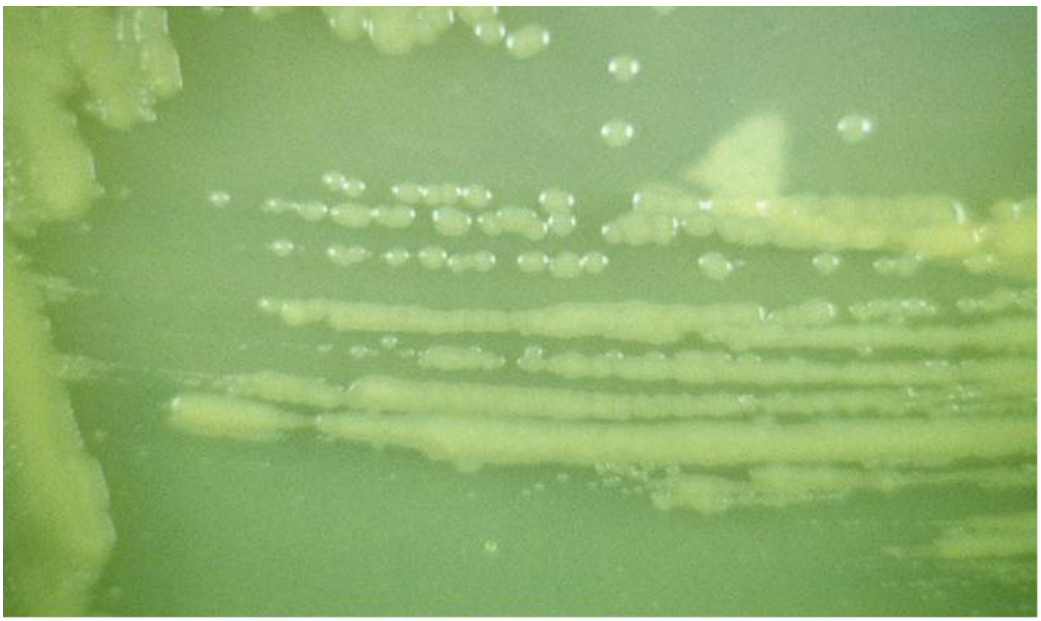


Figure 8. *Pseudomonas aeruginosa* colonies on petri dishes with Luria-Bertani (LB) media. Typical green-yellow color of *Pseudomonas aeruginosa* colonies on solid (agar) LB media.

2. RESEARCH OBJECTIVES

Gut microbiota is the area which provide a lot of opportunities for numerous research. Metabolic capacities of gut microorganisms are very large and not yet being fully known. The key for better public health may also be hidden in knowing, how individual gut organisms react to different conditions. Because there are many, synthetically and naturally produced, halogen-containing compounds, through different paths, daily entering our environment, our body, the question "what happens with these compounds next", arises. Are these compounds accumulating in our body or are they eliminated? Are maybe bacteria involved in removal of halogen containing compounds? There are articles about soil bacteria capacities for degradation one of the environmental, food and water pollutant 3-CPD. As we know, no research has been done about gut bacteria capacities, to perform such degradation.

Pseudomonas aeruginosa strains were previously isolated from fecal samples of human volunteers. Few research about soil strains of *Pseudomonas* spp. ability to degrade 3-CPD has been done. Because of wide range of enzymes and consequently metabolic capacities, our *Pseudomonas aeruginosa* gut strains might also be appropriate for dehalogenation of 3-CPD. Halohydrin hydrogen-halide lyase (haloalcohol dehalogenase, Hhe) are enzymes that over special mechanism can cleave carbon halogen bonds and there are reports about presence of these enzymes in *Pseudomonas* spp..

The aim of our work is to test the ability of human gut *Pseudomonas aeruginosa* strains to degrade 3-CPD. We will test the behavior of *Pseudomonas aeruginosa* strains in the presence of 3-CPD as a sole carbon source with determining bacterial growth spectrophotometrically. We will also determine viable cell number of *Pseudomonas aeruginosa* with evaluating the capacity of bacteria to form colonies (CFU). To perform the reaction of dehalogenation, appropriate enzymes are required. With modifications of growth media, we will try to create special conditions which are sometimes required for enzyme expression. After dehalogenation of 3-CPD, chloride ions should be released. Such release might be the consequence of Hhe activity. We will evaluated the presence of chloride ions in media with modified chemical/spectrophotometric method of Florence and Farrar. Before applying the method in our research, calibration curve will be generated with different chloride concentrations.

3. EXPERIMENTAL WORK

3.1 Materials

3.1.1 Chemicals and substances

- (±)-3-Chloro-1,2-propanediol (98%) Sigma-Aldrich (Steinheim, Germany)
- 1,2-Propanediol (99,5%) Panreac (Barcelona, Spain)
- 4-Aminobenzoic acid Sigma-Aldrich (Steinheim, Germany)
- Agar, Bacteriological European type Panreac (Barcelona, Spain)
- Ammonium chloride Panreac (Barcelona, Spain)
- Ammonium nitrate Sigma-Aldrich (Steinheim, Germany)
- Ammonium sulfate Sigma-Aldrich (Steinheim, Germany)
- Boric acid Merck (Darmstadt, Germany)
- Calcium chloride Sigma-Aldrich (Steinheim, Germany)
- Calcium chloride-dihydrate Merck (Darmstadt, Germany)
- Calcium chloride-hexahydrate Sigma-Aldrich (Steinheim, German
- Calcium sulfate Merck (Darmstadt, Germany)
- Cyanocobalamin (Vitamin B 12) Merck (Darmstadt, Germany)
- Copper(II) chloride-dihydrate Sigma-Aldrich (Steinheim, Germany)
- D(+)-Biotin (vitamin H) Sigma-Aldrich (Steinheim, Germany)
- di-Potassium phosphate Sigma-Aldrich (Steinheim, Germany)
- di-Sodium hydrogen phosphate-dihydrate Merck (Darmstadt, Germany)
- Ethanol absolute (99,5%) Panreac (Barcelona, Spain)
- Folic acid Sigma-Aldrich (Steinheim, Germany)
- D-(+)-Glucose Sigma-Aldrich (Steinheim, Germany)
- Iron(II) chloride-tetrahydrate Sigma-Aldrich (Steinheim, Germany)
- Iron(III) chloride Merck (Darmstadt, Germany)
- Iron(III) nitrate-nonahydrate Sigma-Aldrich (Steinheim, Germany)
- (±)-α-Lipoic acid Sigma-Aldrich (Steinheim, Germany)
- Magnesium sulfate Sigma-Aldrich (Steinheim, Germany
- Magnesium sulfate-heptahydrate Sigma-Aldrich (Steinheim, Germany)
- Manganese(II) chloride-tetrahydrate Sigma-Aldrich (Steinheim, Germany)

- Mercury(II) thiocyanate — Sigma-Aldrich (Steinheim, Germany)
- Methanol — Panreac (Barcelona, Spain)
- Nickel(II) chloride-hexahydrate — Merck (Darmstadt, Germany)
- Nicotinic acid — Merck (Darmstadt, Germany)
- Perchloric acid 70% — Panreac (Barcelona, Spain)
- Potassium phosphate — Sigma-Aldrich (Steinheim, Germany)
- Pyridoxamine dihydrochloride — Sigma-Aldrich (Steinheim, Germany)
- (−)-Riboflavin — Sigma-Aldrich (Steinheim, Germany)
- Calcium D(+)-pantothenate — Sigma-Aldrich (Steinheim, Germany)
- Sodium chloride — Sigma-Aldrich (Steinheim, Germany)
- Sodium molybdate-dihydrate — Sigma-Aldrich (Steinheim, Germany)
- Sodium selenite-pentahydrate — Sigma-Aldrich (Steinheim, Germany)
- Sodium tungstate-dihydrate — Sigma-Aldrich (Steinheim, Germany)
- Thiaminie hydrochloride — Sigma-Aldrich (Steinheim, Germany)
- Tryptone — Panreac (Barcelona, Spain)
- Yeast extract — Panreac (Barcelona, Spain)
- Zinc chloride — Merck (Darmstadt, Germany)

3.1.2 Growth media

Luria-Bertani medium (LB)

- Bacto-tryptone — 10 g
- Bacto-yeast extract — 5 g
- NaCl — 10 g
- H_2O — up to 1000 ml

For solid media add 15g/l of Agar.

Minimal medium (MM)

- Bushnell-Haas Salts — 3,27 g
- Trace elements — 3 ml
- Selenium tungsten solution — 3 ml

- Vitamin solution I 2 ml
- Vitamin solution II 6 ml
- H_2O up to 1000 ml

Bushnell-Haas Salts:

- KH_2PO_4 1 g
- K_2HPO_4 1 g
- NH_4NO_3 1 g
- $FeCl_3$ 0,05 g
- $CaCl_2$ 0,02 g
- $MgSO_4$ 0,2 g

Trace elements:

- HCl 25% 10 ml
- $FeCl_2x4H_2O$ 1,5 g
- $CaCl_2x6H_2O$ 190 mg
- $MnCl_2x4H_2O$ 100 mg
- $ZnCl_2$ 70 mg
- H_3BO_3 63 mg
- $Na_2MoO_4x2H_2O$ 36 mg
- $NiCl_2x6H_2O$ 24 mg
- $CuCl_2x2H_2O$ 17 mg
- H_2O up to 1000 ml

Selenium tungsten solution:

- $Na_2SeO_3x5H_2O$ 3 mg
- $Na_2WO_4x2H_2O$ 4 mg
- MeOH 0,5 g
- H_2O up to 1000 mL

Vitamin solution I:

- 4-Aminobenzoic acid 40 mg
- D(+)-Biotin (vitamin H) 10 mg
- Nicotinic acid 100 mg
- Calcium D(+)-pantothenate 50 mg
- Pyridoxamine dihydrochloride 150 mg
- Thiamine hydrochloride 100 mg
- Vitamin B 12 (cyanocobalamin) 50 mg
- H_2O up to 1000 mL

Vitamin solution II:

- Lipoic acid 10 mg
- Riboflavin 10 mg
- Folic acid 4 mg
- H_2O up to 1000 mL

M9 minimal medium (M9)

- M9 salt 200 ml
- $MgSO_4$ (1M) 2 ml
- $CaCl_2$ (1M) 0,1 ml
- Trace elements 3 ml
- Selenium tungsten solution 3 ml
- Vitamin solution I 2 ml
- Vitamin solution II 6 ml
- H_2O up to 1000 mL

M9 salt:

- $Na_2HPO_4*2H_2O$ 8,5 g
- KH_2PO_4 3 g
- NaCl 0,5 g
- NH_4Cl 1 g
- H_2O up to 200 ml

Mineral medium (MiM)

- $(NH_4)_2SO_4$ 1,5 g
- KH_2PO_4 1 g
- K_2HPO_4 2 g
- $MgSO_4*H_2O$ 0,2 g
- NaCl 0,1 g
- $CaSO_4$ 0,06 g
- $FeCl_3x6H_2O$ 0,001 g
- $CaCl_2$ (1M) 0,1 ml
- Trace elements 3 ml
- Selenium tungsten solution 3 ml
- Vitamin solution I 2 ml
- Vitamin solution II 6 ml
- H_2O up to 1000 mL

3.1.3 Buffer

Potassium phosphate buffer, 0,02 M, pH= 7,0.

- KH_2PO_4 1 M
- K_2HPO_4 1 M

61.5 ml K_2HPO_4 (1 M) and 38.5 ml KH_2PO_4 (1 M) were mixed. 10 ml of this solution was diluted with water to final volume 500 ml.

3.1.4 Bacteria

- *Pseudomonas aeruginosa* strain 1
- *Pseudomonas aeruginosa* strain 2
- *Pseudomonas aeruginosa* strain 3
- *Pseudomonas putida* strain KT2440

3.2 Methods

3.2.1 Growth curve

Strains of *Pseudomona aeruginosa* were inoculated with sterile inoculating loop, in flasks containing pre-prepared and sterilized liquid minimal medium (MM). Stock solutions of 1,2-propane-diol (PD) and 3-chloro-1,2-propanediol (3-CPD) were prepared by diluting these compounds in deionized water. PD (0,5%), 3-CPD (0,5%) or both PD (0,25%) + CPD, (0,25%) were added in flasks as a carbon sources. Bacteria were incubated under 37°C in MaxQ™ 4000 Benchtop Orbital Shaker (Thermo Scientific, USA), with frequency of a rotation-250 revolutions per minute (rpm), overnight. Following day, optical density (OD) of each culture was measured at 600 nm with Synergy HT Multi-Mode Microplate Reader (BioTek, USA), and OD of the inoculum was adjusted to the standard value 0,01 (Eq. *1*). The volume of inoculum was transferred in flasks with appropriate volume (net volume remains constant) of MM and PD, CPD or PD+CPD, and incubated under the same conditions. The OD was checked at different time intervals and recorded. Using measured OD value, standardized growth curve of the organism was plotted (absorbance verses time).

$$OD_1V_1 = OD_2V_2$$

(Equation *1*)

where:

OD1 = OD of the broth culture, inoculated the previous day.

V1 = volume of this broth culture to be added to the inoculum

OD2 = OD of the inoculum (as a standard, this value was adjusted to 0.01)

V2 = volume of the inoculums (in this experiment, 50 ml)

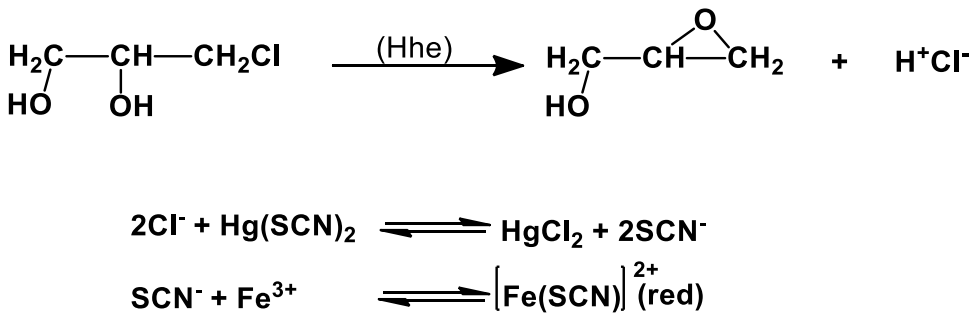
3.2.2 Determination of viable cell number

Testing bacteria culture, using only *Pseudomonas aeruginosa* strain 1 and PD (0,5%) as a sole carbon source, was prepared with the same protocol and apparatus as previous (growth curve) experiment. OD of the inoculum was also adjusted to the standard value 0,01. The continuing of experiment was different. Quantification of cells by viable plating technique was done. The spread plate method was used. In different time intervals OD at 600 nm was measured with Synergy HT Multi-Mode Microplate Reader (BioTek, USA). At the same time, aliquot (1 μl) from sample containing flask, was taken with sterile pipette and serially diluted with MM in the range of 10^{-4} to 10^{-9}. 0,1 ml of each of these dilutions was distributed evenly over the surface of pre-prepared LB agar plates and spread, using a sterile bent glass rod, onto the surface of agar plates. The plates were then incubated under 37°C in sample storage chamber. After 24 h, plates, with between 25 to 250 colony forming units (CFUs), were inspected and used to calculate the number of CFUs/ml. The result was expressed as logCFU/ml and plotted as log/CFU verses OD.

3.2.3 Florence and Farrar method for spectrophotometric determination of chloride ions in solution (43)

Spectrophotometric method for determination of chloride was used to test the release of chloride ions from 3-CPD. Such a release would prove the degradation of 3-CPD by bacteria

strains. The method was described by Florence and Farrar and is based on chemical reaction between Iron (III) nitrate nonahydrate, Mercury (II) thiocyanate and chloride. Chloride ion reacts with Mercury (II) thiocyanate and form a chloromercurate (II) complex ion, with the liberation of thiocyanate ions which then react with Iron (III) to give the familiar red color which could be detected spectrophotometrically at 460 nm (Eq. *2*).



(Equation *2*)

Two liquid reagents need to be formulated for this test: Mercuric (II) thiocyanate solution and Iron (III) nitrate solution.

Iron (III) nitrate solution: 15.1 g of $Fe(NO_3)_3$ X $9H_2O$ needs to be dissolved in 45 ml of 72% perchloric acid ($HClO_4$) (for our experiment we used 70% $HClO_4$) and diluted to 100 ml with water. This solution is 0.375 M in Iron (III), and 5.25 M in perchloric acid.

Mercury (II) thiocyanate: Saturated solution in ethanol (EtOH) needs to be prepared.

Recommended procedure for measuring, is to pipette a 20 ml aliquot of the sample, containing less than 50 μg of chloride, into a 25 ml volumetric flask. Then add 2.00 ml of Iron (III) nitrate reagent, 2.00 ml of mercury (II) thiocyanate reagent, dilute to volume with distilled demineralised water and mix. Absorbance against a reagent blank at 460 nm after 5 min needs to be measured. Calibration curve should be done for evaluation of the results (43).

For our experiments we prepared calibration curve by diluting different weights of chloride ions (using NaCl) in water. Before diluting, the calculation of appropriate NaCl weights was done.

Mm(NaCl)= 58,44 g/mol , Mm(Cl)= 35,45 g/mol

$w(Cl^-)$= 0,606

In 1 μg NaCl there is 0,606 μg Cl^- ions. To get 1 μg/ml of Cl^- ions 1,648 μg of NaCl should be taken and diluted in 1 ml of water.

Stock solution of Cl^- ions with concentrations 10 μg/ml was prepared by diluting 0,1648 mg of NaCl in 10 ml of water and stock solution of Cl^- ions with concentrations 100 μg/ml was prepared by diluting 1,648 mg of NaCl in 10 ml of water. Concentrations 0,1 mg/l, 0,2 mg/l, 0,3 mg/l, 0,4 mg/l, 0,5 mg/l, 0,6 mg/l, 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 6 mg/ml, 7 mg/ml, 8 mg/ml, 9 mg/ml were prepared by diluting appropriate volume of 10 μg/ml stock solution with appropriate volume of water. Concentrations 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml were prepared by diluting appropriate volume of 100 μg/ml stock solution with appropriate volume of water.

When preparing the calibration curve (OD versus chloride ions concentration), to 1 ml of each dilution of chloride ions, 100 μl Iron (III) nitrate reagent and 100 μl mercury (II) thiocyanate reagent was added. Such solution was mixed with pipette. After 5 minutes OD was measured at 460 nm with Synergy HT Multi-Mode Microplate Reader (BioTek, USA).

3.2.4 Procedures of bacteria preparation for the determination of chloride ions release

Due to the optimization of the method few different procedures have been used for preparation of bacteria.

Procedure 1

Bacteria were inoculated with sterile inoculating loop in flask containing appropriate media and appropriate concentration of 3-CPD. The OD of the inoculum was adjusted to the standard value 0,01 (adjustment procedure is described above). Media and concentrations,

that were used, are described in chapter "RESULTS". Flask with bacteria was then incubated in MaxQ™ 4000 Benchtop Orbital Shaker (Thermo Scientific, USA) under specific conditions including modification of temperature and frequency of a rotation. In time intervals, samples were taken out of flask with pipette. In few experiments OD of samples was measured with Synergy HT Multi-Mode Microplate Reader (BioTek, USA) at 600 nm to see the growth of bacteria. For detection of chloride ions release, samples were transferred in to Eppendorf tubes and centrifuged with Eppendorf Centrifuge 5418 with maximum frequency of a rotation (14000 rpm) for 3 minutes. 10 µl of supernatant was taken out with pipette and diluted 1:100 (v/v) with water. Reagents for chloride determination were added and after five minutes OD was measured with Synergy HT Multi-Mode Microplate Reader (BioTek, USA) at 460 nm. pH was regularly checked with Universal Indicator (Panreac, pH Indicators in reels). Charts OD versus time were made in the end.

Procedure 2

Bacteria were grown on LB media overnight. Next day late phase *Pseudomonas* strains were taken out from flask with pipette, transferred in 50 ml centrifuge tubes and centrifuged with Beckman GS 6R centrifuge (Beckman Coulter, USA) with rotation frequency 3750 rpm for 10 minutes. Supernatant was discarded and bacteria were 2 times washed with media- with pipette, media was added to precipitated bacteria, bacteria were resuspended by using Snijders Press-to-Mix shaker 34524 (Gemini BV, Netherland) and centrifuged again. After that bacteria were resuspended in the same media and 3-CPD was added as a sole carbon source. This was followed by incubation under 37°C in sample storage chamber. In different time intervals chloride ions release was measured, with the same protocol as described under "PROCEDURE 1" above, and charts were made. pH was also regularly checked with Universal Indicator (Panreac, pH Indicators in reels).

Procedure 3 – enzyme induction

This procedure was similar as described in Mamma D. et al (30). Bacteria strains were inoculated by transferring cell suspension with inoculating loop from stock culture to 250 ml Erlenmeyer flask containing 50ml pre-prepared and sterilized MiM. Glucose 5,0 g/l was added and then, cells were incubated in MaxQ™ 4000 Benchtop Orbital Shaker (Thermo Scientific, USA) under 30°C and with rotation frequency 250 rpm for 24 h. Next day this preculture was used to prepare a culture. In 500 ml Erlenmeyer flask, 100 ml of MiM, glucose 2 g/l and 3-CPD 250 mg/l, were added. 10% (v/v) of above mentioned preculture

was inoculated in Erlenmeyer flask. Flask with bacteria was incubated in MaxQ™ 4000 Benchtop Orbital Shaker (Thermo Scientific, USA) under 30°C and with rotation frequency-250 rpm for 72 h. After 72 h, bacteria were transferred in 50 ml centrifuge tubes and centrifuged with Beckman GS 6R centrifuge (Beckman Coulter, USA) with rotation frequency 3750 rpm for 15 minutes. Centrifuge tubes were weighed before adding the media with bacteria and after supernatant removal. Bacteria were than washed and resuspended in 0,02 M phosphate buffer to obtain a 1 g dry cell weight/l. 20 ml of cell-suspension was transferred in to 25 ml flasks. Different amounts of 3-CPD, from pre-prepared 3-CPD stock solution, were added in flasks containing cell-suspension, and placed on MaxQ™ 4000 Benchtop Orbital Shaker (Thermo Scientific, USA) under 30°C and with rotation frequency 100rpm. Thereafter in different time intervals chloride ions release was measured and charts were made with the same protocol as described under "PROCEDURE 1" above. pH was also regularly checked with Universal Indicator (Panreac, pH Indicators in reels).

4. RESULTS

4.1. Growth of *Pseudomonas aeruginosa* strains

We have inoculated three different strains of *Pseudomonas aeruginosa* isolated from human gut in test tubes with liquid minimal media (MM) in the presence of 1,2-propanediol (PD, 0,5%), 3-chloro-1,2-propanediol (3-CPD, 0,5%) and in the presence of both (PD, 0,25% + 3-CPD, 0,25%), as a carbon sources, at 37°C. We determined bacterial growth spectrophotometrically at 600 nm. All *Pseudomonas* strains grew in the presence of PD (Figure 9). The strain 1 grew faster in the presence of PD and reach maximum at 7,5 h, followed by strain 3 (10 h) and strain 2 (more than 10 h) (Figure 9). In presence of PD+3-CPD bacteria grew slower, when compared to only PD (data not shown). With only 3-CPD, bacteria grew very slowly (data not shown). Maximal growth was achieved at 0.6 optical density (OD) at 600 nm (Figure 9). On the base of these results we selected strain 1 for further analyzes.

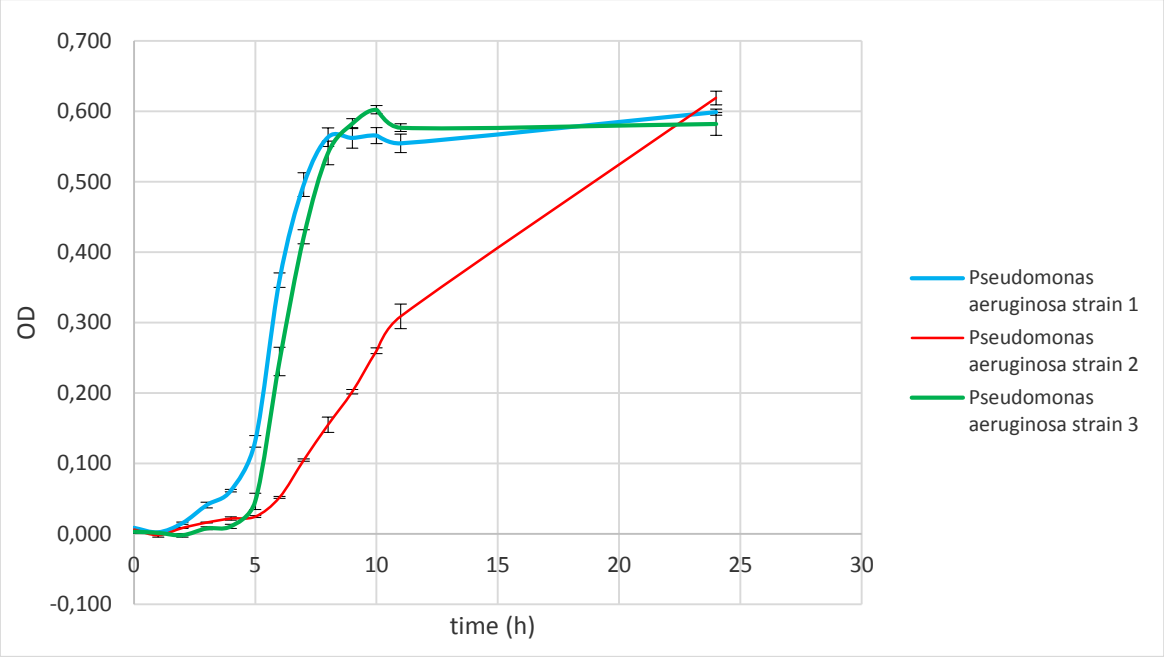


Figure 9. Determination of the growth of *Pseudomonas aeruginosa* strains. Three different strains grown in MM and the presence of 0,5% PD. OD was detected at 600 nm.

4.2. Determination of viability of P*seudomonas aeruginosa* strain 1

We evaluated the capacity of *Pseudomonas aeruginosa* strain 1 to form colonies. The bacteria were cultivated in liquid MM with PD (0,5%) at 37ºC. At several time points, OD was measured and bacteria were diluted and spread on petri dishes with Luria-Bertani media (LB). After 24 h, CFU were counted and expressed as logCFU/ml (Figure 10).

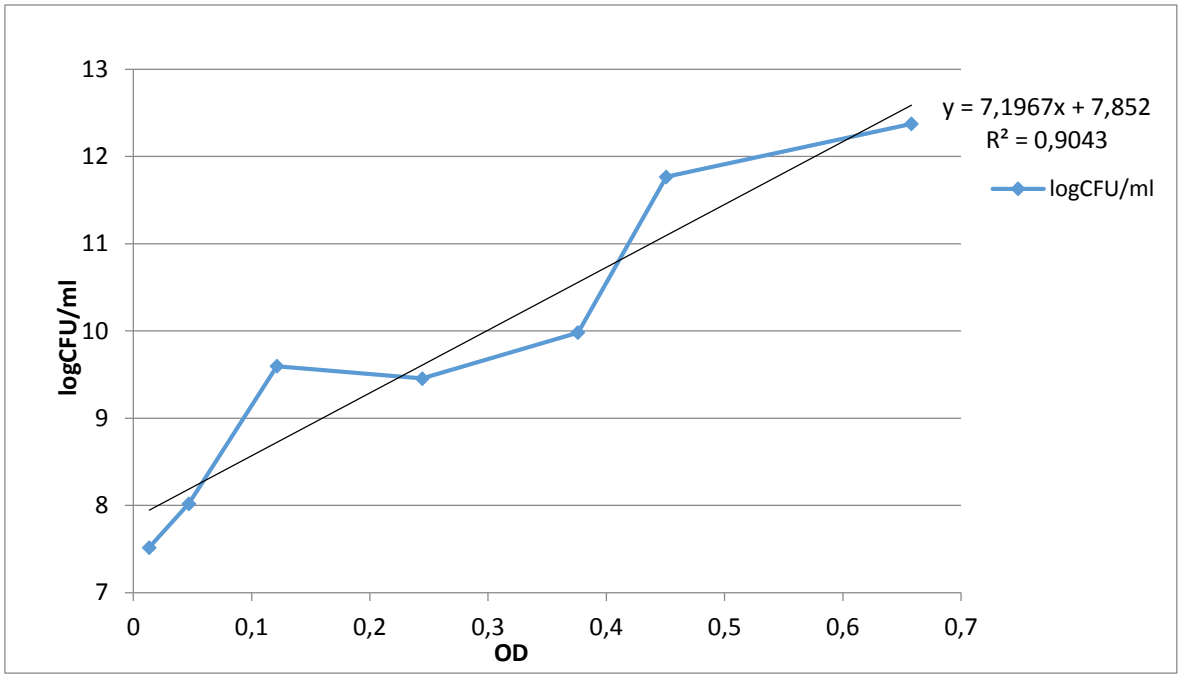
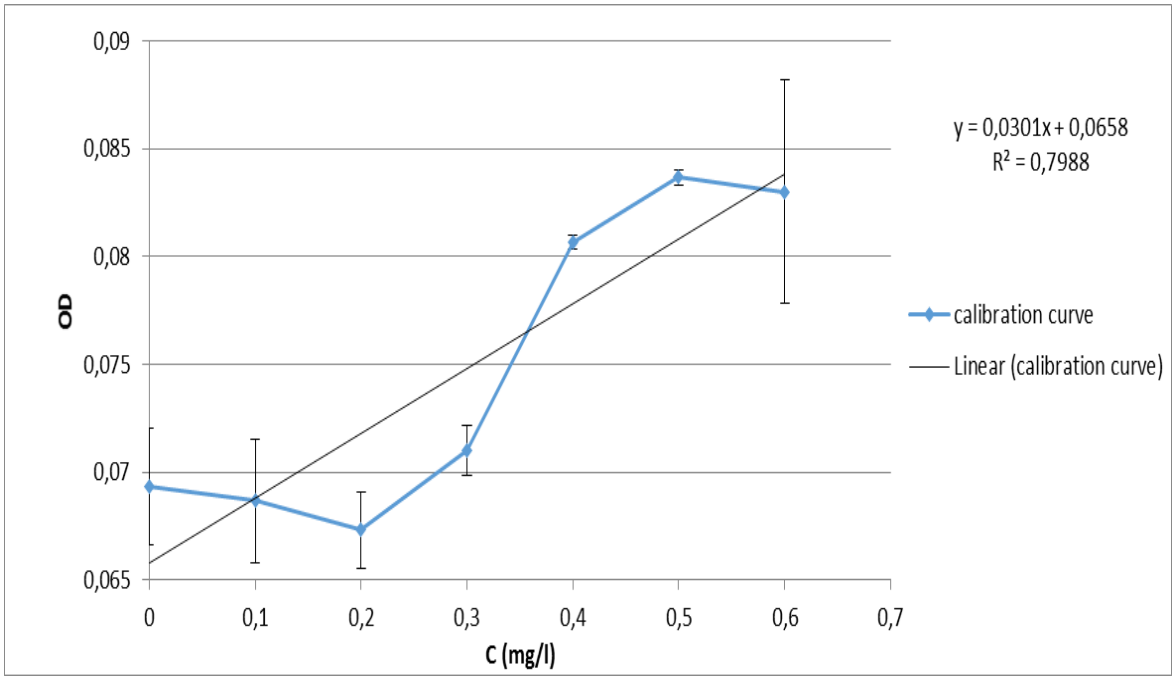


Figure 10. Estimation of logCFU/ml as a function of OD for *Pseudomonas aeruginosa* strain 1. Strain 1 bacteria were diluted and spread on petri dishes with LB after cultivation in liquid MM with PD (0,5%). CFU were counted after 24 h. OD was detected at 600 nm.

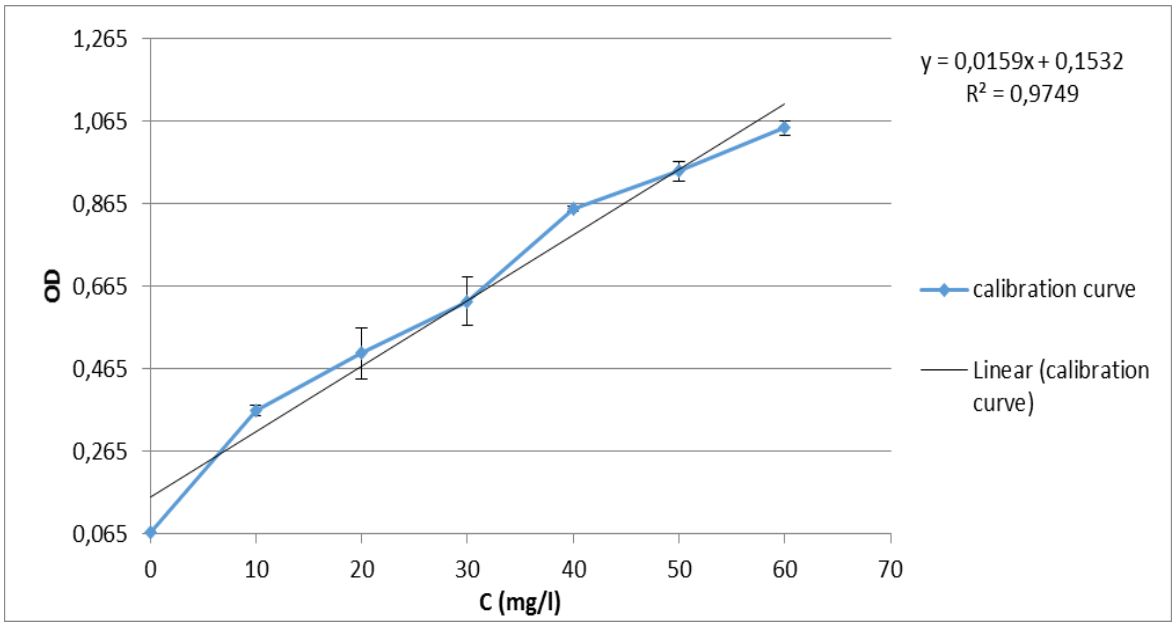
4.3. Spectrophotometric determination of chloride ions in solution

We have previously determined that *Pseudomonas* strains were able to grow slowly on 3-CPD which was a sole carbon source in growth media indicating ability to degrade it. The use of carbon structure requires previous release of chloride ions. To test this release, the method of Florence and Farrar was used (43). A calibration curve was generated with different chloride ions concentrations, and the absorbance was measured spectrophotometrically at 460 nm. The results showed that linearity was achieved between 0 mg/l and 10 mg/l of chloride ions (Figure 11C).

A


0,09
0,085
0,08
0,075
0,07
0,065
OD
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
C (mg/l)
y = 0,0301x + 0,0658
R² = 0,7988
calibration curve
Linear (calibration curve)

B


1,265
1,065
0,865
0,665
0,465
0,265
0,065
OD
0
10
20
30
40
50
60
70
C (mg/l)
y = 0,0159x + 0,1532
R² = 0,9749
calibration curve
Linear (calibration curve)

C

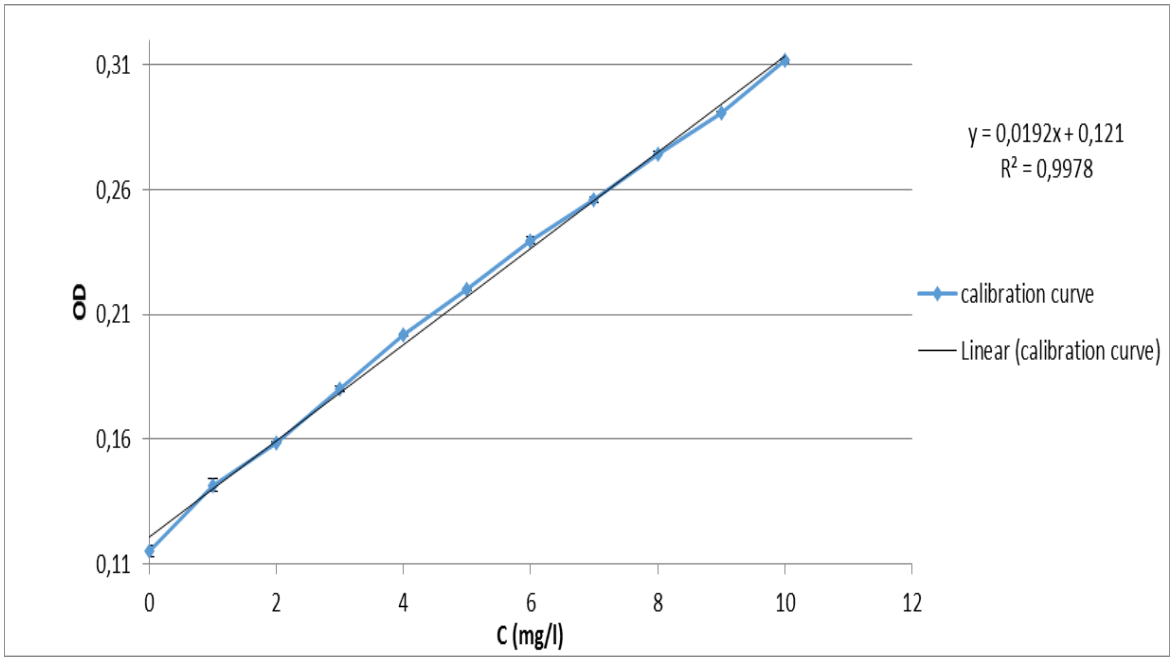


Figure 11. Calibration curve for chloride ions. A; between 0 mg/l and 0,6 mg/l of chloride ions. B; between 0 mg/l and 60 mg/l of chloride ions. C; between 0 mg/l and 10 mg/l of chloride ions. Standard solutions were prepared with diluting NaCl in water. OD was detected at 460 nm.

4.4. Determination of chloride ions release by *Pseudomonas aeruginosa* strain 1

The release of chloride ions from 3-CPD was determined (following procedure 1) in the best growing *Pseudomonas aeruginosa* strain, namely strain 1. The strain was cultured in liquid MM with 0.5% of 3-CPD as a sole carbon source at 37ºC. Strain 1 grew slowly on 3-CPD (Figure 12) while releasing a very small amount of chloride ions. (Figure 13).

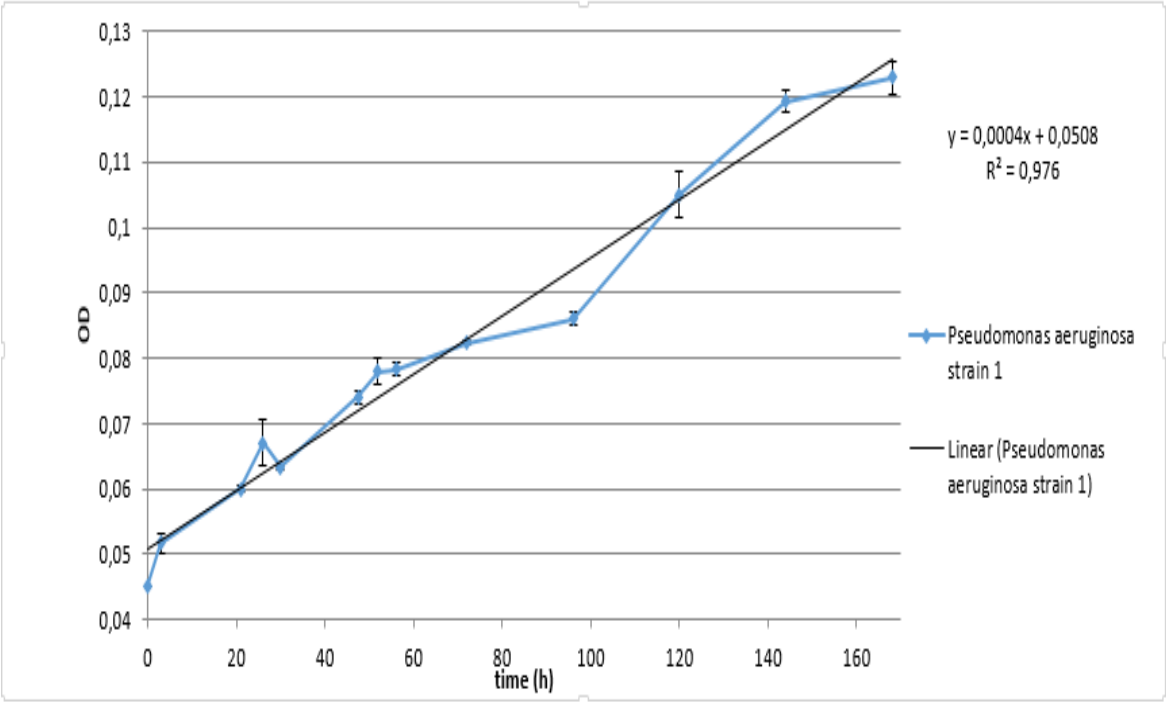


Figure 12. Determination of the growth of *Pseudomonas aeruginosa* strain 1. Strain 1 grown in MM and the presence of 0,5% 3-CPD. OD was detected at 600 nm.

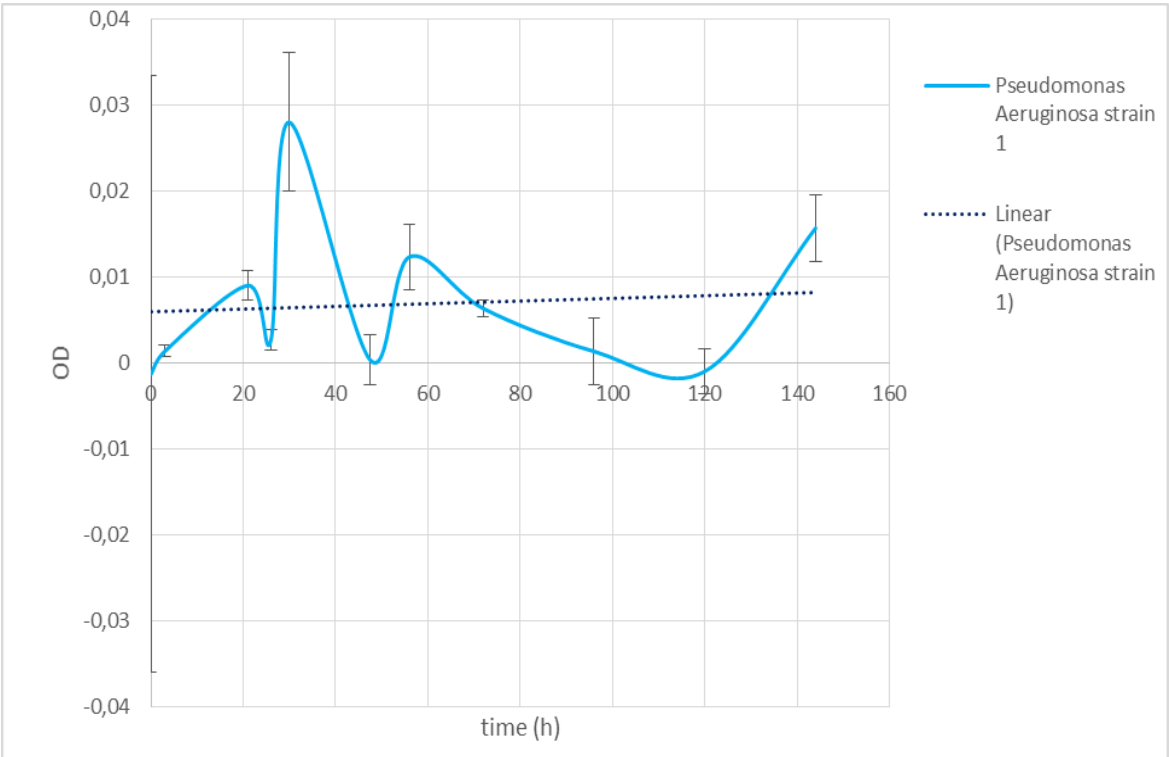


Figure 13. Release of chloride ions by *Pseudomonas aeruginosa* strain 1. Strain 1 was grown in MM and the presence of 0,5% 3-CPD. Chloride was measured with modified procedure of Florence and Farrar method. OD was detected at 460 nm.

4.5. Chloride ions release by *Pseudomonas aeruginosa* strain 1, grown under higher concentrations of 3-CPD

Pseudomonas aeruginosa strain 1 was grown this time under higher concentrations of 3-CPD (1000 mg/l and 2000 mg/l) as a sole carbon source and the release of chloride ions was measured (again following procedure 1) (Figure 14). The results showed that chloride ions release could not be measured, possibly due to the presence of NH_4Cl in culture minimal media.

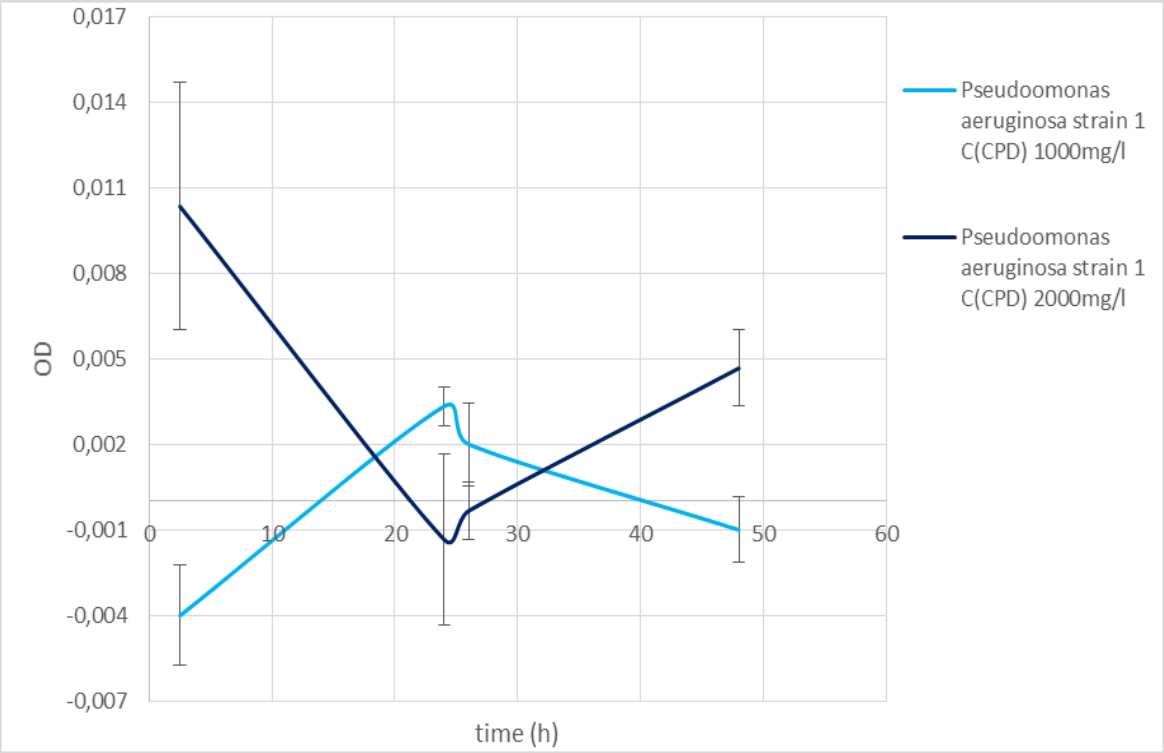


Figure 14. Release of chloride ions by *Pseudomonas aeruginosa* strain 1 grown under higher 3-CPD concentrations. Strain 1 was grown in MM and the presence of 1000 mg/l and 2000 mg/l 3-CPD. Chloride was measured with modified procedure of Florence and Farrar method. OD was detected at 460 nm.

4.6. Chloride ions release by *Pseudomonas putida* KT2440, grown under higher concentrations of 3-CPD

Pseudomonas putida KT2440 with the presence of three putative chlorohydrolases shows the ability for hydrolysing chloride substituents from chloroorganics (30, 42). This strain was used as a positive control in our experiments.

The strain was grown in liquid M9 with different concentrations of 3-CDP (following procedure 1). Our results showed that this strain did not release any significant amount of chloride ions from 3-CPD (Figure 15).

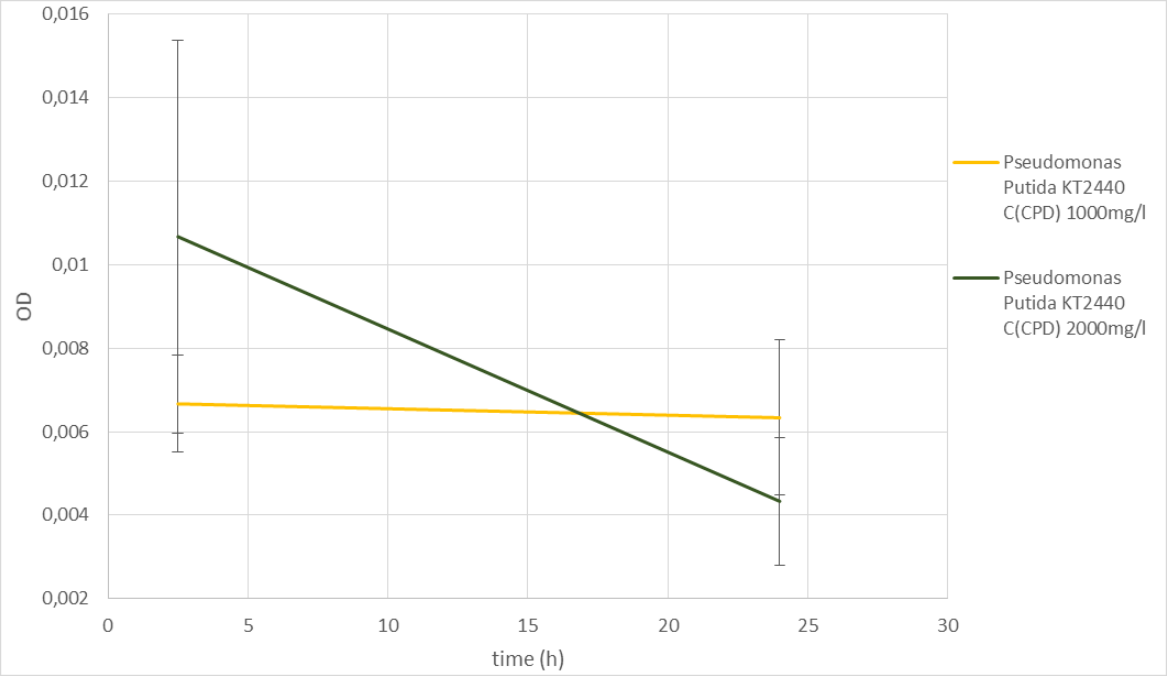


Figure 15. Release of chloride ions by *Pseudomonas putida* strain KT2440 grown under higher 3-CPD concentrations. Strain KT2440 was grown in M9 and the presence of 1000 mg/l and 2000 mg/l 3-CPD. Chloride was measured with modified procedure of Florence and Farrar method. OD was detected at 460 nm.

4.7. Chloride ions release by *Pseudomonas putida* KT2440 and *Pseudomonas aeruginosa* strain 1, grown under lower concentrations of 3-CPD

The release of chloride ions by *Pseudomonas putida* KT2440 and *Pseudomonas aeruginosa* strain 1 was investigated. The experiment was performed with lower concentrations of 3-CPD (following procedure 1). Our results showed that both strains did not release significant amounts of chloride ions (Figure 16).

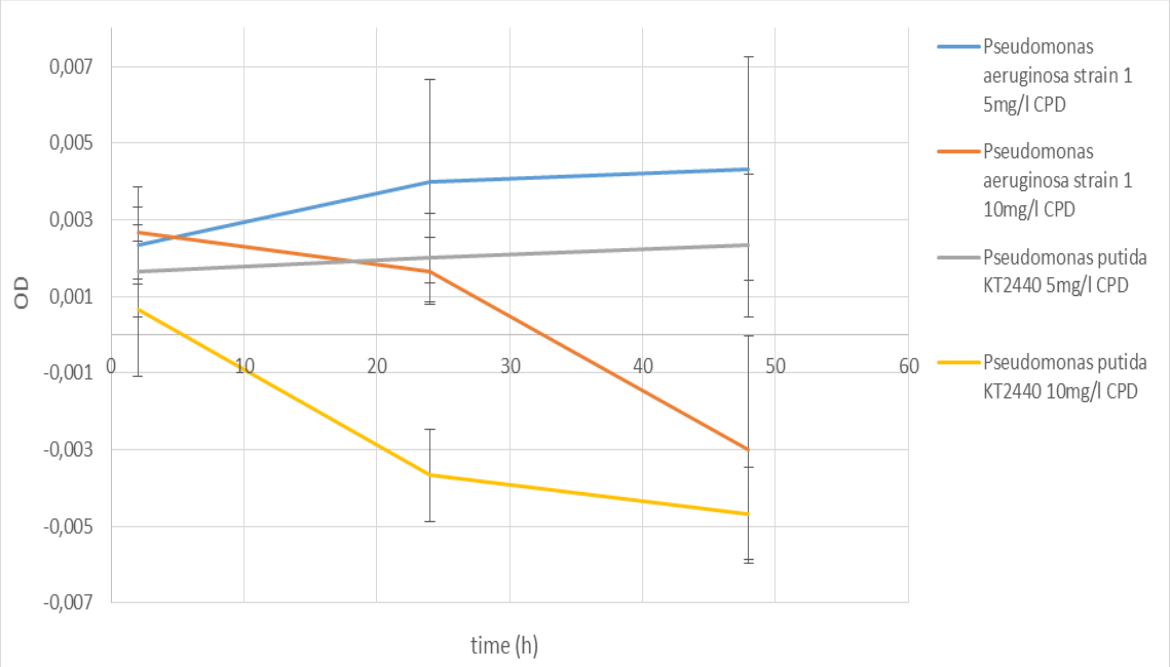


Figure 16. Release of chloride ions by *Pseudomonas putida* strain KT2440 and *Pseudomonas aeruginosa* strain 1 grown under lower 3-CPD concentrations. Strain KT2440 and strain 1 were grown in M9 and the presence of 5 mg/l and 10 mg/l 3-CPD. Chloride was measured with modified procedure of Florence and Farrar method. OD was detected at 460 nm.

4.8. Chloride ions release by late phase *Pseudomonas putida* KT2440 and *Pseudomonas aeruginosa* strain 1, exposed to different concentrations of 3-CPD

As previously shown, the strains did not release significant amounts of chloride ions when grown on 3-CPD as a sole carbon source. However, both strains may have the enzymatic machinery needed for chloride ions release from 3-CPD. To test this hypothesis, late phase *Pseudomonas* strains grown on LB media were resuspended in M9 with 3-CPD and chloride ions release was measured (following procedure 2). Besides, the minimal media was prepared without chloride ions. Our results showed that both strains did not release significant amounts of chloride ions (Figure 17).

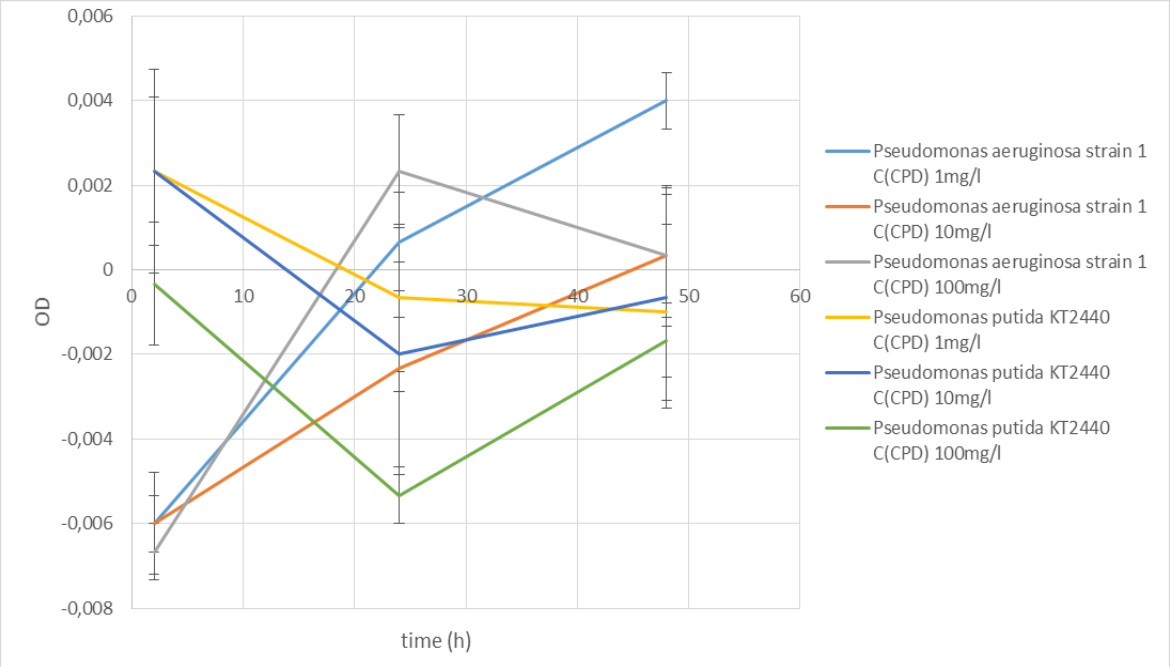


Figure 17. Release of chloride ions by late phase *Pseudomonas putida* strain KT2440 and *Pseudomonas aeruginosa* strain 1. Strain KT2440 and strain 1 were grown in LB overnight and later resuspended in M9 with 1 mg/l, 10 mg/l and 100 mg/l of 3-CPD. Chloride ions release was measured with modified procedure of Florence and Farrar method. OD was detected at 460 nm.

4.9. Chloride ions release by late phase *Pseudomonas putida* KT2440 and *Pseudomonas aeruginosa* strain 1 exposed to different concentrations of 3-CPD after enzyme induction

Effective dehalogenation may require the induction of haloalcohol dehalogenase in *Pseudomonas* strains. For this experiment, the strains were grown in mineral media containing glucose (5 g/l). After 24 h, we subcultured in mineral media (MiM) with glucose (2 g/l) and 3-CPD (250 mg/l). After 72 h, bacteria were centrifuged and resuspended to obtain a 1 g dry cell weight/l. 3-CPD was added (5 mg/l and 200 mg/l) and chloride ions release was measured (the whole experiment was done following procedure 3). Our results showed that chloride ions were effectively released by both strains (Figure 18). At 5 mg/l 3-CPD, there was an initial release of chloride ions. At 200 mg/l 3-CPD, release of 3-CPD was significant after 200 h.

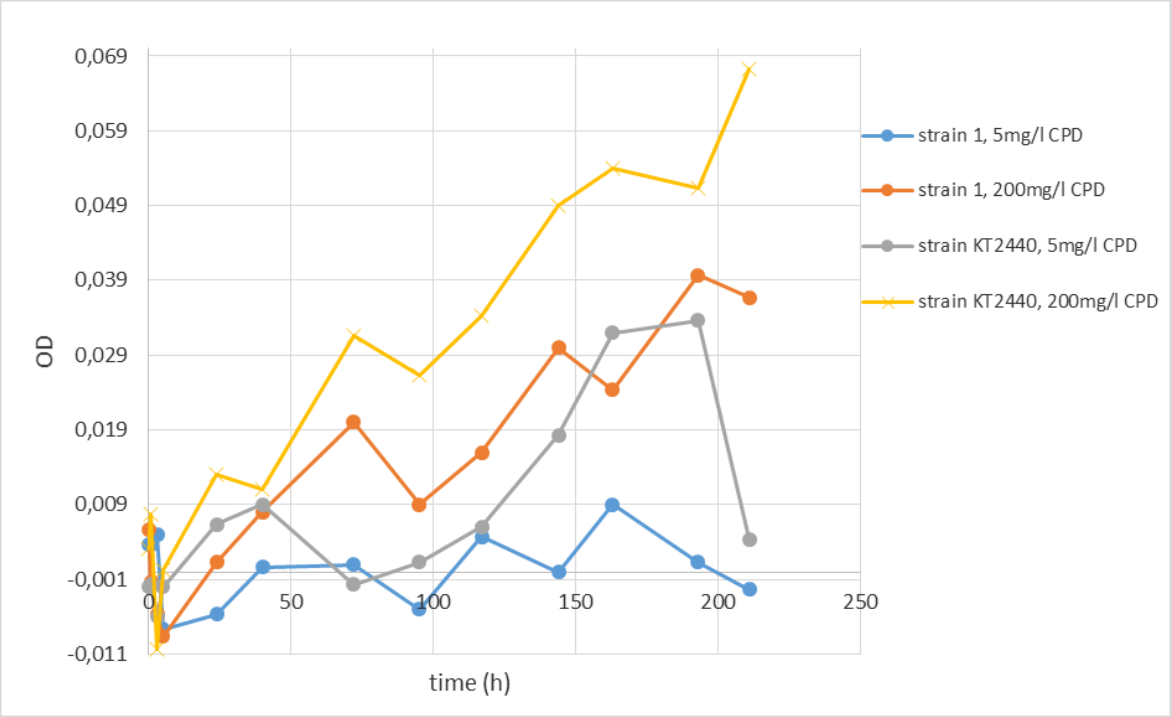


Figure 18. Release of chloride ions by *Pseudomonas putida* strain KT2440 and *Pseudomonas aeruginosa* strain 1 after enzyme induction. Strain KT2440 and strain 1 were grown in MiM with glucose (5 g/l) for 24 h, than in MiM with glucose (2 g/l) and 3-CPD (250 mg/l) for 72 h, than quantity of bacteria that obtain 1 g dry cell weight/l were resuspended in 0,02 M phosphate buffer (pH 7) with 5 mg/l, and 200 mg/l 3-CPD. Chloride ions release was measured with modified procedure of Florence and Farrar method. OD was detected at 460 nm.

5. DISCUSSION

The research work behind this master thesis was performed at the Biomedical Research Center (CIBM) in Granada, Spain. The research has been done within a project which analyses the differences in microbiota metabolism from the distal guts of lean and obese adolescents and their functional capabilities (37). There are few research works describing soil bacteria capabilities for dehalogenation of 3-CPD. No study so far has described any human gut bacteria capabilities for dehalogenation of 3-CPD.

5.1. Growth and determination of viable cell number of *Pseudomonas aeruginosa* strains

The bacteria used in this research were previously isolated from human fecal samples. We can see that all strains of bacteria were able to grow on PD as a sole carbon source. However, strain 1 grew faster, reaching its maximum at 7.5 h. Since other strains grew more slowly (strain 3- 10 h and strain 2 more than 10 h), strain 1 was selected for further analyses. The lag phase, necessary for RNA synthesis, enzymes and other proteins for living in new environment lasted approximately 2 h and it took about 5 h to take over log phase, to achieve stationary phase (Figure 9). The logarithm number of bacteria at specifically OD could be read out of the Figure 10. For estimation of strain 1 population, we used a plate counting method coupled to measuring optical density at 600 nm with a spectrophotometer. Correlation curve was very specific for each strain, because the light scattering of cells depends not only on cell number, but also on size, shape, granularity and other individual factors. When reaching 0,6 OD, there were approximately 10^{12} CFU/ml of culture media. The number of bacteria varied according to the first order kinetics in the exponential phase. We can calculate the constant of exponential growth rate (k) experimentally from the graphs using following equation (Eq. *3*):

$$k = \frac{(logN_2 - logN_1)2{,}303}{t_2 - t_1}$$

(Equation *3*)

where:

logN is logarithm number of bacteria at specifically time t.

The rate of exponential growth of bacteria culture may also be expressed as generation time t_g (Eq. *4*). It is the time interval necessary for the cells to divide.

$$t_g = \frac{ln2}{k}$$

(Equation *4*)

where:

k is constant of exponential growth rate.

5.2. Standard curve of chloride ions

To determinate Cl^- ions, with spectrophotometric method, as a consequence of bacterial degradation of 3-CPD, the standard curve of chloride ions was prepared. Our results showed that linearity was achieved with chloride ions concentrations between 0 mg/l and 10 mg/l (Figure 11 C). This was confirmed by calculation of determination coefficient of linear regression (R^2) which is 0,9978. This value indicated an excellent goodness-of-fit of chloride ions determination. When chloride ions concentration was between 0 mg/l and 60 mg/l or between 0 mg/l and 0,6 mg/l, R^2 value decreased (Figure 3A and Figure 3B). The linearity area shown in our experiment fits with recommendation for calibration curve preparation protocol (43). With 10 mg/l of chloride ions in the media, an OD 0,3 was achieved.

5.3. Determination of chloride ions release by *Pseudomonas aeruginosa* strain 1

The purpose was to grow the best growing strain, *Pseudomonas aeruginosa* strain 1, in 0,5% 3-CPD as unique carbon source and test the dehalogenation reaction by a putative halohydrin hydrogen-halide lyase (haloalcohol dehalogenase, Hhe) activity (44). Strain 1 grew very slowly in the presence of 3-CPD as a sole carbon source, indicating its ability to degrade it. In 160 h OD 0,12 was achieved (Figure 12). When growing *Pseudomonas aeruginosa* strain RW41 with 4-chlorobenzensulfonate, it took 144 h to achieve an OD higher than 0,1 (45). Figure 13 shows that chloride ions concentration slightly increased in media with strain 1.

In order to improve chloride ions release, a new experiment was prepared. Strain 1 was grown in higher 3-CPD concentrations, 1000 mg/l and 2000 mg/l. No significant increase in chloride ions release was measured (Figure 14). We reasoned that the presence of NH_4Cl in culture minimal media might mask chloride ions release. Normally when crystals of ammonium chloride are dissolved in water the compound of NH_4Cl decompound in to component ions (Eq. *5*). Cl^- ions could interfere our measures and reduce sensitivity.

$$NH_4Cl \xrightarrow{H_2O} NH_4^+{}_{(aq)} + Cl^-{}_{(aq)}$$

(Equation *5*)

5.4. Chloride ions release by *Pseudomonas putida* KT2440

Pseudomonas putida KT2440 was used as a positive control. Although strain KT2440 has the necessary enzymes for hydrolyzing chloride substituents in chloro-organics (42), we couldn't obtain significant chloride ions release (Figure 15). In these experiments, we used the high 3-CPD concentrations previously tested (42) but we could not reproduce chloride ions release. It is known that microorganisms may suffer from substrate inhibition. High concentrations of halogenated compounds can inhibit growth and their degradation (30).

5.5. Chloride ions release by *Pseudomonas putida* KT2440 and *Pseudomonas aeruginosa* strain 1

The next experiment was performed with lower concentrations of 3-CPD as a sole carbon source. When testing degradation ability of haloalcohols by different bacteria isolated from freshwater sediment, 5 mM carbon sources successfully supported growth of bacteria strains, one of them was a *Pseudomonas* spp. strain (44). Our results showed that chloride ions release was not detected neither in presence of strain 1 nor in the presence of strain KT2440, when using 5 mg/l and 10 mg/l 3-CPD as a sole carbon source (Figure 16).

Growing on 3-CPD as a sole carbon source did not produce significant chloride ions release. Since *Pseudomonas* strains on minimal media with different 3-CPD concentrations did not

produce chloride ions, we tested whether late phase bacteria growing on LB rich media and then resuspended on 3-CPD minimal media may have dehalogenation activity. Figure 17 illustrates our results. Again we could not detect significant release of chloride ions.

5.6. Enzyme induction

Bacteria usually do not express metabolic pathways in the absence of its substrate. As a response to changes in the composition of the environment, bacteria can increase the production of needed enzymes. A successful removal of halogenated organic compounds was proven when using specific conditions including growing bacteria in media supplemented with 5,0 g/l glucose, subculturing in mineral media (MiM) with glucose (2 g/l) and 3-CPD (250 mg/l) and finally exposing culture of bacteria to different concentrations of 3-CPD (30). We tested these conditions (Figure 18). In contradiction to former results, shown on Figures 12-17, we detected an increase in chloride ions release. In presence of high 3-CPD concentrations (200 mg/l), both strains, strain 1 and KT2440, released a higher quantity of chloride ions in the same time interval, when compared to 5 mg/l 3-CPD media. It is possible that enzyme induction was achieved and our *Pseudomonas aeruginosa* strain 1 could dehalogenate 3-CPD.

6. CONCLUSION

During our work we tested whether *Pseudomonas aeruginosa* strains, previously isolated from human feces, were able to grow in the presence of 3-CPD as a sole carbon source. The results showed that strain 1 grew *in vitro* in the presence of 3-CPD as a sole carbon source, indicating its ability to degrade it. It is true, that the growth was very slowly, but the fact that there was bacterial growth inspired us for further research. In the presence of PD, which is the possible product of 3-CPD degradation, bacteria grew well, reaching maximum at OD 0,6 after 7,5 h and forming 10^{12} CFU/ml. The use of modified Florence and Farrar's spectrophotometric method for chloride ions determination, as result of 3-CPD degradation, was good decision because of simplicity and low costs. Detection of chloride ions was achieved but, for more accurate results, alternative methods, such as gas chromatography (GC) or high-pressure liquid chromatography (HPLC) should be tested.

Our assumptions, that special conditions are required for enzyme expression, have proven to be true. *In vitro* growth of bacteria under 0,5% of 3-CPD as a sole carbon source indicated the ability of strain 1 to degrade 3-CPD, but did not result in significant chloride ions release. Late phase bacteria experiment, including growth of bacteria on LB media overnight and later inoculation in media with 3-CPD as a sole carbon source, did also not result in increase of chloride ions release. The enzyme induction protocol was more successful. For the enzyme induction, media was supplemented with 5,0 g/l of glucose, later bacteria were subcultured in media with both carbon sources glucose (2 g/l) and 3-CPD (250 mg/l) and finally culture was exposed to different concentrations of only 3-CPD. Exposure of bacteria to specific conditions resulted in increased concentrations of chloride ions. Our work also showed a correlation between 3-CPD concentrations and chloride ions release. Exposure of bacteria to 3-CPD and in the same time availability of alternative carbon source (glucose in our case) could protect the enzyme from substrate inhibition and support the expression of haloalcohol dehalogenases. This enzyme should, on the basis of our results, be expressed by *Pseudomonas aeruginosa* spp isolates and should degrade the environmental, food and water pollutant- 3-CPD.

More experiments must be performed to prove this hypothesis. As mentioned above, a more sensible method for qualitative and quantitative evaluation of chloride ions release should be optimized in order to calculate kinetic parameters (V_{max}, K^m, k_{cat}) of enzymatic activity.

The next step should be preparation and optimization of a method for protein evaluation. Electrophoresis of cell-free extracts could be used to visualize the induction of dehalogenases amongst other proteins. Such visualization could help to determine the type of enzyme.

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