Biocatalytic reduction using *Clostridium sporogenes*

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ABSTRACT

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Chemical modification of organic compounds by biological systems is a powerful method to synthesize valuable chemicals, as it provides good yield, requires mild conditions and shows different types of selectivity. Hundreds of reactions and enzymes have been identified, but there is still a great need to discover new types of biocatalysts. Anaerobic bacteria possess unique metabolic pathways which make them a perfect target for screening for new industrially useful enzymatic activities.

Clostridium sporogenes, a Gram-positive obligately anaerobic bacterium, was previously shown to catalyze two types of biocatalytic reduction, the reduction of amides to amines in the presence of hydrogen and hydrogenation of unsaturated carbon-carbon double bonds of activated alkenes.

The first aim of this project was to optimize the reaction conditions for reduction of benzamide to benzylamine. Previous attempts to optimize this reaction were unsuccessful because of the poor method for benzylamine detection. The method was improved by changing the equipment and the conditions of analysis, and the benzylamine detection limit was significantly enhanced. Unfortunately, even this did not allow progress in the project since no benzylamine formation was observed. Further optimization of the reaction such as testing cells from different physiological stages of growth and improvement of the hydrogen delivery system is required.

The second reaction provided by *C. sporogenes*, reduction of nitroalkenes, was proposed to be catalysed by enoate reductase enzyme, however previous attempts to purify this enzyme were unsuccessful. As an alternative strategy to identify the enzyme, *C. sporogenes* enoate reductase knock out mutants were prepared. The mutants were not able to reduce cinnamic acid, an intermediate of phenylalanine fermentation in the Stickland reaction, which had been proposed to be converted by enoate reductase. Moreover, mutated *C. sporogenes* showed decreased biomass production in media containing different energy sources such as glucose or phenylalanine. The mutants were not able to reduce *(E)*-1-nitro-2-phenylpropene, which confirmed the hypothesis that enoate reductase was responsible for reduction of this substrate. On the other hand, reduction of *(E)*-1-phenyl-2-nitropropene was still possible, but with a lower yield compared to the wild type strain. It is possible that this substrate was reduced by two independent enzymes, and that enoate reductase is one of them. In the future the gene encoding enoate reductase will be cloned and the enzyme will be overexpressed in *E. coli* to produce a potential new biocatalyst for industrial biotransformation. Moreover, the mutant will be used for purification of the enzyme responsible for reduction of *(E)*-1-phenyl-2-nitropropene.

DECLARATION

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ABBREVIATIONS

ATP - adenosine-5'-triphosphate

C=C - carbon-carbon double bond

CoA - coenzyme A

DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

e.g. - *exempli gratia* (Latin), for example

ee. - enantiomeric excess

FAD - flavin adenine dinucleotide

FMN - flavin mononucleotide, riboflavin-5'-phosphate

i. e. - *id est* (Latin), that is

mM - millimolar concentration

NAD(H) - nicotinamide adenine dinucleotide (reduced)

NADP(H) - nicotinamide adenine dinucleotide phosphate (reduced)

sp. - species

TBB - *tert*-butylbenzene

TBME - *tert*-butyl methyl ether

1. Introduction

Chemical modification of compounds by biological systems is a powerful and still not thoroughly explored method to synthesize valuable chemicals. Biocatalysts are usually more efficient than chemical catalysts, require mild reaction conditions and provide high stereoselectivity (Faber, 2004). Hundreds of biotransformations have been described in the literature until now but there is a great need for discovery of new enzymes and reactions that could be used in the pharmaceutical, chemical and textile industry (Faber, 2004).

Clostridium sporogenes is an anaerobic bacterium with unique properties among living organisms. From the 1930s it has been known that it is a proteolytic organism that can grow in media without carbohydrates using amino acids as the energy source only, although the presence of carbohydrates can stimulate the growth (Stickland, 1934; Lovitt *et al.*, 1987b). *C. sporogenes* can obtain energy from an unusual reaction between two amino acids, where one of them acts as the electron donor and the second as the electron acceptor (Stickland, 1934). The unusual fermentation pathways make *C. sporogenes* an excellent source of proteins showing new enzymatic activities that can be used in biotransformations.

It was found that *C. sporogenes* is an excellent biocatalyst for organic reductions in synthesis of high value compounds. Whole cell or crude protein extracts were used for reduction of activated alkenes, imines, amides and aromatic nitro compounds (Angermaier and Simon, 1983a; Angermaier and Simon, 1983b; Dipeolu *et al.*, 2005; Fryszkowska *et al.*, 2008; Dipeolu *et al.*, 2009; Fryszkowska *et al.*, 2010).

Biocatalytic reduction of benzamide to benzylamine was described by Dipeolu *et al.* in 2005. In contrast to chemical reactions that require drastic conditions (high temperature and pressure) and strong metal catalysts (lithium aluminium hydride, borane, copper chromite or Pd/Re/zeolite), reduction of amide was performed in mild conditions (Dipeolu *et al.*, 2005). One aim of this project is the optimization of reaction conditions and analytical methods to improve the method described by Dipeolu, which will allow the purification and characterization of the enzyme responsible for this type of catalytic activity in the future.

The second reaction provided by *C. sporogenes* that is useful for biotransformations is hydrogenation of activated alkenes. Reduction of α,β-unsaturated carboxylic acids, esters, lactones, carboxaldehydes, ketones and nitro compounds is

important in chiral synthesis, as it gives up to two new asymmetric carbons in the structure. Many reductases responsible for this type of biotransformation have been described in the literature (enoate reductases, 'Old Yellow Enzyme' family, for a review see Stuermer *et al.*, 2007), but previous experiments showed that enzymatic activities that are present in *C. sporogenes* make this organism unique with respect to the unusual substrate range and stereoselectivity of the products (Fryszkowska *et al.*, 2008; Fryszkowska *et al.*, 2010).

Because previous work on purification of the enzyme or enzymes responsible for this type of bioreduction was not successful, a novel strategy using genetic engineering is proposed to characterize this enzyme activity. Based on the nucleotide sequence of enoate reductase from *C. botulinum* genome (a strain that is closely related to *C. sporogenes*), a genomic knock-out mutant was prepared using the ClosTron knock-out system (Heap *et al.*, 2007). The aim of this project is a full characterisation of the enoate reductase *C. sporogenes* knock-out mutant, its physiology and usefulness in biotransformations.

The main purpose of this project is to use *C. sporogenes* as a novel biocatalyst, that can be used at preparative scale to provide industrially useful biotransformations in the future.

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2. Literature survey

2.1. Biotransformations

Biotransformation is a process where a biological system (a whole organism or an isolated enzyme) is used to make a chemical alteration on a chemical compound. In contrast to a biosynthesis, a xenobiotic biotransformation is used to modify a substrate that is completely alien to the natural reaction pathways in a particular organism. The more common approach, biosynthetically directed biotransformation, is used to transform an analogue of the natural substrate or intermediate. Both ways are valuable tools in research and commercial synthesis of chemicals. Biocatalysts can consist of a whole microorganism or its part. Also inducible or constitutive enzymes can be used in biotransformations (Leuenberger, 1984; Hanson, 1995).

People have been using biotransformations on a daily basis for several thousand years. The first evidence of biotransformation in the history of humans originates from Egypt, Babylon, China and Mexico where alcoholic drinks and vinegar were produced about 5000 years BC. Since then many applications using biocatalysis were described. The 20^{th} century gave considerable progress in the field of biotransformation, *e.g.* biosynthesis of vitamin C or sucrose inversion using invertase (Bornscheuer and Buchholz, 2005). Nowadays biotransformation techniques are mainly used in medicine, therapy and diagnostics, in detergent production and in the textile and starch industries (Bornscheuer and Buchholz, 2005).

2.2. Advantages and disadvantages of biocatalysis

There are many advantages of using biocatalysts in chemical reactions. Biocatalysts are more efficient that chemical catalysts, so only a small amount of the biocatalyst needs to be used in the reaction (from mole percentage of 0.1-1% for chemical catalyst to 10^{-3}-10^{-4}% for biocatalysts). Furthermore, biocatalysts accelerate the reaction by a factor of 10^{8}-10^{10} (Faber, 2004).

Biocatalysts show different types of selectivities. They can modify a selective position in the molecule that is not possible to perform using chemical catalysts. Biocatalysts can be chemoselective, *i.e.* they modify a specific type of functionality and do not react with other sensitive groups. This makes the reaction cleaner and simplifies the downstream processing. They can also be regioselective and diastereoselective, *i.e.*

they distinguish the same type of functionalities located in different areas of a molecule. Biocatalysts are also enantioselective, *i.e.* the biocatalyst reacts with different enantiomers from a racemic substrate with different rates, or synthesizes only one product enantiomer from a prochiral substrate. As enzymes are not bound to their natural roles, they can catalyze a broad spectrum of reactions and accept also manmade substrates (Faber, 2004).

Unlike heavy metal catalysts, enzymes are also environmentally acceptable and biodegradable. They can act under mild conditions with pH usually from 5 to 8 and temperature range from 20 to 40°C. As enzymes are compatible with each other, a multienzyme system can be used where different reactions are carried in a cascade of processes allowing more efficient synthesis. Enzymes can also work in organic solvents, not only in water (Faber, 2004).

On the other hand, the narrow operational parameters of enzymes can make some limitations with the biocatalysis. A change of pH, temperature or salt concentration can deactivate the enzyme. However, although enzymes are also active in other solvents than water, biocatalytic reactions in organic media are usually less efficient.

To become active, enzymes require their natural cofactors such as ATP, NADH or NADPH. Molecules that carry the redox potential or chemical energy are rather unstable and expensive. However they can be replaced by recycling systems based on cheaper and stable energy substrates (Faber, 2004).

Last but not least, the substrate-inhibition and product-inhibition phenomena require that every enzymatic reaction should be optimized with respect to proper substrate addition or product extraction, allowing the reaction to reach the maximum rate of conversion (Faber, 2004).

Taking into consideration all advantages and disadvantages mentioned above, the reaction can be performed using whole microorganisms or more or less purified enzyme. A whole cell biotransformation (fermentation process) requires expensive equipment, but gives a large amount of biomass starting from cheap carbon sources. The biotransformation of growing or harvested cultures can be performed without expensive cofactors and gives high enzymatic activities, but may cause problems with side reactions. Biotransformation using isolated enzymes gives very high enzyme activities and simple apparatus can be used, but it is necessary to provide a suitable cofactor recycling system (Faber, 2004).

2.3. Enzymes and biotransformations in organic chemistry

At present about 3200 different enzymes have been identified. They are divided into six classes depending on their type of catalytic activity (Table 2.1.).

Table 2.1. Classification of enzymes (modified from Faber, 2004).

Class	Number	Reaction type
Oxidoreductases	1	Oxidation and reduction reactions, oxygenation of C–H, C–C, C=C bonds, removal and addition of hydrogen group equivalents.
Transferases	2	Transfer of groups.
Hydrolases	3	Hydrolytic formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydries, glycosides.
Lyases	4	Addition-elimination of small molecules on C=C, C=N, C=O bonds.
Isomerases	5	Isomerization (racemization, epimerization, rearrangement).
Ligases	6	Formation-cleavage of C–O, C–S, C–N, C–C bonds.

In the area of organic chemistry the most widely used class of enzymes are hydrolases, especially lipases, proteases and esterases (Faber, 2004). The methodology of using this class is established very well and collections of hydrolases are commercially available.

For biocatalytic reduction, isolated dehydrogenases or whole cell biocatalysts are used, mainly to reduce ketones to secondary alcohols for chiral synthesis. Enzymatic oxygenation is used for mono- and di-hydroxylation, epoxidation and sulfoxidation. The limitation of using oxidoreductases in biotransformations is the necessity of using cofactor recycling systems. The exceptions are hydrogen peroxide-dependent enzymes, where the substrate can be constantly delivered to the reaction (Faber, 2004).

Other classes of enzymes are not as commonly used as hydrolases and oxidoreductases, but research methods for some enzymes such as aldolases, transketolases, glycosidases or glycosyl transferases are being developed. The

importance of these enzymes becomes more significant as new proteins are made available by using genetic engineering (Faber, 2004).

2.4. Biocatalytic reductions in chiral synthesis

The first attempt to use a biocatalyst to reduce an organic compound was performed in 1911 by Neubauer (cited in Simon *et al.*, 1985). A growing culture of baker's yeast was used to convert phenylglyoxylic acid into mandelic acid. Since then, hundreds of articles were published describing biocatalytic reductions of ketones to chiral alcohols and hydrogenation of unsaturated activated alkenes (Simon *et al.*, 1985).

Biocatalytic reduction can be used to synthesise chiral products by stereospecific hydrogenation of unsaturated compounds (Fig. 2.1.).

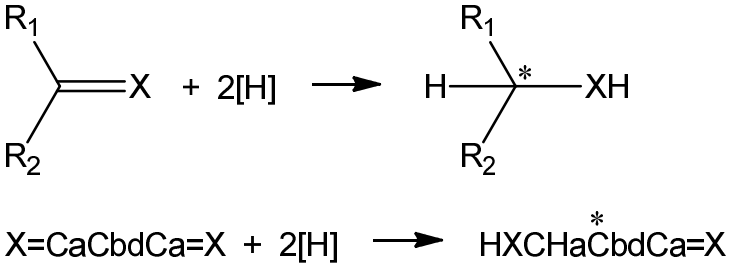


Fig. 2.1. Reduction reactions catalysed by dehydrogenases used in the chiral synthesis. X = O, N–, –C– (Simon *et al.*, 1985).

A few approaches are used to synthesise a chiral substrate using reductases: asymmetric reduction of aldehydes and ketones resulting in primary and secondary alcohols, reduction of C=N double bonds and reduction of activated C=C double bonds in α,β-unsaturated carboxylic acids, esters, lactones, carboxaldehydes, ketones and nitro compounds (Simon *et al.*, 1985; Faber, 2004).

There are a number of routes for reducing organic compounds depending on the type of electron donor and the type of electron transport from the donor to the acceptor (Simon *et al.*, 1985). One of the most commonly used is reduction using NAD(P)H produced in a series of enzyme catalyzed reactions from glucose or other carbohydrates. Whole cells of bakers yeast or bacteria are used as catalysts for NAD(P)H regeneration as well as the final reduction. The method was used for reduction of acetaldehyde and pyruvate using glucose or reduction of nitrobenzene to aniline by *C. sporogenes* using

fructose, starch and glucose (Simon *et al.*, 1985, Dipeolu *et. al*, 2009). This system depends on a whole metabolic pathway to regenerate the cofactor. The disadvantage of this method is the low productivity number caused by side reactions. A better, simpler solution is direct NAD(P)H formation in a two enzyme system, where NAD(P) in converted into NAD(P)H in a single irreversible reaction coupled to the transformation. Formic acid, hydrogen or ethanol can serve as the electron donors where whole cells of different bacteria such as *Pseudomonas ovalis*, *Arthrobacter* sp. or *Clostridium kluyveri* are used for the reduction of organic compounds (Simon *et al.*, 1985). The direct formation of NAD(P)H can be improved by using natural or artificial mediators. In some pathways the electron donor can be used to reduce a natural cofactor (e.g. ferrodoxin), that converts NAD(P) to NAD(P)H inside the cells. Reaction can be further accelerated by using artificial mediators, such as methyl viologen or methyl-4,4'-bipyridinium dication, although the interaction with cellular metabolism is unclear. Using theses systems some α,β-unsaturated carboxylates, nitroalkenes and 2-oxocarboxylates were reduced by whole cells of Clostridia, *Ruminococcus productus, Acetobacterium woodi* and *Proteus vulgaris* (Simon *et al.*, 1985).

In some cases a successful biotransformation was obtained by using two purified enzymes system that were previously isolated from different sources. By using the cofactor regeneration system NAD(P) was converted to NAD(P)H by a NAD-dependent dehydrogenase, *e.g.* using isopropanol or cyclohexanol as substrates (Simon *et al.*, 1985; Kroutil *et al.*, 2004), and then used by the final reductase to reduce the substrate of biotransformation. The most common system uses glucose-6-phosphate dehydrogenase that oxidizes glucose-6-phosphate at the expense of NAD or NADP. The product of the reaction, 6-phosphoglucolactone spontaneously hydrolyzes to phosphogluconate and shifts the equilibrium of the reaction towards the product and improves the NAD(P)H regeneration (Simon *et al.*, 1985; Faber, 2004).

Because some electron mediators can be reduced electrochemically (*e.g.* methyl viologen) biotransformations can also be performed in electrochemical cells. Electrochemically reduced methyl viologen was used to convert NAD(P) to NAD(P)H using viologen-dependent NAD(P)H reductase and then NAD(P)H was used by the final reductase to transform the substrate. Some enzymes, such as enoate reductase or 2-oxocarboxylate reductase, could also accept electrons directly from the electrochemically reduced mediator (Simon *et al.*, 1985).

The system of reduction using biocatalysts should be adjusted to the scale of biotransformation and to the specific properties of the reaction in respect to the substrate range and side products.

Biocatalytic reductions of unsaturated compounds are still being developed as they give a big contribution in chiral synthesis. There are many of tools for discovering new biocatalytic activities. In a directed evolution new enzymes with distinct features are created *in vitro* by changing the nucleotide primary structure of the enzyme encoding gene (Turner, 2003). In metagenomics (environmental and community genomics) microorganisms are tested by the direct extraction and cloning of their DNA followed by function-based or sequence-based screens (Handelsman, 2004). The third, traditional approach is screening of microbes from enriched environmental samples and testing them for useful biotransformations (Asano, 2002). As alternative, a more targeted approach can be used by testing microorganisms which are known to contain unusual enzymes and which grow by unusual metabolic processes.

2.5. Biotransformation using *Clostridium sporogenes*

Clostridium sporogenes is a Gram-positive rod-shaped obligately anaerobic bacterium. Differences in metabolism between aerobic and anaerobic organisms make these bacteria an excellent source of enzymes to catalyze unique chemical reactions.

The first experiments explaining how *C. sporogenes* obtains its energy and describing biotransformations (oxidation and reduction of amino acids) were performed in the early 1930s (Stickland, 1934). Until now over 370 research articles about *C. sporogenes* have been published.

The first report of biotransformation using *C. sporogenes* was published by Aries and Hill in 1970. They reported that enzymes isolated from strains of *C. sporogenes* and other Clostridia were able to oxidise 3α-, 7α- and 12α-hydroxyl groups in the steroid, cholic acid. The reactions were optimized by changing the pH of the reaction mixtures and characterized by their K_m values. Since then a number of other unusual reactions have been discovered (Aries and Hill, 1970).

The extracts of *C. sporogenes* reduced uracil to dihydrouracil in the presence of NADPH but not NADH (Hilton *et al.*, 1975). Cytosine was also metabolized to dihydrouracil indicating that the reduction was preceded by deamination (for structures

of substrates and product, see Fig. 2.2.). Other pyrimidines were reduced to the corresponding dihydro derivatives.

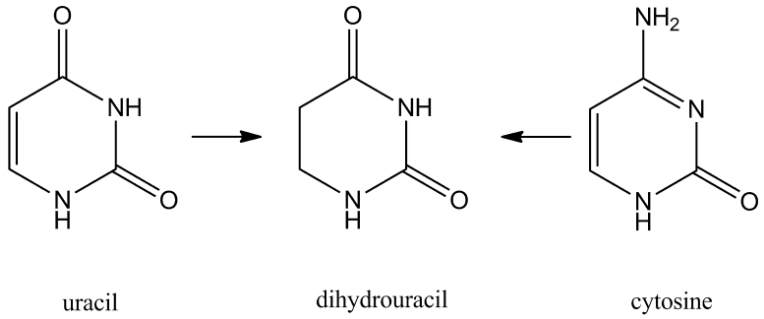


Fig. 2.2. Biotransformation of uracil and cytosine to dihydrouracil by *C. sporogenes*.

A further C=C bond reduction was discovered when seven strains of *C. sporogenes* isolated by Verhulst *et al.* in 1985 were found to reduce linoleic acid into transvaccenic acid (Verhulst *et al.*, 1985). The reaction followed in two steps, at first isomerization of the 12-*cis* double bond into 11-*trans* double bond followed by reduction of the 9-*cis* double bond (Fig. 2.3.). This is one of few examples of reduction of non-activated C=C bonds.

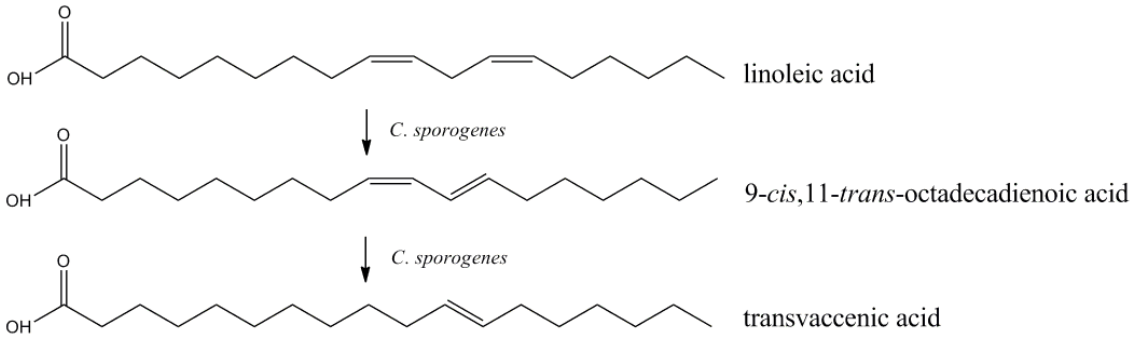
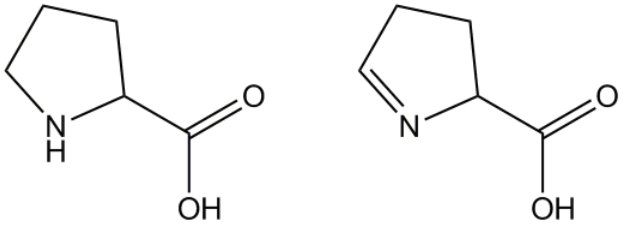


Fig. 2.3. Reduction of linoleic acid to transvaccenic acid by *C. sporogenes*. The intermediate of the reaction was 9-*cis*,11-*trans*-octadecadienoic acid.

C. sporogenes was also able to reduce proline to δ-aminovaleric acid and the imine, Δ'-pyrroline-5-carboxylic acid (PCA; Fig. 2.4.). The enzyme activities responsible for these types of bioreduction were purified and characterized by their isoelectric points. The proline dehydrogenase activity was dependent on nicotinamide adenine dinucleotide and L-proline whereas the Δ'-pyrroline-5-carboxylic acid reductase required L-Δ'-pyrroline-5-carboxylic acid and reduced nicotinamide adenine dinucleotide (Costilow and Cooper, 1978). Reduction of proline was also observed by

Lovitt *et al.* in 1986. The reaction was coupled to transmembrane proton translocation (Lovitt *et al.*, 1986).



proline Δ'-pyrroline-5-carboxylic acid

Fig. 2.4. Structures of proline and Δ'-pyrroline-5-carboxylic acid.

In 2004 a novel imine reductase activity was discovered using whole cell catalyst of *Acetobacterium woodii* and *C. sporogenes* (Li *et al.*, 2004). This type of catalysis could play an important role in α-chiral amine synthesis. A virtual library of imines was created by self-assembly of different amines (aniline and butylamine) and aldehydes (benzaldehyde and butanal). The reaction was started by application of H_2 (an electron donor for hydrogenation). Some samples were also treated with caffeate, inducer of enzyme responsible for C=C reduction suspected of mediating the imine reduction too. Two reduced products were detected in samples catalyzed by *Acetobacterium woodii* induced with caffeate (N-butylaniline and phenylbenzylamine) and one product in a reaction mixture of uninduced cells (N-butylaniline). Also, side products such as benzyl alcohol and benzoic acid were detected. These results suggested that *A. woodii* contains two different imine reductases – one of them is induced by caffeate and the second one is produced constitutively. Imine reduction using *C. sporogenes* requires further optimization of the biotransformation and analytical methods (Li *et al.*, 2004; Li, 2003, report, University of Manchester).

Dehalogenases are relatively rare enzymes responsible *e.g.* for microbial debromination of brominated ethenes. Cell free extracts of *C. sporogenes* showed debrominating activity in the presence of FMN and NAD(P)H (Kitamura *et al.*, 1999). (α-Bromoiso-valeryl)urea was reduced to (3-methylbutyryl)urea, but the reaction was inhibited by sodium arsenite or potassium cyanide. The reaction was proposed to proceed in two steps. At first flavins were reduced by flavin reductase in the presence of NAD(P)H. Then reduced flavins were used as an electron donor to reduce the (α-bromoiso-valeryl)urea (Kitamura *et al.*, 1999). In similar reaction conditions, cell free extracts of *C. sporogenes* reduced stilbene oxide and styrene oxide to the corresponding

alkenes (Kitamura *et al.*, 2000). There are no other precedents for this unusual reaction and normally epoxides are hydrolyzed by other organisms.

Amides also tend to be hydrolyzed to acids in normal metabolism. However, the first publication about biocatalytic amide to amine reduction was published by Dipeolu *et al.* in 2005. During oxidative respiration amide reduction seems not to be energetically favourable, whereas in anaerobic bacteria this reaction may play a role in ATP synthesis. It was shown that whole cells of *C. sporogenes* were able to reduce benzamide to benzylamine using H_2 as the electron donor (Dipeolu *et al.*, 2005). Benzylamine production was detected after 2 h with maximum rate of formation and reached a yield of 21% after 24 h. By that time 93% of substrate was consumed which suggested the presence of side reactions, such as benzamide hydrolysis. Benzamide reduction showed saturation kinetics increasing linearly up to 1 mM and reaching saturation at about 5 mM. The benzylamine production yield decreased exponentially from 73% at 0.25 mM to 1% at 20 mM benzamide. On the other hand the production yield of benzoic acid, product of benzamide hydrolysis, increased linearly at that range of substrate.

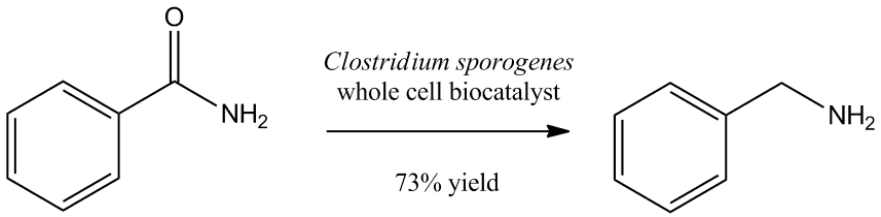


Fig. 2.5. Benzamide to benzylamine reduction by whole cell biocatalysts of *C. sporogenes* (Dipeolu *et al.*, 2005).

A two hour latency before benzylamine production and the fact that a part of substrate underwent unidentified side reactions could be explained by the presence of two step biotransformation: from amide to imine and from imine to amine, where side reactions acted at the imine intermediate stage.

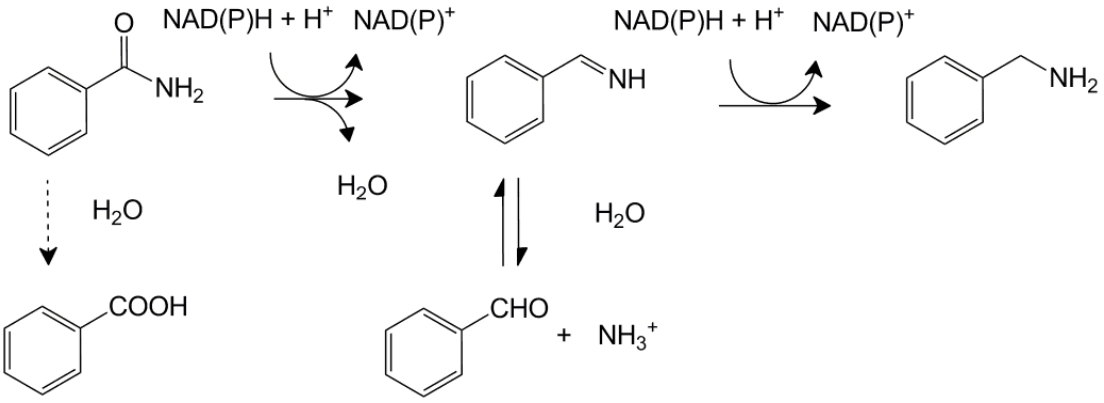


Fig. 2.6. Proposed reaction scheme for benzamide to benzylamine reduction with an imine intermediate (Dipeolu *et al.*, 2005).

Further work such as enzyme purification and elimination of side reactions is needed to test this biotransformation (Dipeolu *et al.*, 2005).

Another biotransformation observed in *Clostridium sporogenes* is reduction of C=C double bonds. The *Clostridium* extract can be used for enantioselective reduction of C=C double bonds of β,β-disubstituted and α,β-disubstituted nitroalkenes to nitroalkanes, very important chemical compounds in organic synthesis (Fig. 2.7.; Fryszkowska *et al.*, 2008).

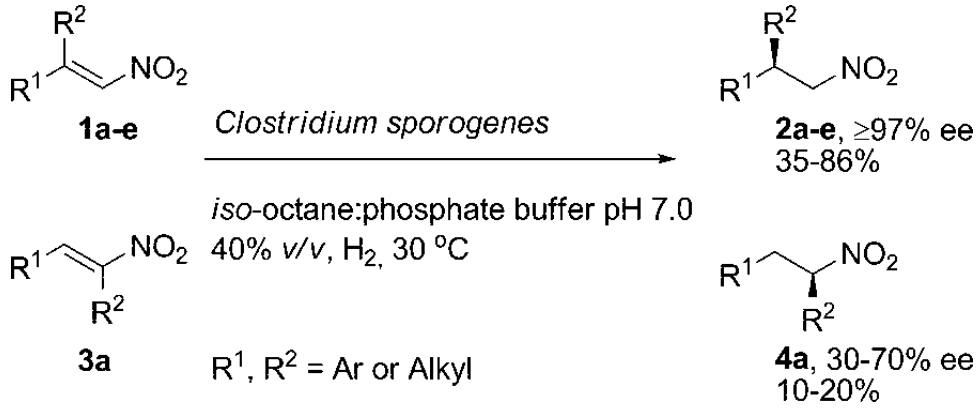


Fig. 2.7. The enantioselective reduction of C=C double bonds of β,β-disubstituted and α,β-disubstituted nitroalkenes (Fryszkowska *et al.*, 2008).

It was shown that the enoate reductase from anaerobic bacteria reduced α,β-unsaturated carboxylic acids, ketones, aldehydes and nitroalkenes with the reaction driven using H_2. The stereoselective reduction of aromatic and heteroaromatic β,β-disubstituted nitroalkenes was efficient and highly enantioselective in 35-86% yield

depending on the substrate. By contrast, α,β-disubstituted nitroalkenes were transformed in lower yield and their optical purity was low (Fryszkowska *et al.*, 2008).

C. sporogenes was also used to reduce aromatic nitro compounds. Angermaier and Simon used protein crude extracts to reduce the nitro group of *p*-nitrobenzoate. The reaction was catalyzed by the NADH-dependent reductase. During the purification process two different enzymatic activities were found to reduce aromatic nitro groups. The first reaction step led to the nitro radical anion with transfer of one electron (Angermaier and Simon, 1983b). It was also found that using whole cell biocatalysts nitrobenzene can be reduced to aniline with yields of 8% in aqueous ethanol (4% v/v) and 45% in a biphasic heptane/aqueous system.

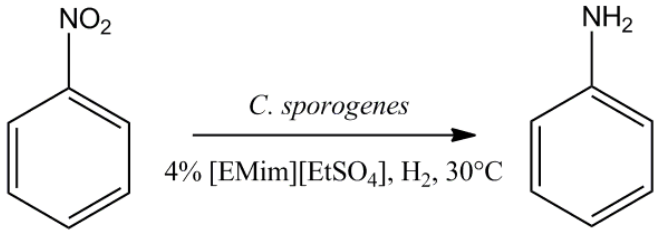


Fig. 2.8. The reduction of nitrobenzene to aniline by *C. sporogenes* (Dipeolu *et al.*, 2009).

The reaction could be improved by using water miscible ionic liquids as the solvent in the biotransformation, *e.g.* 1-ethyl-3-methyl imidazolium ethylsulfate ([EMim][$EtSO_4$] (Fig. 2.8.). However it inhibited growth by 58%, but allowed reduction of nitrobenzene with 79% yield (Dipeolu *et al.*, 2009).

C. sporogenes can also reduce aliphatic nitro compounds. Angermaier and Simon observed reduction of 2-nitroethanol by protein extracts in the presence of hydrogen, however the reaction rate was much smaller than for reduction of aromatic nitro compounds (Angermaier and Simon, 1983a)

All theses examples of biotransformations showed that *C. sporogenes* possesses a number of enzymes catalyzing unusual reactions. This can be explained by the specific fermentation routes in which *C. sporogenes* obtains the energy. To date very few of the enzymes have been isolated and characterized, and the scope to use them for preparative biotransformations has rarely been explored.

2.6. The Stickland reaction

One of the reasons that *C. sporogenes* may have great potential as biocatalyst is that it uses some very unusual metabolic processes to obtain carbon and energy for growth. Aerobic organisms use oxygen as the final functional hydrogen acceptor during oxidative deamination of amino acids. However, Stickland observed that *C. sporogenes* and other bacteria can obtain their energy from deamination between two amino acids acting as donor and acceptor of hydrogen respectively (Stickland, 1934; Nisman, 1954). *C. sporogenes* growing with amino acids which played a role as hydrogen donors reduced methylene blue and brilliant cresoylblue and, with amino acids which act as hydrogen acceptors reoxidized reduced phenosafranin and benzylviologen. It was shown that interaction of a donor and acceptor amino acid (*e.g.* alanine and glycine) results in mutual deamination as follows:

$$CH_3CHNH_2COOH + 2NH_2CH_2COOH + 2H_2O \rightarrow 3CH_3COOH + 3NH_3 + CO_2.$$

A number of proteolytic bacteria belonging to the family *Clostridiae* are able to metabolize amino acids by the way of the Stickland reaction. Some bacteria can ferment amino acids nonreductively such as *Diplococcus glycinophilus* (fermentation of glycine) and *C. propionicum* (fermentation of alanine and other amino acids; Nisman, 1954).

The metabolic pathway of L-phenylalanine fermentation was proposed (Fig. 2.9.). In the first step the amino group is transferred from phenylalanine to 2-oxoglutarate resulting in phenylpyruvate formation (Dickert *et al.*, 2000). In the oxidative branch phenylpyruvate is converted to phenylacetyl-CoA, and then ATP and phenylacetate are produced in the substrate level phosphorylation reaction. In the reductive branch phenylpyruvate is reduced to (R)-phenyllactate. Phenyllactate is then dehydrated to *(E)*-cinnamate followed by reduction to 3-phenylpropionate by enzyme called enoate (or cinnamate) reductase.

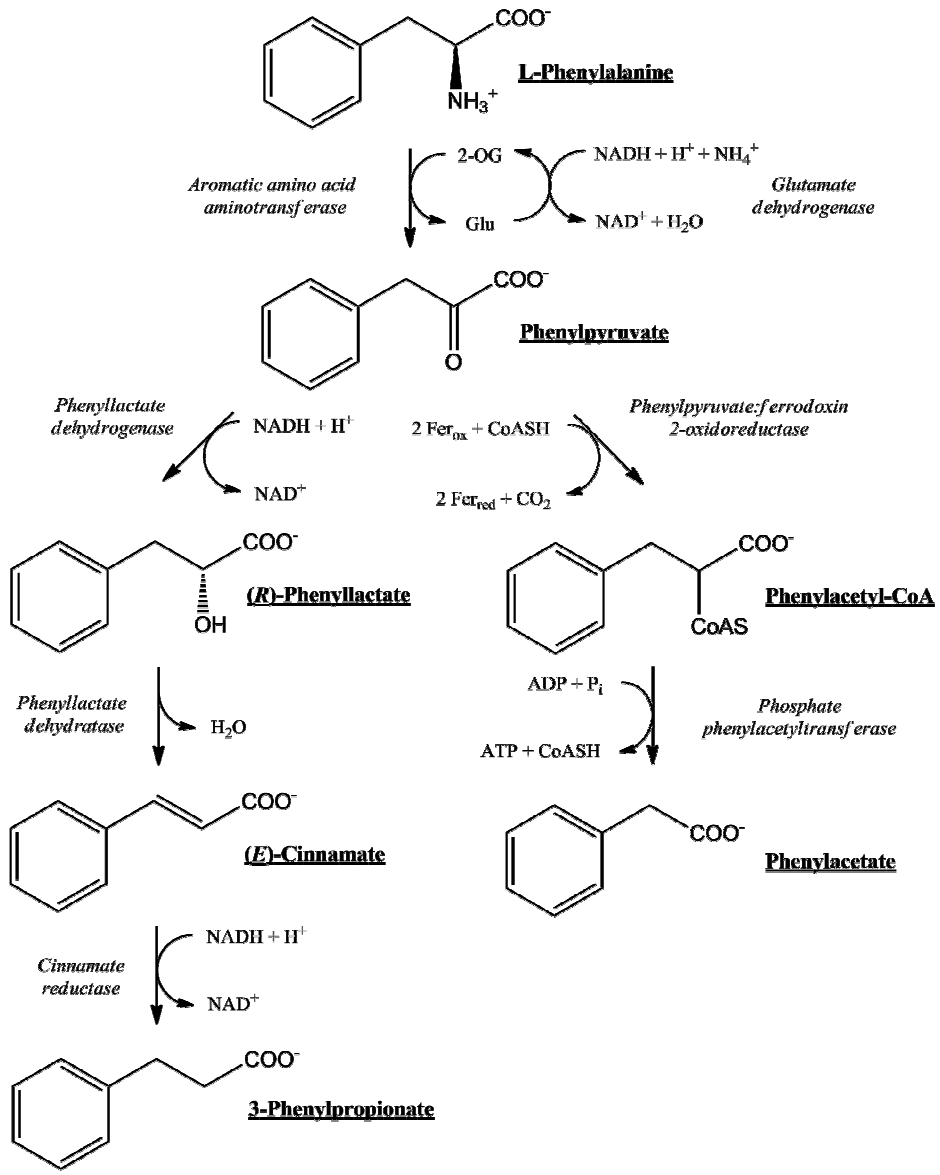


Fig. 2.9. L-phenylalanine fermentation by *C. sporogenes* (Dickert *et al.*, 2000).

It was also shown that amino groups from glycine, isoleucine, leucine, valine and phenylalanine were transferred to 2-oxo-acids as a result of transaminase activity (Bader *et al.*, 1982).

The enzymes of the Stickland metabolic pathway were identified by Dickert *et al.* in 2000. The enzyme complex responsible for the reversible *syn*-dehydration of (R)-phenyllactate to *(E)*-cinnamate was purified from *C. sporogenes* grown on medium supplemented with phenylalanine (Dickert *et al.*, 2000). The complex was composed of three subunits: FldA, FldB and FldC. The FldA subunit was not oxygen sensitive and showed the activity of cinnamoyl-CoA:phenyllactate CoA-transferase. Other subunits contained one [4Fe-4S] cluster and showed oxygen sensitive phenyllactate dehydratase

activity. FldA subunit was homologous to CaiB protein from *E. coli* that is involved in the metabolism of carnitine. The dehydration of phenyllactate proceeded in two steps, CoA-transfer from cinnamoyl-CoA to phenyllactate followed by the dehydration of phenyllactyl-CoA and regeneration of cinnamoyl-CoA.

Phenyllactate dehydratase activity required presence of ATP, $MgCl_2$ and the oxygen sensitive initiator protein FldI (Dickert *et al.*, 2002). Genes encoding all four proteins, FldA, FldB, FldC and FldI were identified and cloned. The gene cluster *fldAIBC* showed similarity to a family of CoA-transferases. FldI protein was overexpressed in *E. coli* and exhibited homodimeric structure containing one [4Fe-4S] cluster. In the oxidized state the protein showed ATPase activity that seemed to be essential for the dehydratase activity. FldBC showed high identity to 2-hydroxyglutaryl-CoA dehydratase (HgdAB) from *Acidaminococcus fermentas*. The *fldAIBC* gene cluster showed similarities to *hadAIBC* gene cluster from *C. difficile* and *hadABC,I* gene cluster from *C. botulinum* (Dickert *et al.*, 2002).

In the next step, the cinnamate is reduced to phenylpropionate by an enoate reductase. This is also coupled to ATP formation according to the reaction:

$$RCH{=}CHCOOH + 2H + ADP + P_i \rightarrow RCH_2CH_2COOH + ATP + H_2O.$$

Whole cells of *C. sporogenes* incubated with cinnamate under an atmosphere of hydrogen produced ATP from AMP, whereas the ADP level did not change. When cells were incubated under an atmosphere of nitrogen ATP was still formed, but with a 5-fold lower rate. ATPase inhibitors, N,N'-dicyclohexylcarbodiimide (DCCD) and carbonyl cyanide *p*-[trifluoromethoxy]-phenyl-hydrazone (FCCP), strongly inhibited ATP formation suggesting that ATP formation was coupled to the electron transport from hydrogen to cinnamate. ATP formation was only observed when freshly harvested cells with intact membranes were used for the assay. On the other hand, ATP synthesis coupled to reduction of 2-oxo acids was also observed with crude cell extracts or cells treated with ultrasound (Bader and Simon, 1983).

Whole cells of *C. sporogenes* reduced the proposed intermediates *(E)*-cinnamate, (2R)-phenyllactate, phenylpyruvate and L-phenylalanine to phenylpropionate. (2S)-phenyllactate was not a substrate showing that the enzyme catalysing the biotransformation was enantioselective. On the other hand assay using protein crude extracts gave only cinnamate reduction to phenylpropionate and decarboxylation of

phenylpyruvate to phenylacetate. 4-methyl-2-pentanoate, which may be derived from leucine, was a good substrate for the tested enzyme (Buhler *et al.*, 1980).

The enoate reductase may determine the substrate range of the Stickland fermentation. It was known that some species of *Clostridia* such as *Clostridium sporogenes*, *Clostriudium bifermentas* and toxigenic types A, B, D and F of *Clostridium botulinum* can produce hydrocinnamic and cinnamic acids when growing on L-phenylalanine (Moss *et al.*, 1970). In presence of mixtures of phenylalanine and isoleucine *C. sporogenes* reduced phenylalanine and oxidized isoleucine resulting in accumulation of 3-phenylpropionate and 2-methylbutanoate. When phenylalanine or isoleucine were incubated alone both of them were able to enter reductive and oxidative branches. Phenylpyruvate consumption in the oxidative branch was coupled to formation of ATP and phenylacetate formation. As *(E)*-3-methylpentenoate and *(Z)*-3-methylpentenoate are not substrates for enoate reductase it was postulated that this enzyme determined the behaviour of amino acids with respect to reductive and oxidative branches in Stickland reaction (Bader *et al.*, 1982).

2.7. Enoate reductases

The enoate reductase from *C. sporogenes* appears to be an unusual enzyme with several differences from classical enoate reductases. Enoate reductases, which are members of the flavin containing 'old yellow enzyme' family, catalyses the stereoselective reduction of activated alkenes in NAD(P)-dependent reaction that is extremely useful in chiral synthesis (Fig. 2.10.).

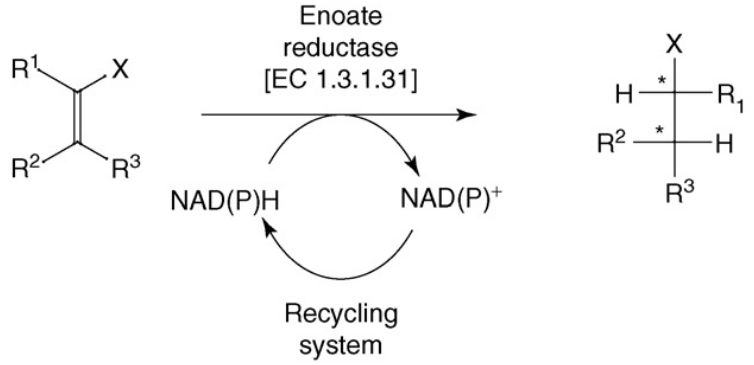


Fig. 2.10. Stereoselective hydrogenation of activated alkenes. X – electron withdrawing group such as keton, aldehydes, carboxylic acid, ester, anhydride, lactone, imide or nitro (Stuermer *et al.*, 2007).

Enoate reductases have been found in microorganisms (in bacteria and lower fungi) and in plants. They can reduce activated C=C bonds in ketones, aldehydes, carboxylic acids, esters, anhydrides, lactones, imides and nitro compounds. Enoate reductases are involved in many metabolic pathways such as enoyl-CoA reductase in fatty acid synthesis, morphinone reductase in morphinone biosynthesis, 12-oxophytodienoic acid reductase in synthesis of jasmonic acid and in the Stickland reaction in amino acid fermentation by anaerobic bacteria (Bühler *et al.*, 1980; Stuermer *et al.*, 2007). Enoate reductases were also found to reductively cleave nitroesters resulting in alcohol and nitrite, reduce aromatic nitro groups to amines and transform aromatic nitro compounds to non-aromatic Meisenheimer complex (Stuermer *et al.*, 2007).

The mechanism of asymmetric reduction by isoform OYE1 from *Saccharomyces carlsbergensis* was described by Kohli *et al.* in 1998 (Fig. 2.11.).

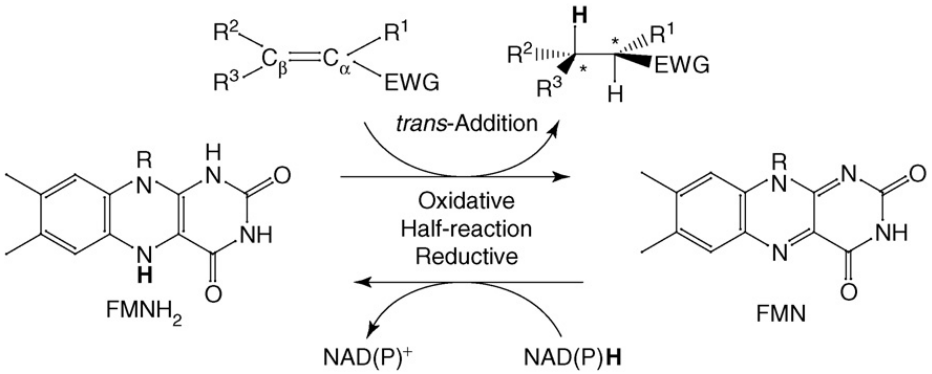


Fig. 2.11. The catalytic mechanism of enoate reductase (Stuermer *et al.*, 2007).

A hydride is derived from $FMNH_2$ and transferred to C_β in a stereoselective way. Then the tyrosine 196 adds a proton to C_α and recovers it from the solvent. The reduction is characterized by absolute stereoselectivity and proceeds in a *trans* fashion. When tyrosine 196 was mutated to phenylalanine, the enzyme was still able to bind ligands and to catalyse the reductive half-reaction (NAD(P)H oxidation to $NAD(P)^+$), but the oxidative half reaction was slower by 6 orders of magnitude (Kohli *et al.*, 1998; Stuermer *et al.*, 2007).

The clostridial enoate reductase was purified from *C. kluyveri* with 88-fold enrichment of its specific activity by fractionation and chromatography. Two different strains, *Clostridium kluyveri* and Crotonate strain La 1 (now known as *C. tyrobutyricum*), growing on medium supplemented with *(E)*-2-butenoate (crotonate)

were able to reduce *(E)*-2-methyl-2-butenoate to 2-methylbutyrate or *(E)*-cinnamate to 3-phenylpropionate in a NADH-dependent reactions (Fig. 2.12.). The stereochemical course of this biotransformation was different than the previously described reaction driven by butyryl-CoA dehydrogenases, so an occurrence of novel enzymatic activity was postulated (Tischer *et al.*, 1979).

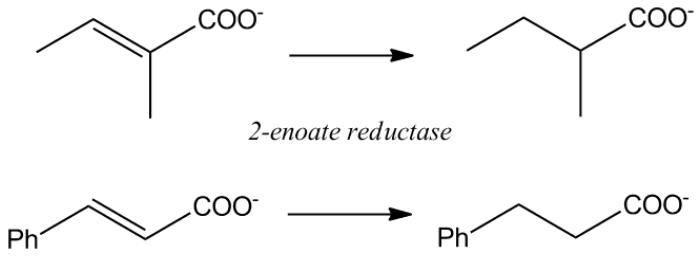


Fig. 2.12. Reduction of *(E)*-2-methyl-2-butenoate or *(E)*-cinnamate by enoate reductase from *C. kluyveri* (Tischer *et al.*, 1979).

The enzyme was characterized by the amino acid composition with isoelectric point about 8.4, the molecular weight 450 kDa and subunit weight of 72 kDa. One subunit of the enzyme contained 1 FAD molecule, 3.5-3.8 atoms of iron and 4.0 labile sulphur atoms. Analysis of the absorption spectrum showed that enoate reductase had a typical flavin spectrum with maximum at 450 nm that disappeared when the enzyme was reduced with NADH. Dichloroindophenol, hexacyanoferrate III, oxygen, *(E)*-2-methyl-2-butenoate and *(E)*-cinnamate reoxidized the enzyme. The enzyme activity was inhibited by 0.1 mM *p*-hydroxymercuribenzoate, a compound reacting with sulphide sulphur of iron-sulphur clusters as well as with sulfhydryl groups, or 1 mM mersalyl. Kinetics data showed a small K_m value for NADH indicating that this can be a physiological cofactor for enoate reductase. The enzyme showed the highest activity at pH 6.0 (Tischer *et al.*, 1979).

Enoate reductase was purified from *C. tyrobutyricum* (Crotonate strain La 1) with a high yield about 90%. The enzyme complex was a homomultimer with a molecular mass 920 ± 20 kDa. The purified fragment of the protein was able to reduce acetylpyridine adenine dinucleotide in the presence of NADH. Further analysis showed that enoate reductase is a dodecamer (tetramer of trimers) with tetrahedral symmetry (Kuno *et al.*, 1985).

By comparing substrate specificity of enoate reductases form different species, it was shown that *Clostridium* sp. La 1 and *C. kluyveri* reduced a broad range of enoates

whereas enoate reductase of *C. sporogenes* acted as a cinnamate reductase with yield of 70-100%.

Cinnamate reductase activity was also found in another anaerobic bacterium *Peptostreptococcus anaerobius* (Buhler *et al.*, 1980). Comparison of enoate reductases from Clostridia and *P. anaerobius* showed that although theses enzymes have a very similar range of substrates, they have opposite enantioselectivities. *C. sporogenes* reduced cinnamate in a *trans* fashion to the *si*-faces of both C-2 and C-3 of cinnamate resulting in (2S,3R)-3-phenyl-[2,3-^{2}H]propionate, whereas *P. anaerobius* added to the *re*-faces giving (2R,3S)-3-phenyl-[2,3-^{2}H]propionate (Giesel *et al.*, 1981).

However, although the saccharolytic *C. butyricum*, *C. pasteurianum* and *C. propionicum*, were able to metabolize *(E)*-2-butenoate, they could not hydrogenate *(E)*-2-methyl-2-butenoate or cinnamate. The enoate reductase from *C. tyrobutyricum* was immunologically similar to enoate reductase from *C. kluyveri*, but different from *C. sporogenes*, further emphasising that there are significant differences between enoate reductases form different families of Clostridia (Giesel and Simon, 1983)

Enoate reductase activity depended on the compounds that growing medium were supplemented with. Addition of glucose raised the activities of enzymes from *C. kluyveri* and *C. tyrobutyricum* to the highest values whereas supplementation with isoleucine decreased the activity to the lowest value. There was no difference in the activity for cells growing on medium with cinnamate and *(E)*-4-methyl-2-pentenoate (Giesel and Simon, 1983).

The highest activity of enoate reductase was observed for hydrophobic enoates that are not branched in the *β*-position. The exception was 2-butenoate, which showed the highest activity with enoate reductase from Crotonate La1, but was not a substrate for enzymes from other Clostridia species (Giesel and Simon, 1983).

The *C. tyrobutyricum* enoate reductase gene (*enr*) was cloned and sequenced by Rohdich *et al.* in 2001. By sequence similarity the enoate reductase was also identified in the *C. thermoaceticum* and *C. kluyveri* genomes. The nucleotide sequence analysis showed, that enoate reductase genes are similar to flavoproteins such as 2,4-dienoyl-CoA reductase from *E. coli* and to yeast old yellow enzyme family. Overexpression of the *C. thermoaceticum* enoate reductase in *E. coli* expression system under anaerobic conditions afforded an soluble, enzymatically active enzyme with the specific activity 0.1 μmol min^{-1} mg $^{-1}$ for reduction of *(E)*-methylbutenoate using NADH as the cofactor. When the enzyme was expressed in aerobic conditions, the product was an insoluble

protein. Overexpressed enoate reductase from *C. tyrobutyricum* was found to be inactive (Rohdich *et al.*, 2001).

2.8. ClosTron – the gene knock-out system for Clostridia

Attempts have been made to purify enoate reductase from *C. sporogenes* but have met with limited success. Therefore other approaches are needed to obtain large quantities of the enzyme for use in industrial biotransformations. The best strategy for that is identification of the gene that encodes enoate reductase and overexpression of the protein using *E. coli* host. Because the sequence of *C. sporogenes* enoate reductase is not known, knock-out mutants of *C. sporogenes* were prepared based on the gene sequence from closely related *C. botulinum* str. A. Then mutants were tested for the enoate reductase activity.

2.8.1. Genomic mutants in Clostridia genus

Large progress in genome sequencing for all major Clostridia species led to a need for new DNA transfer methods for this specific genus. In case of production strains with knock-out mutated genes only few examples were presented with successful insertional inactivation of target DNA sequence, mainly for these four species: *C. acetobutylicum*, *C. beijerinckii*, *C. perfringens* and *C. difficile* (Heap *et al.*, 2007).

Double crossover mutants were reported only in *C. perfringens* as the consequence of the high frequency of the DNA transformation into this bacterium. In 1995 Awad *et al.* developed an allelic exchange method to provide a genetic evidence for the role of alpha-toxin in gas gangrene or clostridial myonecrosis caused by *C. perfringens*. Bacteria were transformed with a suicide plasmid containing a gene insertionally inactivated with an erythromycin-resistance gene. Two different genes were inactivated: *pfoA* (encoding an oxygen-labile haemolysin called theta-toxin or perfringolysin O) and *plc* gene (the alpha-toxin, phospholipase C). The knock-out mutants were not able to produce detectable theta-toxin or alpha-toxin activity. Mutations could be complemented by transfection with plasmids with transcriptionally active wilt-type *pfoA* or *plc* genes (Awad *et al.*, 1995).

In *C. perfringens* allelic exchange was also used in the research project determining the precise conjugative deletion of the chloramphenicol-resistance

transposon Tn4451 from its parent plasmid plP401. By the inactivation of *tnpX*, the largest gene in Tn4451, it was shown that this gene is required for the excision of the transposon from the plP401 plasmid. Allelic exchange was used to put the mutated *tnpX* delta 1 allele into p1P401. It was concluded that *tnpX* was also required for the conjugative excision of Tn4451 in *C. perfringens*. The ability to excise the transposon was restored by expression of the wild-type *tnpX* gene from a compatible plasmid (Bannam *et al.*, 1995).

A few single crossover mutants brought by Campbell-like integration were also reported, but they are segregationally unstable. The first demonstration of gene mutation by homologous recombination in *Clostridium acetobutylicum* NCIMB8052 was reported by Wilkinson and Young in 1994. Transfection using plasmids containing inactivated fragments of *gutD* and *spo0A* genes resulted in inability of the strain to metabolise sorbitol as the energy source or form endospores, respectively (Wilkinson and Young., 1994). Green and Bennett in 1996 injected a nonreplicative plasmid carrying inactivated aldehyde/alcohol dehydrogenase (*add*) gene to *C. acetobutylicum* ATCC824 what led to production of stable generations of integrants, that were not able to produce butanol (Green and Bennett, 1996). A similar experiment in the same ATCC824 strain allowed to disrupt metabolic pathways of acetate and butyrate production. Mutated phosphotransacetylase (*pta*) or butyrate kinase (*buk*) gene fragments were integrated by homologous recombination to the bacterial chromosome. Mutants were characterized with reduced production of acetate or butyrate, respectively (Green *et al.*, 1996). *Buk* and *aad* mutations could be complemented by overexpression of replicative plasmids pTHAAD and pTHBUT encoding aldehyde/alcohol dehydrogenase and butyrate operon respectively. Complementation of the *buk* mutant restored butyrate kinase activity and butyrate production during exponential growth whereas complementation of *aad* mutant with pTHAAD restored NAD(H)-dependent butanol dehydrogenase activity, NAD(H)-dependent butyraldehyde dehydrogenase activity and butanol production during solventogenic growth (Green and Bennett, 1998).

The SolR gene was found to encode the repressor of the sol locus containing *add*, *ctfA*, *ctfB* and *adc* genes involved in butanol and acetone formation in *C. acetobutylicum* ATCC824. Overexpression of SolR resulted in a mutant with solvent-negative phenotype. Two mutants B and H were created by inactivation of SolR *via* homologous recombination. They showed deregulated solvent production, activation of

aad, lower overall acid production, improved and prolonged solvent production and increased biomass accumulation (Nair *et al.*, 1999).

The first report of gene inactivation in *C. difficile* and *C. beijerinckii* was presented by Liyanage *et al.* in 2001. *C. beijerinckii* insertion mutant BR54, when compared to the wild-type, was more sensitive to methylglyoxal (MG) and contained more free-MG in cells suggesting the reduced ability to metabolise this compound. Locus of the mutation was found in *gldA* gene encoding glycerol dehydrogenase. Insertional inactivation of this gene in *C. beijerinckii* and *C. difficile* gave the same phenotype as BR54 proving that glycerol dehydrogenase plays an essential role in detoxification process (Liyanage *et al.*, 2001).

By using the *E. coli-C. perfringens* shuttle vector that is unstable in *C. difficle*, an insertional inactivation of target genes was performed with high reproducibility. *C. difficile* genes *rgaR* and *rgbR*, showing a high homology to *C. perfringens VirR* (a gene responsible for transcriptional regulation of other genes involved in the toxin synthesis), were knocked out. It was shown that inactivation of *rgaR* caused a change in the expression of four other genes (O'Connor *et al.*, 2006).

However DNA transfer systems to clostridial genomes using homologous recombination were developed, they are not efficient and universal for all strains. In 2007 a new system for insertional inactivation of genes in *Clostridia* using a bacterial group II intron was described (Heap *et al.*, 2007).

2.8.2. Introns group II in genetic engineering

Introns group II were discovered in the 1980s during sequencing of fungi and plant genomes. They were found in rRNA, tRNA, mRNA of organelles in fungi, plants, protists and mRNA in bacteria. They are divided into two subgroups IIA and IIB with different distinctive structural features. Group II introns showed catalytic activity (they are ribozymes about 0.6-2.5 kb) and can self-splice *in vitro* (described for the first time in yeast mitochondrial group II intron, mid-1980s). The primary structure group II introns can encode polypeptides essential for splicing *in vivo* (intronic ORF with homology to reverse transcriptases, endonucleases, RNA maturases and helicases). The secondary structure of intron group II consists of six domains (dI – dVI) and a central core (Fig. 2.13.).

The catalytic centre involved in self-splicing process is formed by two highly conserved elements located in domains I and V (Fig. 2.13., blue nucleotides with tertiary interaction ζ -ζ'). Domain dIV encodes functions for splicing and mobility (intronic ORFs). Domain dVI contains a bulged adenosine (shown in red) that plays a crucial role in the splicing. Positioning of 5' and 3' splice sites is determined by base-pairing between EBS1, EBS2 and EBS3 sequences located in domain dI of the intron (Exon Binding Sites) and IBS1, IBS2 and IBS3 sequences (Intron Binding Sites) in exons (Bonen and Vogel, 2001).

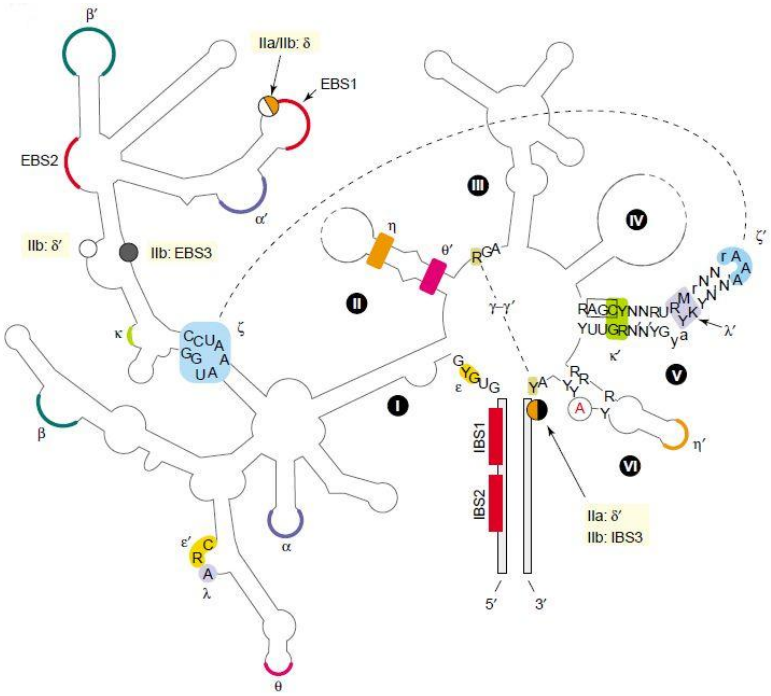


Fig. 2.13. Predicted secondary structure of intron group II. Domains are marked from I to VI. Bulged adenosine in domain VI is shown in red. Tertiary interactions are shown by colour Greek symbols (Bonen and Vogel, 2001).

The intron group II splicing proceeds in two transesterification steps. At first a 2'hydroxyl of bulged adenosine attacks the 5' splicing site, then 3'OH of the upstream exon from 5' splice site attacks 3' splice site. The products of splicing process are the excised intron with a lariat structure with 2'-5' linkage and a 6-7 nucleotide tail and two spliced exons. However group II introns can self-splice *in vitro*, splicing *in vivo* requires the protein machinery encoded by intronic ORFs or by the host genome (Bonen and Vogel, 2001).

Group II introns can also move within a genome or invade new genomes by a process called 'retrohoming'. The site of intron integration is determined by complementarity between intron and exon sequences (EBS and IBS; Fig. 2.14.).

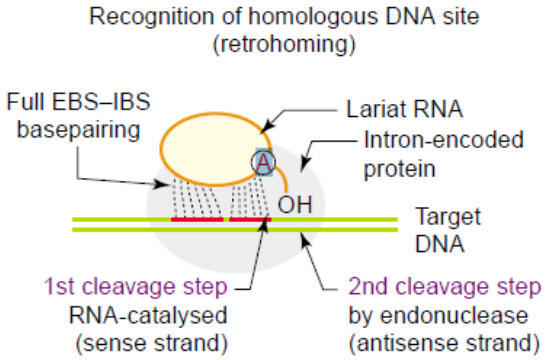


Fig. 2.14. Recognition of homologous DNA site by RNP composed of excised intron and intron encoded protein (from Bonen and Vogel, 2001).

The RNP composed of excised intron (lariat RNA) and intron-encoded protein catalyses the first cleavage step of sense strand by reverse splicing into DNA. The second anti-sense strand is cut by an endonuclease and the intron is used as a template in reverse transcription-cDNA synthesis (Fig. 2.15.). Then a recombination-independent system repairs the nicks in the first strand and synthesises the second DNA strand. The retrohoming process in bacteria results in no co-conversion of markers. The efficiency of integration can approach 100% (Bonen and Vogel, 2001).

Fig. 2.15. Retrohoming process in bacteria. RNP cleaves homologous DNA site and reverse splice into DNA. First DNA strand is synthesised by reverse transcription. Repair system synthesises the 2nd DNA strand (from Bonen and Vogel, 2001).

Karberg *et al.* (2001) showed that mobile group II introns can be retargeted to insert into any DNA target in a wide host range. *Lactococcus lactis* L1.LtrB intron was used for specific insertional inactivation of genes in *E. coli*, *S. flexneri* and *S. typhimurium*. thymidylate synthetase. A screening system was developed, where the combinatorial intron library was tested for integration into the *thyA* (thymidylate synthetase) gene located in a plasmid (Karberg *et al.*, 2001). Positive integration of the intron caused activation of the Tet^R gene giving the resistance to tetracycline. Group II

introns were also used for disruption of chromosomal genes in *E. coli* with efficiency from 0.1 to 10% by screening without selection. A convenient method for generation of retargeted introns by PCR and a technique that allowed an introduction of point mutations into wild-type chromosomal genes were also developed. All of that made group II introns a useful tool for genetic engineering and functional genomics in different bacteria.

An insertional inactivation system based on group II intron was developed by introduction of a retrotransposition-activated selectable marker (RAM) to the L1.LtrB intron, which enabled one-step bacterial gene disruption at near 100% efficiency after selection in medium with antibiotic. An antibiotic resistance gene interrupted by a self-splicing group I intron in opposite orientation was placed in the L1.LtrB group II intron. A successful integration of the L1.LtrB intron to a target gene when the group I intron spliced from RAM gave a resistance to antibiotic in selective medium (Zhong *et al.*, 2003).

2.8.3. The ClosTron system

A gene knock-out system based on group II intron retrohoming developed by Zhong *et al.* (2003) used an IPTG-inducible T7 promoter and RAM with trimethoprim resistance. A similar system constructed by Sigma-Aldrich, TargeTron® Vector pACD4K-C, relied on kanamycin resistance. However none of them could be used with Clostridia as most clostridial genera are naturally resistant to both antibiotics and the T7 promoter tends not to work in their cells.

In 2007, Heap *et al.* developed a system with equivalents of these elements working in clostridial cells, the ClosTron, a universal knock-out system. The main component of the system is plasmid pMTL007 (Fig. 2.16.).

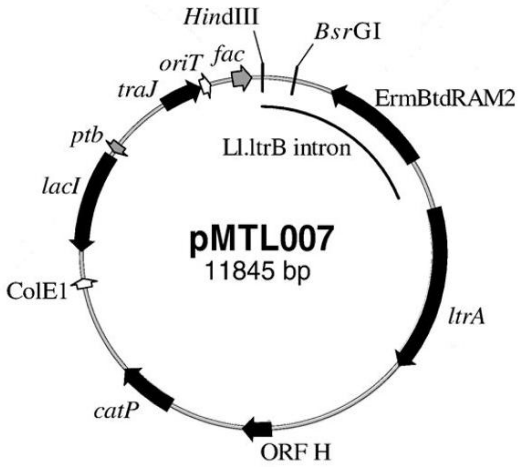


Fig. 2.16. pMTL007 vector from ClosTron system (Heap *et al.*, 2007).

Plasmid pMLT007 contains the *Lactococcus lactis* L1.LtrB intron under transcriptional regulation of the IPTG-inducible 'fac' promoter composed of *C. pasteurianum* ferrodoxin gene promoter and *E. coli lacZ* operator. Transcription of the intron sequence is repressed by *E. coli lacI* repressor gene product under the *ptb* promoter from *C. acetobutylicum* phospohotransbutyrylase gene. *LtrA* gene located upstream of L1.LtrB intron contains intron ORF encoding elements required for the retrohoming process. The retrotransposition-activated selectable marker located in the L1.LtrB intron was constructed using the *ermB* gene from *Enterococcus faecalis* containing a linker between the *C. acetobutylicum thl* promoter and the *ermB* ORF of 36 base pairs with the group I *td* intron from phage T4 (Fig. 2.17.).

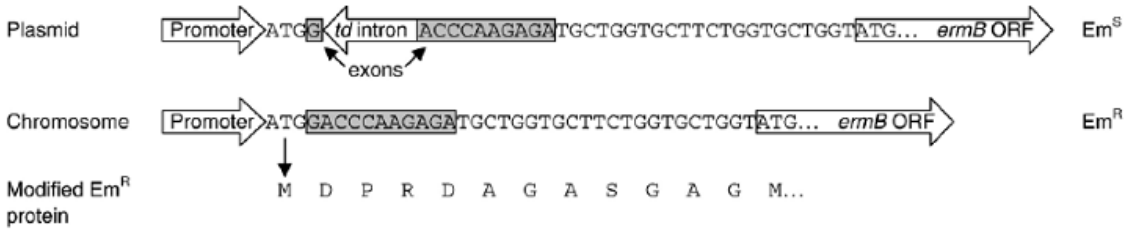


Fig. 2.17. Retrotransposition-activated selectable marker in pTML007 plasmid (Heap *et al.*, 2007).

The presence of the *td* intron in *ermB* prevents the erythromycin resistance whereas splicing it out gives an *erm*B protein with 12 amino acid extension in the N-terminus. The mutant generation process using ClosTron is shown in Fig. 2.18.

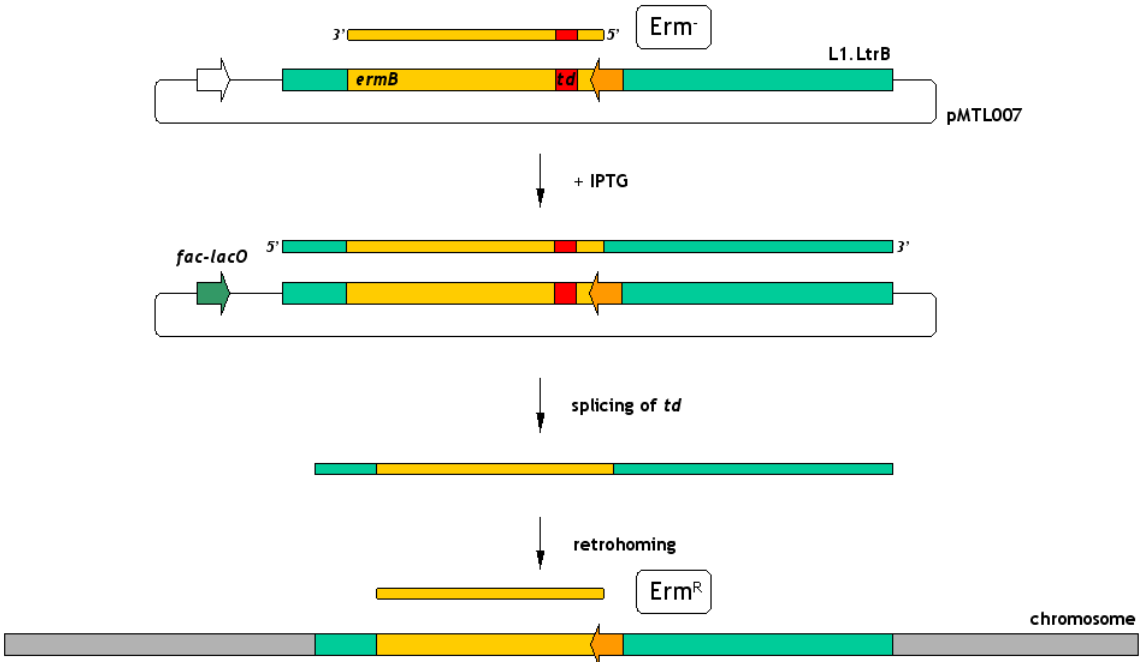


Fig. 2.18. Genomic mutant generation using ClosTron system (description in the text).

When the IPTG-inducible promoter is not active, the *ermB* gene yields an inactive product and the strain is not erythromycin resistant. In presence of IPTG, the L1.LtrB intron is transcribed and *td* intron group I splices out, allowing the expression of *ermB* ORF after integration of L1.LtrB intron into targeted site in the host genome.

To demonstrate the functionality of the ClosTron system, genomic mutants of *pyfF* and *spo0A* in three clostridial species (*C. difficile*, *C. acetobutylicum* and C. *sporogenes*) were produced. Mutant analysis showed very high frequencies of insertion into the chromosome (from 7% for *C. acetobutylicum pyrF* 345s to 100% *C. sporogenes spo0A* 249s, *pyrF* 595s and *C. difficile spo0A* 178a). Analysis of mutant phenotypes showed that all *pyrF* knock-outs were unable to grow in minimal medium without 50µg/L uracil and *spo0A* did not form endospores, confirming the inactivation of these genes.

It was demonstrated that the ClosTron insertional inactivation system is highly efficient and specific. ClosTron is minimally dependent on host factors, the mechanism requires only excised intron and protein product of the intronic ORF, and can be used in wide host range (both Gram-positive and Gram-negative bacteria). The system can be retargeted to insert efficiently into virtually any desired DNA target and yields extremely stable genomic mutants, what makes it an excellent tool in genetic engineering of Clostridia (Heap *et al.*, 2007).

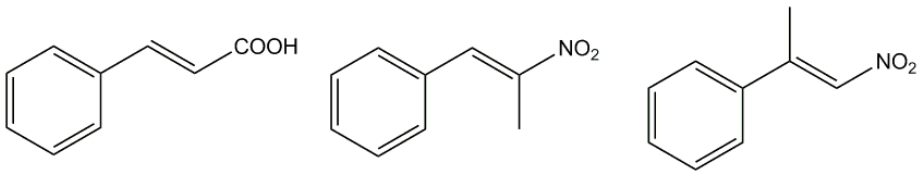
3. Objectives of the study

The main objective of this project is to test *C. sporogenes* as a novel biocatalyst for two types of reduction, amide reduction to produce amines and nitropropene hydrogenation to produce chiral compounds.

Because *C. sporogenes* is a bacterium classified as a Biological Safety Level 2 organism, cultivation and using it for biotransformation is connected to a serious risk for the researcher and other people in proximity. The health and safety policy at the University of Manchester requires a special attitude to any work using dangerous bacteria. As the aim of this project, Risk Assessment, COSHH forms and Standard Operating Procedures for working with *C. sporogenes* were prepared and validated by series of preliminary experiments.

Studies on amide reduction by *C. sporogenes* (Dipeolu *et al.*, 2005) showed that the reaction is complex and its further optimization is needed. The analytical method described by Dipeolu based on GC-MS analysis was not sensitive enough to detect trace amounts of amines. A new analytical method for detection of benzylamine and benzamide was developed and tested for the detection limits. Afterwards, degradation of benzylamine and benzamide by *C. sporogenes* cell suspensions was tested. Finally, the whole cell biotransformation technique was used to reduce benzamide to benzylamine.

The reduction of C=C double bonds of unsaturated nitroalkenes by *C. sporogenes* was described by Fryszkowska *et al.* (2008). The reaction conditions were tested and optimized for a wide range of substrates, but purification of the enzyme responsible for this type of bioreduction was not successful. Based on the literature data it was suspected that enoate reductase may be involved in this process. A change of strategy was proposed and the ClosTron gene knock-out system was used to produce enoate reductase knock-out mutants of *C. sporogenes* (fldZ::CTermB). The aim of this part of the project was to test the growth of the mutant in media with different energy sources and compare it to the wild type *C. sporogenes* strain DSM795. The mutant was also tested for the ability to reduce two phenylnitropropene isomers: *(E)*-1-nitro-2-phenyl-propene and *(E)*-1-phenyl-2-nitro-propene using the whole cell biotransformation (structures, Fig. 3.1.).



(E)-cinnamic acid *(E)*-1-phenyl-2-nitro-propene *(E)*-1-nitro-2-phenyl-propene

Fig. 3.1. Structures of substrates that were tested in biotransformations.

Subsequently proteins were extracted and separated using SDS-PAGE electrophoresis looking for the differences between the wild type strain and the mutant. Protein extract were also used for reduction of *(E)*-cinnamic acid using the spectrophotometric assay and *(E)*-1-nitro-2-phenyl-propene and *(E)*-1-phenyl-2-nitro-propene using biotransformation in biphasic system.

4. Materials and methods

4.1. Materials

4.1.1. Reagents:

Acros: benzamide, sodium selenite pentahydrate, *tert*-butylbenzene;
Aldrich: cinnamic acid, resazurin;
Analar: manganese sulphate tetrahydrate;
BDH: ammonium molybdate, magnesium chloride;
BOC: H_2, N_2, ultra-high purity He;
Fermentas: PageRuler^{TM} Prestained Protein Ladder;
Fisher: dipotassium hydrogen orthophosphate, glycine, magnesium sulphate, SDS;
Fluka: glycerol, sodium thioglycollate;
Foremedium: peptone, tryptone;
Lab M Limited: cooked meat medium;
Melford: agar, Tris, yeast extract;
Novexin: Instant Blue Stain;
Rectapur: iron sulphate heptahydrate;
Roche: Complete EDTA-free protease inhibitor;
Sigma: 2-β-mercaptoethanol, acrylamide:N,N'-methylenebisacrylamide (37:5:1), APS, benzylamine, biotin, bromophenol blue, butan-2-ol, calcium chloride, dithiothreitol, erythromycin, FAD, *iso*-octane, L-cysteine HCL, NADH, p-aminobenzoic acid, phenylalanine, potassium phosphate monobasic, riboflavin, TEMED, *tert*-butylmetylether.

4.1.2. Substrates: Substrates for phenylnitropropene reduction assay ((*E*)-1-Nitro-2-phenyl-propene and (*E*)-1-phenyl-2-nitropropene) were synthesised by Dr. A. Fryszkowska (Manchester Interdisciplinary Biocentre, University of Manchester) according to methods described in Fryszkowska *et al.*, 2008.

4.1.3. Bacterial strains: *Clostridium sporogenes* DSM795 culture was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). *Clostridium sporogenes* fldZ::CTermB genomic mutant was prepared by Prof. Nigel Minton group (University of Nottingham).

4.2. Methods

4.2.1. Maintenance of *C. sporogenes* DSM795

The medium was composed of cooked meat medium (5 g/L), KH_2PO_4 (5 g/L), L-cysteine HCl (0.5 g/L), resazurin (1 mg/L of 1% w/v stock solution) and agar (20 g/L) with final volume 200 mL in 0.5 L Duran bottles. The solution was sparged with oxygen-free N_2 for at least 20 min. The suba seals caps were held in place with copper wire and the medium was sterilized by autoclaving using a desk top autoclave (Omega MEDIA, Prestige Medical). The medium was left to cool down to 50-60°C and agar plates were poured in the anaerobic cabinet (MARK3 Anaerobic Workstation, Don Whitley) when the medium was still warm and fluid.

4.2.2. Maintenance of *C. sporogenes* fldZ::CTermB

The TYG medium was composed of tryptone (30 g/L), yeast extract (20 g/L), sodium thioglycollate (1 g/L), resazurin (1 mg/L of 1% w/v stock solution) and agar (20 g/L) with final volume 100 mL in 0.5 L Duran bottles. The solution was sparged with oxygen-free N_2 for at least 20 min. The suba seals caps were held in place with copper wire and the medium was sterilized by autoclaving using a desk top autoclave. The medium was left to cool down to 50-60°C. In the anaerobic cabinet, erythromycin was dissolved in ethanol and added to final concentration 2.5 μg/mL and agar plates were poured when the medium was still warm and fluid.

4.2.3. Preparation of growing medium (Giesel medium)

The solution (1 L) was composed of basal medium (900 mL; containing peptone, 20 g/L; yeast extract, 5 g/L; 1 mL resazurin 1% w/v stock solution), trace element solution (10 mL; $MgCl_2 \cdot 6H_2O$, 3.3 g/L; $CaCl_2 \cdot 2H_2O$, 4 g/L; $MnSO_4 \cdot 5H_2O$, 0.04 g/L; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 g/L; $F_2SO_4 \cdot 7H_2O$ 2.93 g/L), salt solution (20 mL; KH_2PO_4, 34 g/L; K_2HPO_4, 131 g/L; $NaSeO_3 \cdot 5H_2O$, 0.02 g/L), vitamin solution (10 mL; *p*-aminobenzoic acid, 0.08 g/L; biotin, 0.004 g/L; riboflavin, 0.02 g/L), reducing agent solution (10 mL; sodium thioglycollate, 30 g/L) and energy source (L-phenylalanine, final concentration 12.1 mM or D-glucose, final concentration 27 mM). All solutions were degassed by sparging with N_2 for 20-60 min. Basal medium, reducing agent solution and energy source solution were prepared freshly every time. Salt solution and

trace element solution were stored in a fridge (+4°C) whereas vitamin solution was frozen at -20°C. Basal medium, trace element solution salt solution and reducing agent solution were sterilized by autoclaving in bottles sealed with the suba seals caps held in place with copper wire. Vitamin solution and energy source solution were sterilized by filtration with a 0.2 μm plastic filter in the anaerobic work station. All solutions were mixed together in the anaerobic work station. Medium was pre-reduced by overnight incubation in the anaerobic cabinet.

4.2.4. Preparation of cultures

In the anaerobic work station, agar medium in Petri dishes was inoculated with *Clostridium sporogenes* taken from a single colony of the main stock culture growing on a plate using an inoculation plastic loop and re-cultured every week.

Pre-cultures were grown in universal bottles containing about 20 mL of Giesel medium and inoculated from the single bacteria colony grown in Petri dish. The culture was incubated in an anaerobic work station with shaking (200 rpm) in 30°C overnight. 500 mL Giesel medium was inoculated by pouring 20 mL pre-culture. Culture was cultivated until it reached the early stationary phase of growth based on the OD measurements and used for biotransformation.

4.2.5. Microscopic examination of *C. sporogenes*

In the anaerobic cabinet 1 mL of culture was poured into a 1.5 mL tube. 20 μL of culture was applied on a basal slide and cover with cover-slip. Immersion fluid was applied on the cover-slip and bacteria were observed under 100x magnification a using microscope (BA300, Motic).

4.2.6. Measuring the OD of *C. sporogenes*

In the anaerobic cabinet 1 mL of medium was taken out the culture, diluted 10x with deionized water in a plastic cuvette and sealed with parafilm. OD was analyzed at 660 nm using the spectrophotometer (UVmini 1240, Shimadzu).

4.2.7. Harvesting of *C. sporogenes*

The cell culture was transferred in the anaerobic cabinet into centrifuge pots (250 or 500 mL) which were centrifuged at 6000 rpm for 10 minutes using Jouan KR25i centrifuge. The supernatant was discarded and the cells re-suspended in degassed phosphate buffer (50 mM potassium phosphate buffer, pH 7) to give an OD of 200. The washing procedure was repeated to remove the residual medium before the cells were used for the biotransformations.

4.2.8. Assay and analytical method for amide reduction using whole cells

In the anaerobic cabinet, 1 mL of harvested cells solution (OD 200) was mixed with 9 mL of degassed 50 mM potassium phosphate buffer and the required volume of substrate (1 mM; benzamide or benzylamine) solution in ethanol in a 25 mL universal bottle. The bottle was closed with a silicon-PTFE cap and removed from the cabinet.

To start the biotransformation, H_2 was passed through the headspace for 2 min using a gas manifold. The addition of the electron donor initiated the biotransformation and the reaction mixtures were incubated at 30°C shaking upside down to avoid hydrogen loss at 200 rpm for the required time (usually 24 hours).

After biotransformation a sample of reaction mixture (5 mL) was basified with 1 M NaOH (250 μL) checking that the pH>11. Residual substrate and products were extracted with 0.5 mM *tert*-butylbenzene (the internal standard of the analytical method) in *tert*-butylmetylethe (5 mL). 1.5 mL of the upper organic phase was transferred to a new 2 mL tube. The sample was dried by addition of magnesium sulphate (one third of the tube volume), thoroughly mixing for 1 min using a vortex and centrifuging for 1 min at 13000 rpm (Eppendorf Centrifuge MiniSpin).

The supernatant was transferred to a vial and used for gas chromatography analysis using Varian CP-3800 equipment. Ultra-high purity helium (99.995%) was used as carrier gas at a constant flow of 1.6 mL/min. Samples were analyzed by auto injection of sample (1 μL) onto the column (Agilent CAM, 30 m, 0.32 mm, 0.25 μm). Samples were resolved using a temperature programme comprising isothermal chromatography at 80°C for 3 min, followed by a linear gradient of 15°C/min to reach the final temperature of 220°C which was maintained for 7 min. The sample components were detected using a flame ionization detector (FID) at 250°C.

Compounds were identified and quantified based on the retention times and peak areas of analytical standards.

4.2.9. Assay and analytical method for phenylnitropropene reduction

The biotransformation was performed in 30 mL screw top vials with silicon-PTFE caps. Each assay contained substrate (1.7 mM final concentration) in 4.8 mL of anaerobic *iso*-octane, 7.2 mL anaerobic 50 mM potassium phosphate buffer containing *C. sporogenes* cells (OD 1.0) and *tert*-butylbenzene (25 μL) as an internal standard. Reactions were shaken in the anaerobic cabinet at 200 rpm at 30°C for 72 h.

Samples were analyzed using HPLC (Agilent). The samples (15 μL) of the organic phase were auto-injected to a Chiralcel OD column (diameter 4.6 mm × 250 mm) and products were separated by hexane/isopropyl alkohol (95:5) and detected using UV spectrophotometer (by wavelengths 220 and 254 nm). Product quantification was based on calibration curves, which had been previously prepared by Dr. Anna Fryszkowska.

4.2.10. Preparation of cell free protein extracts

Harvested cells were resuspended anaerobically with degassed 100 mM phosphate buffer, pH 7.0, containing 0.1 g/L dithiothreitol, 10 μM FAD and protease inhibitor cocktail (Complete, EDTA-free protease inhibitor cocktail tablets, according to the producer instructions) at the approximate ratio of 1 g dry weight cells to 2.0 mL of buffer. Cells were lysed by passage through a French pressed cell pressure. The protein extract was collected under a flow of N_2. The quality of proteins was determined by SDS-PAGE electrophoresis technique (see 4.2.13). Extracts were used in the cinnamate spectrophotometric assay.

4.2.11. Protein quantification using *DC* Protein Assay (Bio-Rad)

Protein extracts were diluted 10-times. A sample (100 μL) was pipetted to a clean, dry test tube. *RC* Reagent I (500 μL) was added into tube, vortexed and incubated for 1 min at room temperature. *RC* Reagent II (500 μL) was added into tube, vortexed and centrifuged at 13000 rpm for 5 min. The supernatant was discarded and pellet was allowed to dry. Reagent A’ (510 μL) was added to tube and vortexed. The sample was

incubated at room temperature for 5 min and vortexed again. 4 mL of *DC* Reagent B were added and the sample was vortexed immediately. After incubation (15 min) absorbance at 750 nm was read using UVmini 1240 spectrophotometer (Shimadzu). Protein concentration was calculated based on the calibration curve for BSA protein standard.

4.2.12. Spectrophotometric cinnamate reduction assay

Cinnamate reduction activity of *C. sporogenes* crude extracts was determined using spectrophotometric assay. The reaction was set up in anaerobic conditions in quartz cuvettes containing protein extract (20 μL), 10 mM cinnamic acid dissolved in water (100 μL), 4 mg/mL NADH (50 μL) and 50mM potassium buffer pH 7.0 (830 μL). The reduction of the substrate was observed by measuring the absorbance at 340 nm connected to consumption of NADH. The reaction was continued for 5 min reading the absorbance every 2 s.

4.2.13. SDS-PAGE protein electrophoresis

The separating buffer (100 mL) was prepared of 18.15 g Tris base and 0.4 g SDS and the pH was set to 8.8. The separating gel was composed of: 40% acrylamide:N,N'-methylenebisacrylamide (37.5:1) water solution (8 mL), deionised H_2O (6.8 mL), separating buffer (5 mL), 10% APS (100 μL) and TEMED (10 μL). The stacking buffer (100 mL) was prepared of 6.04 g Tris and 0.4 g SDS and the pH was adjusted to 6.8. The stacking gel was prepared of 40% acrylamide:N,N'-methylenebisacrylamide (37.5:1) water solution (1.3 mL), deionised H_2O (6.1 mL), separating buffer (2.5 mL), 10% APS (50 μL) and TEMED (5 μL). The running buffer (1 L) contained 14.4 g glycine, 3 g Tris and 1 g SDS. The loading buffer was composed of 2.6% SDS, 1.3% 2-β-mercaptoethanol, 6% glycerol, 0.2% bromophenol blue, 0.05 M Tris-HC1, pH 6.8.

Plates, spacers, combs and gaskets were washed with ethanol and allowed to dry. Plates were assembled and checked with deionised water. The separating gel was poured straight away and cover with water saturated butan-2-ol. After 45 min butan-2-ol was removed and the headspaces of plates were washed with water five times. The stacking gel was poured, the comb was inserted and gel was left for 1 h. Then the gel cassette was assembled, the comb was removed and the inner and outer tanks were filled with the running buffer. The loading buffer was added to samples to a final volume of 30 μL.

Samples were incubated in a water bath at 95ºC for 5 min and loaded onto the gel. PageRuler^{TM} Prestained Protein Ladder was used as a molecular weight marker. Electrophoresis was run at 35 mV for 2 h. The gel was washed with water twice and stained with Instant Blue Stain overnight (Novexin).

5. Results

5.1. Safe handling of *C. sporogenes*

According to the DSMZ culture collection, *Clostridium sporogenes* DSM795 is a microorganism possessing the Biological Safety Level 2 (http://www.dsmz.de/microorganisms/html/strains/strain.dsm000795.html). It means that work with this strain is connected with moderate individual risk and low community risk. "*C. sporogenes* can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventative measures are available and the risk of spread of infection is limited" (WHO Classification of Infective Microorganisms by Risk Group, http://www.absa.org/riskgroups/index.html).

As a part of the safe and healthy training, the Standard Operating Procedures (SOPs) for working with *C. sporogenes* were prepared and the growth conditions for *C. sporogenes* DSM795 were determined. Bacteria transferred from the vacuum dried stock were successfully grown in Petri dish plates with two different media: cooked meat medium (see Materials and Methods, 4.2.1) and TYG medium (4.2.2). No contamination of other bacteria species was found in the cultures.

5.1.1. Standard Operating Procedures (SOPs) for working with *C. sporogenes*

To establish the methodology required by the safety regulations, Standard Operating Procedures for working with *C. sporogenes* were prepared.

Table 5.1. Standard Operating Procedures for working with *C. sporogenes*.

SOP1. Culture in Petri dishes.

1. The medium was composed of cooked meat medium (5 g/L), KH_2PO_4 (5 g/L), L-cysteine HCl (0.5 g/L), resazurin (1 mg/L of 1% w/v stock solution) and agar (20 g/L) with final volume 200 mL in 0.5 L Duran bottles.
2. Solution was sparged with oxygen-free N_2 for at least 20 min (see SOP3).
3. The suba-seals caps were held in place with copper wire and the medium was sterilized by autoclaving using a desk top autoclave.
4. The medium was left to cool down to 50-60°C.
5. Agar plates were poured in the anaerobic cabinet when the medium was still warm and fluid.

SOP2. Preparation of the Giesel medium.

1. The 1 L solution was composed of:
   - 900 mL basal medium (containing peptone, 20 g/L; yeast extract, 5 g/L; 1mL resazurin 1% w/v stock solution);
   - 10 mL trace element solution ($MgCl_2 \cdot 6H_2O$, 3.3 g/L; $CaCl_2 \cdot 2H_2O$, 4 g/L; $MnSO_4 \cdot 5H_2O$, 0.04 g/L; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 g/L; $F_2SO_4 \cdot 7H_2O$ 2.93 g/L);
   - 20 mL salt solution (KH_2PO_4, 34 g/L; K_2HPO_4, 131 g/L; $NaSeO_3 \cdot 5H_2O$, 0.02 g/L);
   - 10 mL vitamin solution (*p*-aminobenzoic acid, 0.08 g/L; biotin, 0.004 g/L; riboflavin, 0.02 g/L);
   - 10 mL reducing agent solution (sodium thioglycollate, 30 g/L);
   - energy source (usually phenylalanine, final concentration 12.1 mM).
2. All solutions were degassed by sparging with N_2 (described in SOP3) for 20-60 min.
3. Basal medium, reducing agent solution and energy source solution were prepared freshly every time. Salt solution and trace element solution were stored in a fridge (+4°C) whereas vitamin solution was frozen at -20°C.
4. Basal medium, trace element solution salt solution and reducing agent solution were sterilized by autoclaving in bottles sealed with the suba-seals caps held in place with copper wire. The basal medium is divided into 250 ml portions and sterilized in 500 ml bottles.
5. Vitamin solution and energy source solution were sterilized by filtration with 0.2 μm plastic filter in the anaerobic work station.
6. All solutions were mixed together in the anaerobic work station just before the new culture of bacteria was started.

SOP3. Degassing of solutions.

1. The nitrogen was provided to the medium with a manifold connected to a needle going through the suba-seals cap into the bottle.
2. The second needle was used as a safety-valve to allow the exit of the gas.
3. Solutions were sparged with oxygen-free N_2 from 20 to 60 min depending on their volume.
4. After sparging the safety-valve was taken out at first, then the needle providing N_2 into the bottle.

SOP4 Preparation of cultures.

1. All activities connected to the *Clostridium sporogenes* cultures were performed in a lab coat and gloves.
2. In the anaerobic work station Agar medium in Petri dishes was inoculated with *Clostridium sporogenes* taken from single colony of the main stock culture growing on a plate using an inoculation plastic loop and re-cultured every week.
3. Pre-cultures were grown in universal bottles containing about 20 mL of Giesel medium and inoculated from the single bacteria colony grown in Petri dish. The culture was incubated in an anaerobic work station with shaking (200 rpm) in 30°C overnight.
4. 500 mL Giesel medium was inoculated by pouring 20 mL pre-culture.
5. Culture was cultivated until it reached the late log phase of growth based on the OD measurements and used for biotransformation.
6. In the anaerobic cabinet liquid media and spare cell suspensions were poured into a beaker with Virkon solution (final concentration 1%) and kept overnight, then disposed to hazardous material bin in general lab.
7. Petri dishes with bacteria in Agar medium were placed to an autoclave bag, taken out of the cabinet and sterilized in the top-desk autoclave in general lab 3.039 (121°C, 20 min). After sterilization plates were placed in biohazard bag.
8. Hands were washed always when leaving the lab area.

SOP5 Microscopic examination of *C. sporogenes.*

1. All activities connected to the *Clostridium sporogenes* cultures were performed in a lab coat and gloves.
2. In the anaerobic cabinet 1mL of culture was pour to a 1.5 mL tube.
3. 20 μL of culture was applied on a basal slide and covered with cover-slip.
4. The slide was placed in a lidded box and taken out of the anaerobic work station to the general lab 3.039.
5. Immersion fluid was applied on the cover-slip and bacteria were observed under 100x magnification.
6. Slides with bacteria were placed in a beaker containing 1% Virkon solution for a night and then thrown away to the sharp-bin.
7. Hands were washed always when leaving the lab area.

SOP6 Measuring the OD of *C. sporogenes.*

1. All activities connected to the *Clostridium sporogenes* cultures were performed in a lab coat and gloves.
2. In the anaerobic cabinet 1 mL of medium was taken out of the culture, diluted 10x in a plastic cuvette and sealed with parafilm.
3. The cuvettes were placed in a plastic box, the box in an autoclave bag, the bag was sealed with a tape and taken out of the anaerobic work station to the general lab 3.039.
4. OD was analyzed using the spectrophotometer (λ_{660nm}).
5. The cuvettes with bacteria culture were placed in a beaker containing Virkon solution (final concentration 1%) for a night and then thrown away to the biohazard bin.

SOP7 Centrifugation of *C. sporogenes.*

1. The culture was transferred to 500 mL centrifuge pots under anaerobic conditions in the anaerobic work station.
2. The pots were tightly sealed and the 1% Virkon solution was applied on their surfaces next to the seal.
3. Pots were placed in an autoclave bag and taken out the anaerobic work station to the general lab 3.039.
4. Tubes with medium were balanced using a scale.
5. Cultures were centrifuged at 6000 rpm for 10 min at +4°C using Jouan KR25i centrifuge.
6. The pots were placed in the anaerobic workstation again where supernatant of the culture was removed to a beaker containing Virkon solution (final concentration 1%) and cells were washed with degassed 50 mM potassium phosphate buffer pH 7.0.
7. Cells were centrifuged again at the same speed.
8. Then in the anaerobic work station the buffer was discarded to a beaker containing Virkon solution (final concentration 1%), cells were re-suspended in a proper volume of degassed 50 mM potassium phosphate buffer pH 7.0 and used for biotransformation.
9. The rotor of the Jouan KR25i centrifuge was cleaned with 1% Virkon solution after each use and the operation was recorded in the centrifuge log book.

SOP8 Using anaerobic cabinet.

1. All activities connected to work in the anaerobic cabinet were performed in suitable protection equipment: a lab coat, gloves, safety specs.
2. Before working in the cabinet it was ensured that all the alarm switches are kept in the "ON" position. The pressures were checked in the gas cylinders, and the 'O' rings on the port bungs were lubricated.
3. It was also checked that there are 1% Virkon solution, a proper waste container (a bottle or jar with sealed cap) and an autoclave bag inside the work station.
4. The chamber was loaded with necessary equipment via the Interchange by opening the outer door, placing the equipment in the Interchange, closing the outer door and initialising the automatic sequence by pressing the "Automatic Sequence Start" button and holding it until a vacuum was drawn on the lock.
5. When the sequence was finished the hands and forearm were passed through the rubber sleeves. The gas from the sleeves was evacuated and replaced by mixed gases from cylinders and the N_2 line three times. Then the bungs were removed and hands were passed into the chamber.
6. The inner door of Interchange was opened and the equipment was lifted into the chamber. Then the inner door was sealed.
7. When the experiment was finished all contaminated equipment (e.g. tips, loops, tubes, etc.) were placed in the container with Virkon solution and incubated there overnight.
8. On the following day the container was placed in an autoclave bag, taken out of the work station and sterilized by autoclaving in the top-desk autoclave in general lab 3.039, then thrown away to the biohazard bin.
9. Cultures and other cell-containing wastes were disposed of as described in the related SOPs for growth and handling of *Clostridium sporogenes*.

SOP9 Servicing of anaerobic cabinets

1. All activities connected to work in the anaerobic cabinet were performed in suitable protection equipment: a lab coat, gloves, safety specs.
2. The cabinet should be serviced and the catalyst and Anatox® sachets changed when required.
3. Electrical checks of the residual current circuit breaker and examination of the pipework from the condenser plate to the pump (looking for blockages) should be performed every month by the senior member of the research group.
4. Three times per week a soap test should (by sprinkling vessels with soapy water and looking for bubbles) be performed to check if the cylinder is not leaking. The sleeves should be examined for holes. The water tank in the bubble trap should be filled up if necessary.
5. Naked flames must not be used in the same room as the cabinet and all personnel should be informed of this by appropriate signs on the door.
6. Good ventilation is provided in the room by the air handling system and the portable oxygen depletion alarm is turned on.
7. The alarm switches are kept in the "ON" position. The pressures are checked in the gas cylinders (hydrogen: 0-30 psi, nitrogen: 0-100 psi and carbon dioxide: 0-100 psi), and the 'O' rings on the port bungs are lubricated with talc if necessary.
8. While using electrical equipment inside the box special precautions were performed to eliminate electric shock or fire: the contact of liquids with electrical apparatus was avoided.
9. In case of oxygen depletion alarm, gas leaking alarm or electrical faults whilst operating the cabinet the operator should leave the glove box and call for emergency assistance.
10. After work the pressure in gas cylinders should be checked and it should be ensured that there is no leak in glove ports.
11. Check that gas admittance is not occurring on a regular basis while box is not in use. If there is an escape of gas for more than five minutes a warning buzzer will sound and the gas supply to the workstation will be cut off.

For Risk Assessments of procedures and COSHH (Control of substances hazardous to health) forms of compounds that were used, see Appendix 7.

5.1.2. Validation of microbiological methods

To determine the growth rate of *C. sporogenes* in Giesel medium supplemented with 27 mM glucose, the medium (0.5 L) was inoculated with a single bacterial colony taken from the cooked meat medium plate. The optical density (λ = 660 nm) of the culture was measured every hour for 24 hours (Fig. 5.1.).

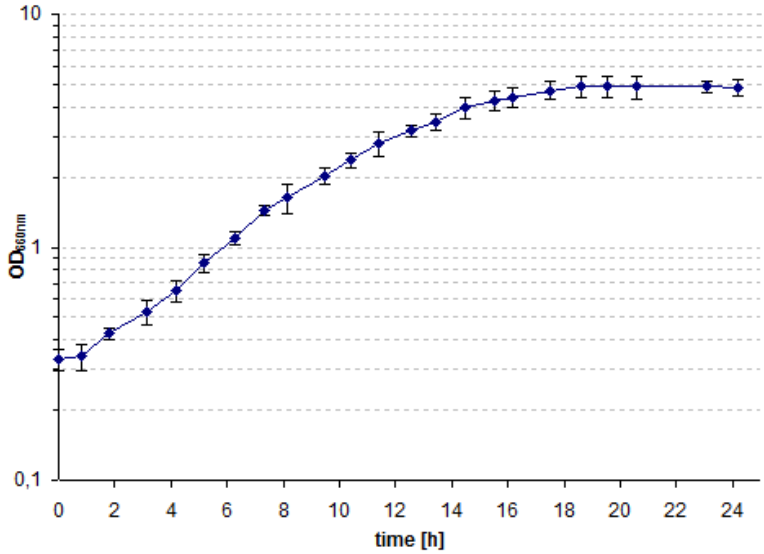


Fig. 5.1. The growth curve in the logarithmic scale for *C. sporogenes* DSM795 in Giesel medium supplemented with 27 mM glucose. Error bars represent standard deviation of three independent experiments.

A short lag phase was observed in the first hour of the culture. The specific growth rate ($\mu = \ln(OD_2 - OD_1)\cdot(t_2 - t_1)^{-1}$) was calculated and it equalled 0.184 h^{-1}, what gave the mean generation time 3.77 h. These parameters were similar with the data obtained previously by Hatziantoniou, 1998 and Chang, 2004 (reports, University of Manchester).

To determine the standard biomass calibration curve of *C. sporogenes* DSM795 a stationary phase culture was centrifuged and cells were resuspended in deionised water to different optical density values. OD_{660nm} of samples were measured using a UV spectrophotometer and samples were transferred to pre-dried and pre-weighed glass vials. Vials were dried to constant weight (Fig. 5.2.).

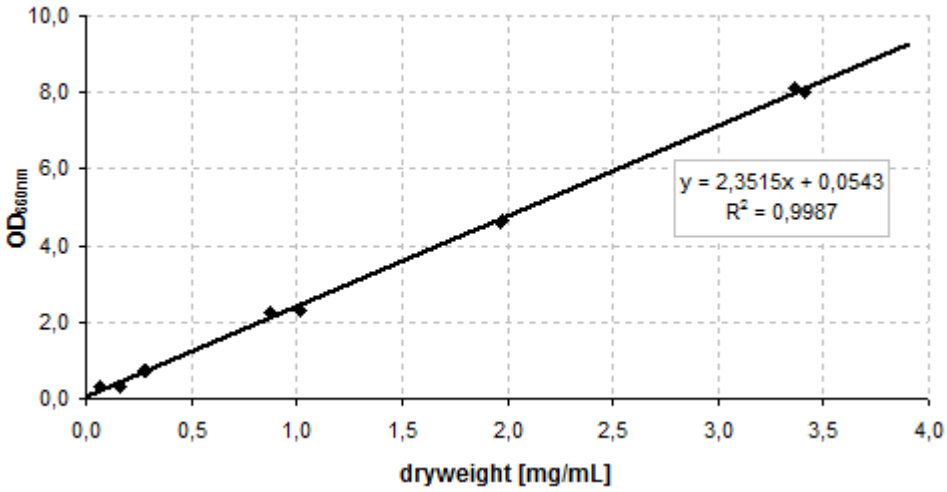


Fig. 5.2. Standard biomass curve for *C. sporogenes* DSM795.

It was shown that 1 mg/mL of the *C. sporogenes* dry biomass equals to 2.35 units of OD_{660nm}. This value is similar to the data obtained by Dipeolu, 2005.

Determination of the specific growth rate and the dry weight showed that the SOPs prepared as a part of this project allowed to cultivate *C. sporogenes* in a reproducible and safe manner without contamination with other bacterial strains.

5.1.3. Conclusions

Standard Operating Procedures for working with *C. sporogenes* and other microorganisms with Biological Safety Level 2 were proposed. The procedures were prepared based on the literature data (medium recipes from Giesel and Simon, 1983; Fryszkowska *et al.*, 2008), laboratory experience of senior members of Gill Stephens' group (Manchester Interdisciplinary Biocentre, University of Manchester) and guidelines provided by the Manchester Interdisciplinary Biocentre Safety Team (University of Manchester). SOPs were tested in practice by running preliminary experiments concerning the growth of *C. sporogenes* and positively accepted by the MIB Biological Safety Officer.

Cultivation of *C. sporogenes* according to directives enclosed in SOPs avoided any contamination of the laboratory by hazardous bacterial strains. At the same time, they guaranteed the sterility of prepared media and avoided any cross contamination of *C. sporogenes* cultures by other bacteria strains or fungi.

This part of the project gave many useful insights on how to handle microorganisms Biological Safety Level 2 to minimize the risk for the researcher and other people. Subsequently it also showed a basic code of practice that should be applied to all types of work when microorganisms are used, to obtain reproducible results of experiments.

5.2. Benzamide reduction

5.2.1. Improved analytical method for benzamide reduction assay

Initially, an analytical method for determination of benzylamine, benzoic acid and benzamide was tested. It had been established by Andrea Dallabona in 2006 (Gill Stephens group, Manchester Interdisciplinary Biocentre). This method involved splitting the reaction mixture into two, acidifying one portion for analysis of acids and basifying

the other portion to analyse the amines. The samples were extracted into dichloromethane (DCM), dried and analyzed on a CP-Sil column. Results of analysis of 0.5 mM benzylamine and 0.5 mM benzamide standard solutions in DCM are presented in Fig. 5.3.

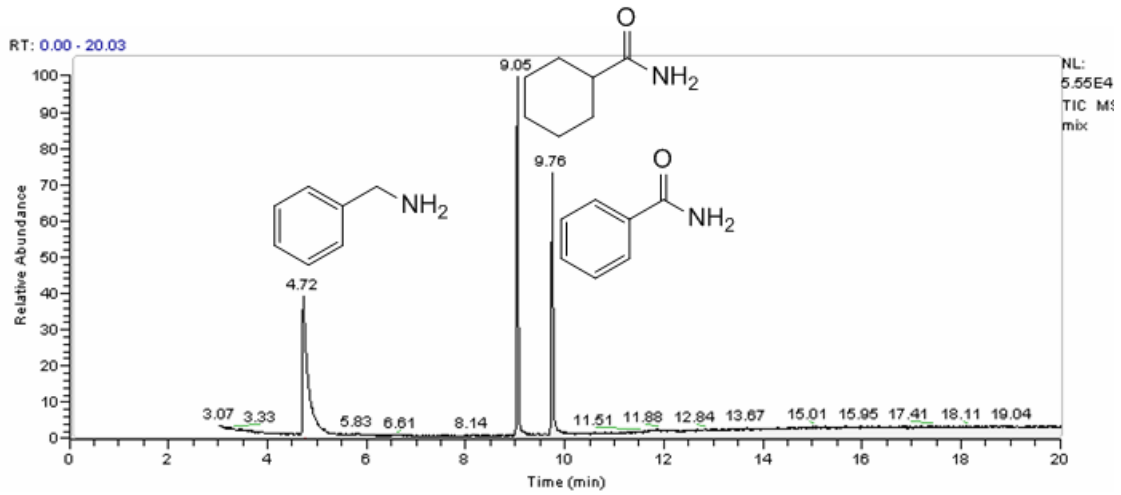


Fig. 5.3. GC-MS analysis of benzylamine and benzamide on CP-Sil column. 0.5 mM cyclohexanecarboxamide was used as the internal standard. Retention times: 4.72 min – benzylamine, 9.05 min – cyclohexanecarboxamide, 9.76 min – benzamide. After biotransformation 10mL of reaction mixture was divided into two equal parts (5 mL each). One of them was basified with 350 µL NaOH to pH>10 (for benzylamine quantification) and the second one was acidified with 1120 µL HCl to pH<3 (analysis of benzoic acid). Compounds were extracted with DCM (2.5 mL) containing 0.5 mM cyclohexanecarboxamide. Samples were dried by passing through a glass pipette containing magnesium sulfate (3 cm layer). 1 µL of each sample was injected to gas chromatograph with connected mass spectrometer.

There were many limitations of the analytical method. First of all the benzylamine peak in the chromatogram was asymmetric with a large tail which caused poor sensitivity. When the sample was diluted, the detection limit was 100 µM benzylamine. Moreover the reproducibility was poor. Secondly, the correlations between the benzylamine and benzamide concentrations and areas of peaks were not linear, but rather polynomial (see Appendix 1). Moreover, DCM was not a suitable solvent for benzylamine extraction, since the extraction efficiency was less than 30-35 %. Furthermore formation of an emulsion was observed; addition of NaCl did not break the emulsion.

Because the expected range of benzylamine concentration in reaction mixtures was from 10 to 100 µM, a new analytical method was needed. Two different strategies for method improvement were possible. First was the derivatization of amines by acylation, silyation or carbamate formation (Nakovich, 2003). The second was using a

GC column dedicated to amine analysis which prevents the adsorption of amines on the column and reduces the tailing of peak.

The latter strategy was chosen since it is simpler, requiring fewer sample preparation steps. Samples were analyzed using a CAM column (Agilent, details in method section; the column was recommended by Dr. Valentin Kohler, personal communication). Because this column is not compatible with mass spectroscopy, an FID detector was used and this also guaranteed higher sensitivity of detection limit (Dr. Valentin Kohler, personal communication). The internal standard (cyclohexanecarboxamide) was replaced with a compound with a lower boiling temperature, *tert*-butylbenzene (TBB). *Tert*-butyl methyl ether (TBME) was used for extraction instead of DCM, because this gave higher extraction efficiency and the solvent did not form emulsion with the reaction mixture. Results of analysis of TBB, benzylamine and benzamide standard solutions in TBME are presented in Fig. 5.4.

The gas chromatogram showed symmetrical peaks for all tested compounds. There was no tailing in the benzylamine peak and the detection limit decreased to 5-10 µM. By testing solutions with different TBB, benzylamine and benzamide concentrations it was proved that the new method is reproducible and the correlation between the area of peaks and compound concentrations is linear (data in Appendix 1). This method was used for analysis of all biotransformations.

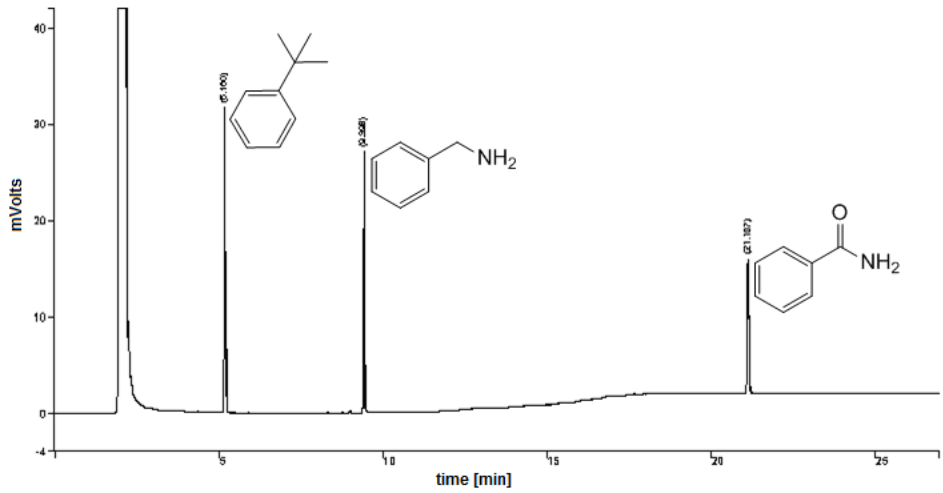


Fig. 5.4. GC-FID analysis of 0.5 mM benzylamine and 0.5 mM benzamide standard solution in TBME analysis. 0.5 mM *tert*-butyl methyl ether was used as the internal standard. Retention times: 5.16 min – tert-butylbenzene, 9.4 min – benzylamine, 21.11 min – benzamide.

5.2.2. Consumption of benzylamine and benzamide by *C. sporogenes*

To check whether benzylamine and benzamide are potential substrates in biotransformations driven by *C. sporogenes*, a whole cell biotransformation (8.5 mg of dryweight/mL) was prepared. Separate reaction mixtures with different substrate concentrations were used for each compound. *C. sporogenes* cells were harvested in the early stationary phase. For a control biotransformation cells were inactivated by autoclaving for approximately 20 min at 121˚C. Biotransformations were prepared as described in Materials and Methods (4.2.8). Results of the analysis for benzamide consumption are shown in Fig. 5.5.

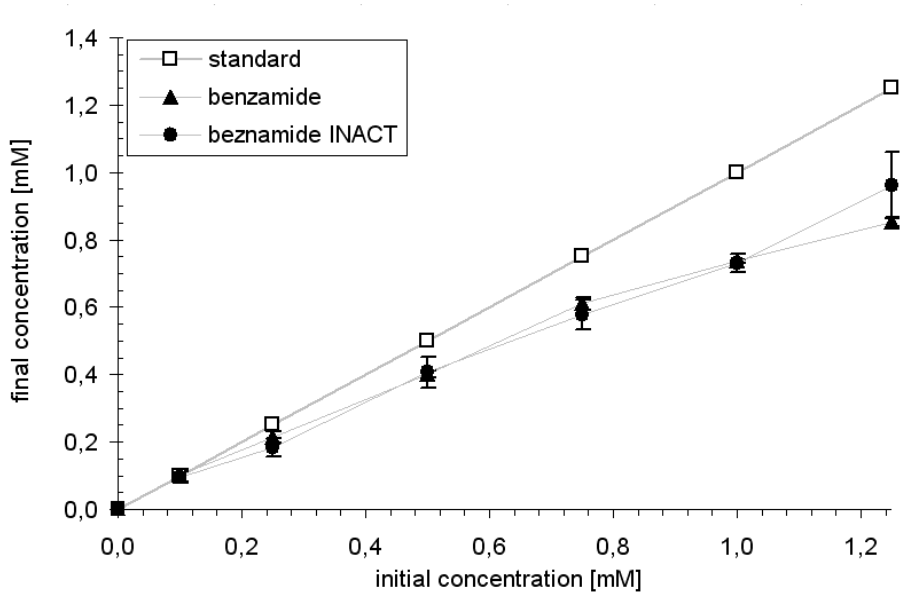


Fig. 5.5. Analysis of benzamide consumption by *C. sporogenes*. Benzamide was extracted from the reaction mixtures after 24 h incubation. Data present average of three independent replicates, error bars correspond to standard error of mean.

Benzamide consumption was observed with a conversion depending on the initial substrate concentration. The higher the initial concentration, the greater the consumption from 10% for 0.1 mM to 30% for 1.25 mM. There was no statistically significant difference between living and heat inactivated cells, indicating that this may have been an abiotic reaction.

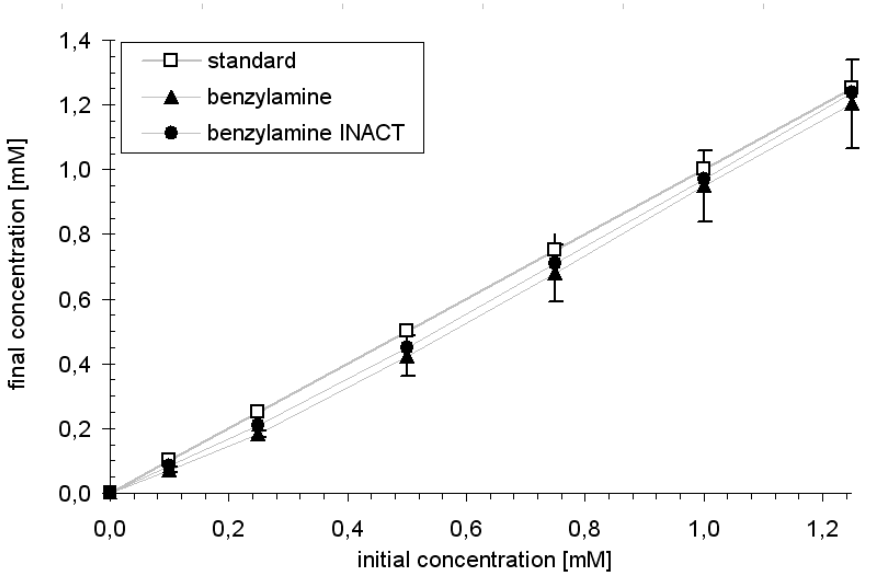


Fig. 5.6. Analysis of benzylamine consumption by *C. sporogenes*. Benzylamine was extracted from the reaction mixtures after 24h incubation. Data present average of three independent replicates, error bars correspond to standard error of mean.

A similar experiment using benzylamine as the substrate showed that this reagent was not consumed by either living or inactivated cells (Fig. 5.6.), what did not confirm previous experiments by Dallabona, 2007 (report, Manchester Interdisciplinary Biocentre).

5.2.3. Benzamide reduction assay

Finally a benzamide reduction assay was performed. *C. sporogenes* cells were harvested in the early stationary phase. The final concentration of cells in the reaction was 8.5 mg dryweight/mL. Five different initial benzamide concentrations were used. Reaction mixtures were examined for the presence of benzamide and benzylamine after 24 h (Fig. 5.7.).

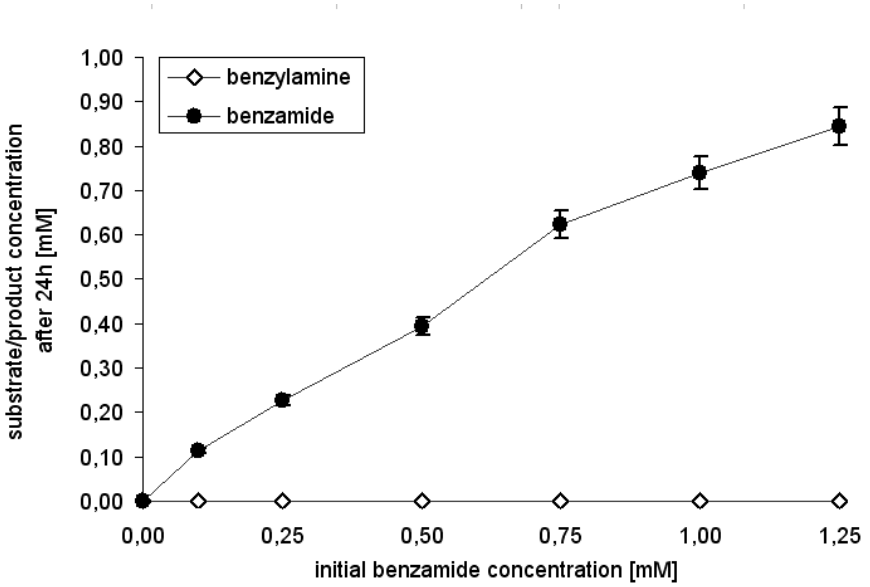


Fig. 5.7. Results of benzamide reduction assay (whole cells, 8.5 mg dryweight/mL). Benzamide and benzylamine were detected using the improved analytical method using GC-FID. Data present average of three independent replicates, error bars correspond to standard error of mean.

No product formation was observed in any sample. Benzamide was consumed during the incubation time with the same yield as observed in previous experiment (see 5.3.; Fig. 5.5). Further optimisation of the amide reduction assay is required. In the future, more efficient hydrogen delivery system will be used. As cells from different phases of growth show differences in their enzyme activities, cells harvested in different stages of growth will be also used to reduce benzamide to benzylamine.

5.3. Phenylnitropropene reduction by *C. sporogenes*

The reduction of phenylnitropropenes using *C. sporogenes* was shown by Fryszkowska *et al.*, 2008. The aim of this part of the project was characterization of enzymes from *C. sporogenes* responsible for this type of biocatalysis. Because the attempt to purify the enzyme failed, the enzymatic activity was lost during the purification procedure (Dr. Karl Fisher, personal communication), an approach based on production of genomic knock-out mutants was proposed. The first enzyme that was chosen to be inactivated, was an enoate reductase, the enzyme responsible for reduction of cinnamic acid to 3-phenylpropionate in the Stickland reaction, since cinnamic acid and phenylnitropropenes tested previously have very similar structure.

5.3.1. Erythromycin resistance of DSM795 and fldZ::CTermB

FldZ::CTermB knock-out mutant of *C. sporogenes* DSM795 strain was prepared by Prof. Nigel Minton group at the University of Nottingham using the ClosTron gene knock-out system. When the L1.LtrB intron from *Lactococcus lactis* has been successfully integrated into the genome the mutants are supposed to be erythromycin resistant, due to the presence of the Em^R gene in the intron. The growth of both DSM795 and fldZ::CTermB was checked in presence of erythromycin (final concentration 2.5 μg/mL) on agar plates with either TYG or cooked meat medium (Table 5.2.).

Table 5.2. The growth of DSM795 and fldZ::CTermB strains in TYG and cooked meat medium with erythromycin (2.5 μg/mL). Results present date from two independent repetitions.

strain	**TYG medium**		**Cooked meat medium**
	-	**erythromycin**	-
DSM795	+	–	+
fldZ::CTermB	+	+	+

Wild type *C. sporogenes* DSM795 was not resistant to erythromycin since its growth was observed only in TYG and cooked meat medium agar plates without the antibiotic. On the other hand, the fldZ::CTermB mutant was able to grow in all media tested, what confirmed a correct integration of L1.LtrB intron into the genome and the presence of active Em^R gene.

To check if the erythromycin affects the growth of DSM795 and fldZ::CTermB, liquid cultures with Giesel medium supplemented with 27 mM glucose were prepared. Cultures (20 mL) were inoculated with overnight pre-culture (1 mL) and OD_{600nm} values were measured over the next 24 h, every hour (Table 5.3. and Appendix 2).

Table 5.3. Growth of DSM795 and fldZ::CTermB in Giesel medium supplemented with glucose (27 mM) in the presence of erythromycin. Results present date from three independent repetitions with standard deviations.

Strain	Growth rate		Max. OD_{660nm}	
	-	erythromycin	-	erythromycin
DSM795	0.23 ± 0.007	0.00 ± 0.000	5.11 ± 0.10	0.59 ± 0.07
fldZ::CTermB	0.21 ± 0.001	0.21 ± 0.003	3.06 ± 0.07	3.05 ± 0.05

There was no significant difference between the growth of fldZ::CTermB in medium with and without erythromycin. Growth rates and maximum OD values of both cultures were similar. As expected wild type DSM795 strain was not able to grow in the presence of erythromycin (the growth rate was close to 0 and the maximum OD below 0.6).

5.3.2. Growth of DSM795 and fldZ::CTermB with different substrates

Because the *fldZ* gene was predicted to encode an enoate reductase enzyme that is involved in the metabolic pathway for L-phenylalanine reduction coupled with ATP formation (Bader and Simon, 1983), the physiology of wild type DSM795 and fldZ::CTermB mutant was checked in different variants of Giesel medium. L-phenylalanine (12.1 mM) or glucose (27 mM) were used as the energy source in the media. Cultures were inoculated with an overnight pre-culture (1 mL; Fig. 5.8. and Appendix 3).

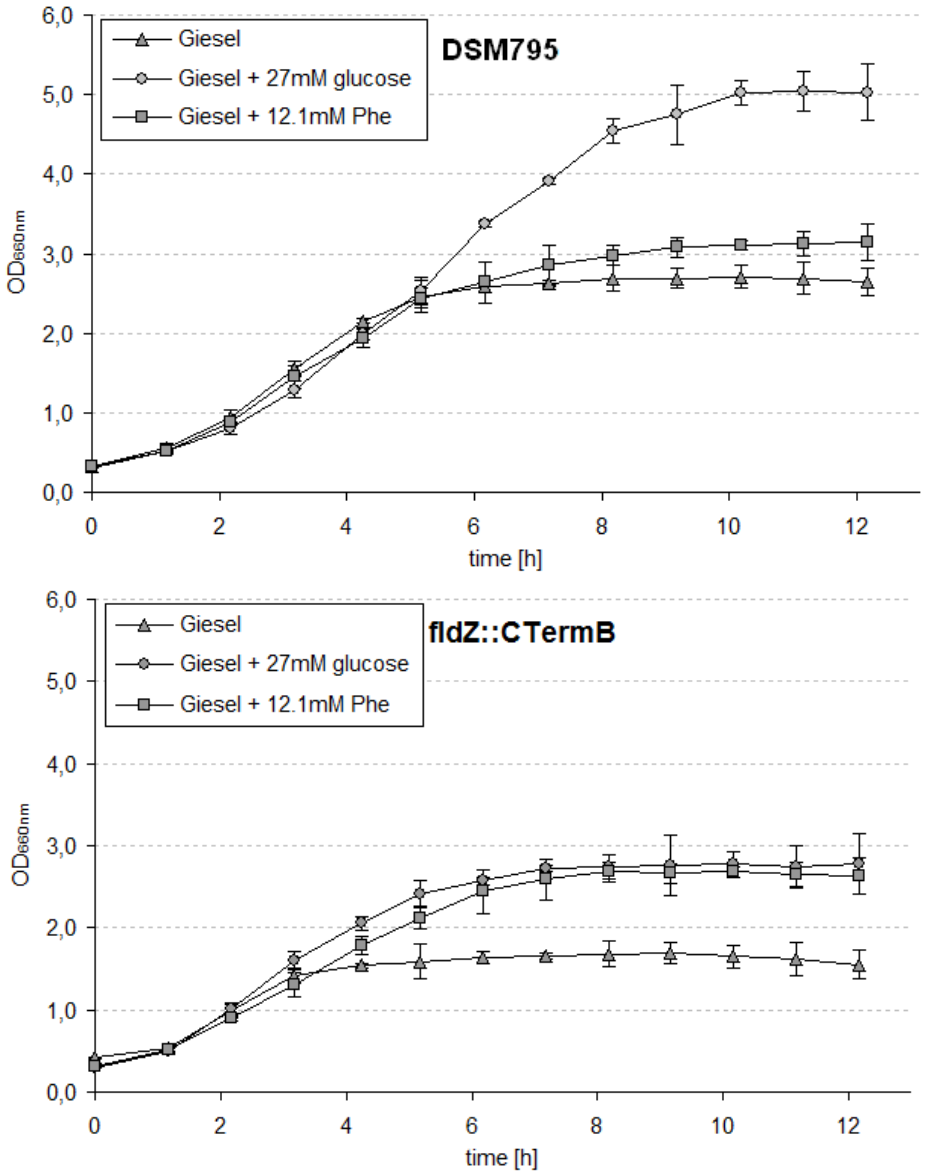


Fig. 5.8. Growth of DSM795 (top) and fldZ::CTermB (bottom graph) in basal Giesel medium (▲) and Giesel medium supplemented with 27 mM glucose (●) or 12.1 mM L-phenylalanine (■). Average of three independent replicates. Error bars correspond to standard deviations.

Growth rates in all samples were similar (Table 5.4; raw data are shown in Appendix 3). No significant differences between DSM795 and fldZ::CTermB were observed for Giesel medium with L-phenylalanine. Maximum OD_{660nm} values decreased from 5.04 and 3.14 (DSM795) to 2.78 and 2.68 (fldZ::CTermB) in basal medium and Giesel supplemented with glucose, respectively.

Table 5.4. Physiology of DSM795 and fldZ::CTermB in different variants of Giesel medium. Data present means of three independent replications with standard deviations.

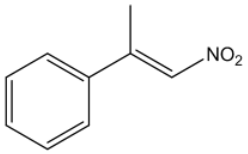
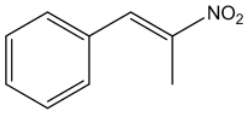
Medium	Specific growth rate		Max. OD_{660nm}	
	DSM795	**fldZ::CTermB**	**DSM795**	**fldZ::CTermB**
Giesel	0.21 ± 0.005	0.19 ± 008	2.71 ± 0.14	1.70 ± 0.05
Giesel + 27 mM glucose	0.20 ± 0. 007	0.24 ± 008	5.04 ± 0.25	2.78 ± 0.24
Giesel + 12.1 mM L-Phe	0.21 ± 0. 003	0.20 ± 004	3.14 ± 0.23	2.68 ± 0.25

Results of this experiment showed unexpected physiological behaviour. There was a dramatic change in the biomass yield between DSM795 and fldZ::CTermB for medium supplemented with glucose and only minor difference, when bacteria were growing using L-phenylalanine as the substrate. *FldZ* gene should encode enoate reductase that was thought to be essential for amino acid metabolism. No role for this type of enzymatic activity was proposed in metabolic pathways of carbohydrates. Further analysis of the DSM795 and fldZ::CTermB strains including the metabolome analysis is needed.

5.3.3. Reduction of phenylnitropropene using harvested cells

To check if the protein encoded by *fldZ* gene is involved in reduction of C=C bonds in phenylnitropropenes, whole cells of DSM795 and fldZ::CTermB strains were used to reduce two phenylnitropropenes *(E)*-1-nitro-2-phenyl-propene and *(E)*-1-phenyl-2-nitro-propene. Cells growing in Giesel medium supplemented with 12.1 mM L-phenylalanine were harvested in the early stationary phase of growth. The reaction mixture was prepared using biphasic system (*iso*-octane:phosphate buffer, 1:2) with hydrogen as an electron donor. After 72 h incubation the organic phases were analyzed using HPLC (Table 5.5 and Appendix 4).

Table 5.5. Whole cell biocatalyst reduction of *(E)*-1-nitro-2-phenyl-propene and *(E)*-1-phenyl-2-nitro-propene using DSM795 and fldZ::CTermB. Data present means of three independent replications with standard errors of mean.

Substrate	Sample	Yield [%]	ee. [%]
 *(E)*-1-nitro-2-phenyl-propene	DSM795	19.5 ± 0.81	≥99.9 ± 0.00
	fldZ::CTermB	0.0 ± 0.00	-
 *(E)*-1-phenyl-2-nitro-propene	DSM795	4.7 ± 0.43	3.0 ± 1.6
	fldZ::CTermB	13.5 ± 2.37	1.0 ± 1.4

(E)-1-nitro-2-phenyl-propene was reduced by DSM795 with 19.5% yield and almost 100% enantiomeric excess, whereas no product was found in samples with fldZ::CTermB cells. DSM795 reduced *(E)*-1-phenyl-2-nitro-propene with 4.7% yield and 3% ee. When fldZ::CTermB cells were used, the yield was higher (13.5%) although the ee. was slightly lower (1%).

Results showed that enoate reductase is responsible for the reduction of C=C bond in *(E)*-1-nitro-2-phenyl-propene, whereas the reduction of *(E)*-1-phenyl-2-nitro-propene may be driven by some other enzyme(s).

5.3.4. DSM795 and fldZ::CTermB protein extracts

Based on nucleotide sequence the molecular weight of *C. sporogenes* fldZ reductase enzyme was predicted to be ~73 kDa. To check if there is a difference in protein pattern between DSM795 and fldZ::CTermB, protein extracts were produced from these cells growing on Giesel medium with L-phenylalanine using the French pressure cell. Protein concentration was quantified using *DC* Protein Assay. Samples were analyzed by SDS-PAGE gel electrophoresis (Fig. 5.9.).

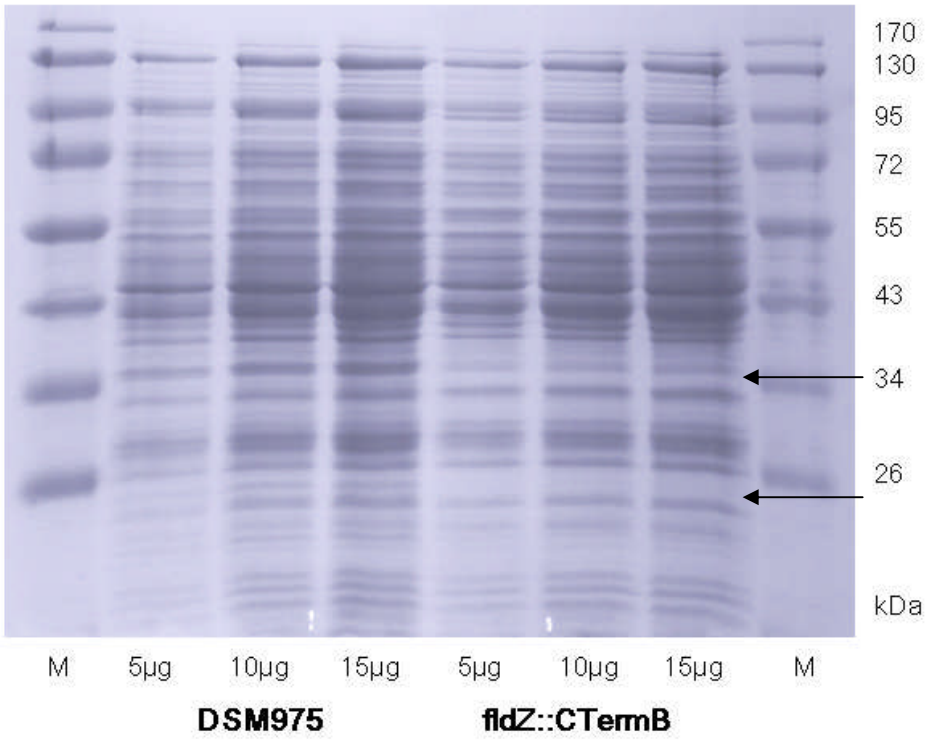


Fig. 5.9. SDS-PAGE gel electrophoresis of proteins extracted from DSM795 and fldZ::CTermB. 5 µg, 10 µg and 15 µg of each sample were applied. M – molecular weight marker, PageRuler™ Prestained Protein Ladder (Fermentas). Differences in the band pattern between DSM795 and fldZ::CTermB are marked with arrows.

Gel electrophoresis confirmed the good quality of the extracts since the bands were distinct and clear. No difference in the band pattern was shown in range 73kDa. Thus, although the bands were present in all samples their intensity was decreased in the fldZ::CTermB sample.

Probably, the expression level of enoate reductase in DSM795 is too low to observe differences in the band pattern between these samples using SDS-PAGE gel electrophoresis. The western blot technique with anti-fldZ antibody cross-reaction would be more sensitive and should be used in the future to verify the mutant.

Then, protein extracts were used in the spectrophotometric cinnamic acid and phenylnitropropene reduction assays.

5.3.5. Spectrophotometric cinnamic acid reduction assay

The last step in the L-phenylalanine metabolism pathway in proteolytic Clostridia is reduction of *(E)*-cinnamic acid to 3-phenylpropionate driven by enoate reductase as shown in Fig. 5.10.

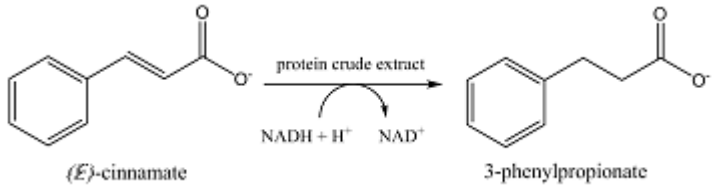


Fig. 5.10. Reduction of *(E)*-cinnamic acid to 3-phenylpropionate by enoate reductase.

To test the hypothesis that *fldZ* encodes reductase is responsible for cinnamic acid reduction, protein extracts from DSM795 and fldZ::CTermB strains were used in spectrophotometric cinnamic acid reduction assays. Under anaerobic conditions extracts were mixed with *(E)*-cinnamic acid and NADH. The reaction was observed by consumption of NADH causing decrease of absorbance at 340 nm (Fig. 5.11. and Appendix 5).

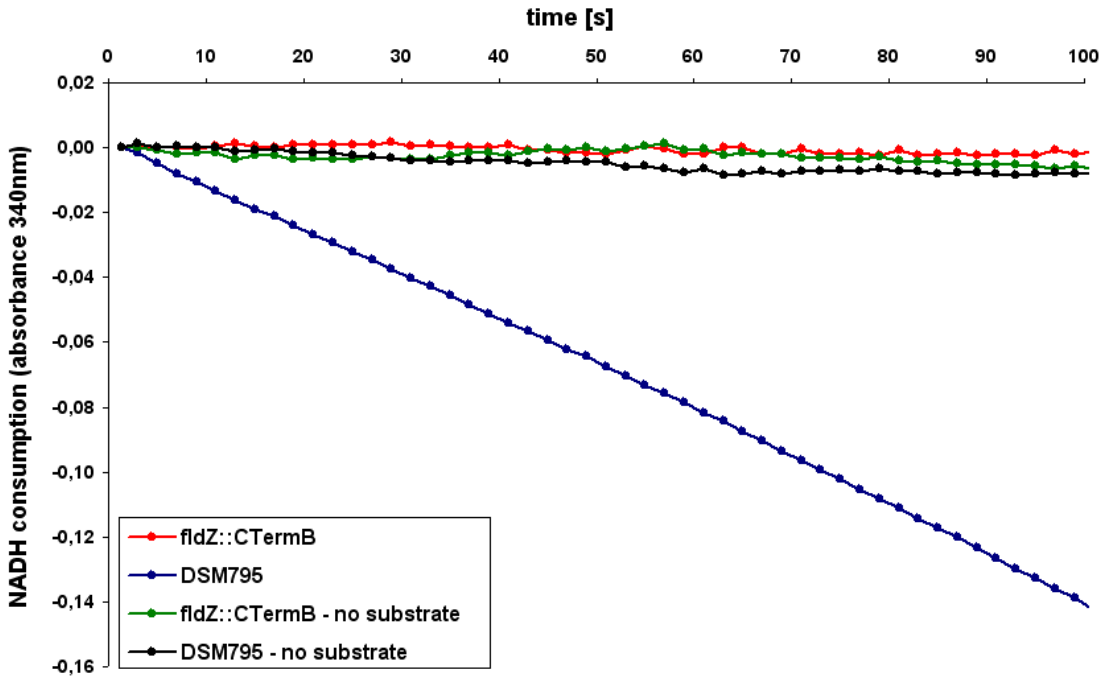


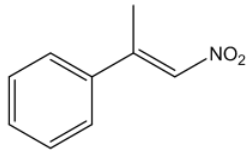
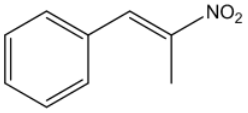
Fig. 5.11. Spectrophotometric cinnamic reduction assay. *(E)*-cinnamic acid was reduced to 3-phenylpropionate coupled with consumption of NADH.

Reduction of cinnamic acid was observed only in samples with protein extract from DSM795 (with NADH consumption 13.8 µmol/min). No decrease of absorbance was shown with protein extract from fldZ::CTermB and in control samples without the substrate, thus fldZ::CTermB does not express an active enoate reductase.

5.3.6. Phenylnitropropene reduction assay using protein extracts

The protein extracts produced as described above were also used to reduce the two phenylnitropropene isomers *(E)*-1-nitro-2-phenyl-propene and *(E)*-1-phenyl-2-nitro-propene. In the reaction NADH was used as the cofactor. Samples were incubated at 30˚C for 72 h and analyzed using HPLC (Table 5.7. and Appendix 6).

Table 5.6. Reduction of *(E)*-1-nitro-2-phenyl-propene and *(E)*-1-phenyl-2-nitro-propene using DSM795 and fldZ::CTermB protein extracts. Data present means of three independent replications with standard errors of mean.

Substrate	Sample	Yield [%]	ee. [%]
 *(E)*-1-nitro-2-phenyl-propene	DSM795	97.8 ± 0.59	≥99.9 ± 0.00
	fldZ::CTermB	0.0 ± 0.00	-
 *(E)*-1-phenyl-2-nitro-propene	DSM795	55.9 ± 0.49	9.5 ± 0.50
	fldZ::CTermB	27.6 ± 0.10	4.0 ± 0.01

Using the protein extract the yield of reduction *(E)*-1-nitro-2-phenyl-propene by DSM795 was improved to almost 100% and almost 100% enantiomeric excess, when compared with whole cell biocatalysts assay. Again, no product was found in samples of fldZ::CTermB mutant.

DSM795 reduced *(E)*-1-phenyl-2-nitro-propene with about 55% yield and 10% ee. When fldZ::CTermB extract was used, the yield was lower (27.6%) with a different enantiomers composition (ee. 4%). *(E)*-1-phenyl-2-nitro-propene can be reduced by the enoate reductase, but in the mutant the activity of other reductases remained and allowed for reduction of this compound.

6. Discussion and future work

6.1. Amide reduction

Attempts to use the benzylamine detection method described by Dallabona in his Transfer Report (Manchester Interdisciplinary Biocentre, 2006) have met with limited success. By changing the type of detector and using a different column the method was significantly improved.

Different concentrations of benzamide incubated with *C. sporogenes* cell suspensions showed that this compound is converted by both living and heat inactivated biocatalysts. The most probable reactions that may have occurred in the samples was hydrolysis of benzamide to benzoic acid by a heat-stable enzyme or cell component, or an abiotic and spontaneous reaction. These mechanisms were previously proposed by Dipeolu *et al.* (2005). On the other hand incubation of benzylamine with whole cells of *C. sporogenes* showed that this primary amine was not metabolized. That made it a perfect product for the amide reduction biotransformation as it was not converted to any other compound in further reactions.

Attempts to reduce benzamide to benzylamine were not successful. Benzamide was consumed during the incubation time with the same yield as previously observed, but there was no product formation in any of the samples that were tested. There were a few possible reasons why the biotransformations did not proceed as described by Dipeolu *et al.* (2005). The amount of the biocatalysts in samples may have been too low and the reaction time too short to observe benzylamine formation. Dipeolu *et al.* (2005) observed some unusual kinetics of the reaction where the product was detected after 2 h latency, however the substrate was consumed from the beginning of the reaction. This observation was explained by hypothetical side reactions on the reaction intermediate (hydrolysis of imine followed by further consumption of products that were formed). Moreover, the biotransformation was started with injection of the hydrogen to the flasks with reaction mixtures. Because the reduction of amide to amine is connected to a great change in the oxidation level of the substrate, it is possible that the amount of the hydrogen serving as an electron donor used in the reaction was not adequate to support the biotransformation. Last but not least, cells from different phases of growth are characterized by different enzyme activities. Dallabona in 2006 observed that there was no amide reductase activity when cells were harvested in the early exponential phase of growth, but benzylamine formation was detected when cell were harvested in the late

log phase. In this project cells were harvested in the early stationary phase of growth. This suggests that amide reduction may have been determined by a dehydrogenase that played a role in the some fermentation pathways during the growth of the culture and its expression could be terminated when culture used all the nutrition compounds from the medium and reached the stationary phase. Alternatively, although in the experiments described by Dipeolu *et al.* (2005) and in this project the same strain of *C. sporogenes* was used and both stock cultures were purchased from the same source (the DSMZ collection), there may have been some differences between the microorganisms tested in the biotransformations caused by genetic diversity processes among one species.

The future work on amide reduction will be focused on the further optimization of the biotransformation method. Cells from different physiological stages of growth will be tested and the amount of the biocatalysts used in the biotransformation will be increased. More efficient hydrogen delivery systems will be created to allow a constant flow of the electron donor to the samples.

6.2. Phenylnitropropene reduction

The hydrogenation of unsaturated C=C bonds in phenylnitropropenes by *C. sporogenes* was described and optimized by Fryszkowska *et al.* (2008). It was shown that, using *C. sporogenes*, the highly enantioselective reduction of nitroalkenes can be observed with good yields. However, the enzyme or enzymes responsible for this type of activity were unidentified. It has been known from the 1980s that *C. sporogenes* possesses an enoate reductase, an enzyme of the 'Old Yellow Enzyme' family, that has been implicated in the Stickland metabolism of amino acids (Buhler *et al.*, 1980; Giesel *et al.*, 1981; Bader *et al.*, 1982; Bader *et al.*, 1983). Because of the structural similarities between physiological substrates of enoate reductase and phenylnitropropenes tested in the biotransformations, this enzyme was suspected to be responsible for the enzyme activity in the biocatalyst.

In previous projects, the purification of the phenylnitropropene reductase had been unsuccessful as the enzyme activity was lost during the protein chromatography steps. Therefore, the approach using production of genomic enoate reductase knock-out mutants was used to confirm that this enzyme is responsible for reduction of those substrates.

The growth experiments in agar plates on medium containing erythromycin showed that the L1.LtrB intron positively integrated to the *C. sporogenes* genome and fldZ::CTermB mutant had a resistance to this antibiotic. Cultures in flasks containing Giesel medium with glucose as the energy source and erythromycin showed that the antibiotic had an inhibitory effect on growth of DSM795, whereas fldZ::CTermB mutant gave significant biomass production. Moreover there were no differences in the growth rate and maximum OD of the culture for mutants growing with and without erythromycin showing that the antibiotic had no effect on the growth of fldZ::CTermB.

Using the spectrophotometric assay it was shown that the enoate reductase mutant was not able to reduce cinnamic acid to 3-phenylpropionate. This experiment confirmed that the gene which was knocked-out using the ClosTron system encodes the enzyme showing the activity of enoate reductase.

The experiments on the growth of DSM795 and fldZ::CTermB in media supplemented with different fermentation substrates gave intriguing results. Although there were not big differences between growth rates of cultures in different media, significant variations of the biomass production were observed. The basal Giesel medium was rich in peptides (from peptone and yeast extract) and carbohydrates (from yeast extract) and could support the growth of bacteria to some extent. Inactivation of the enoate reductase decreased the max OD value which was probably caused by limitation of substrates that could be used in fermentation process.

FldZ::CTermB mutant growing in medium containing glucose showed lower biomass production when compared to the wild type strain. This was surprising since the involvement of enoate reductase was not postulated in the glycolysis pathway. The reduction of the biomass production for fldZ::CTermB mutant could be explained in two ways. In the presence of carbohydrates, *C. sporogenes* can produce energy *via* a mixed Stickland metabolism, where glucose serves as the electron donor and amino acids as the electron acceptors. In the wild type, several amino acids would normally serve as electron acceptors in reductive branch of Stickland metabolism involving enoate reductase, *e.g.* phenylalanine, tyrosine, leucine or isoleucine (Barker, 1981). In the mutant, this would not be possible and instead growth of the culture would have to be supported by reduction of proline and glycine only, the amino acids possessing their reduction pathways. On depletion of theses amino acids the mutant would have to switch to butyrate production as the main electron acceptor. This would result in decreased ATP production and decreased growth yield.

It is difficult to study the physiology of bacteria using a complex media such as Giesel medium. As the next step of the project the growth of wild type *C. sporogenes* DSM795 and enoate reductase knock-out mutant fldZ::CTermB will be examined in the minimal defined medium containing only necessary nutrients and supplemented with different combinations of electron donors and acceptors to test the hypothesis described above (Lovitt *et al.*, 1987a). Furthermore, the fermentation products and the *C. sporogenes* metabolome will be analyzed to determine which metabolic pathways are interrupted by inactivation of enoate reductase expression.

The putative molecular weight of enoate reductase in *C. sporogenes* was predicted based on the molecular weights of enoate reductases form *C. tyrobutyricum* and *C. kluyveri* (Tischer *et al.*, 1979; Kuno *et al.*, 1985). The analysis of the protein extracted from DSM795 wild type and fldZ::CTermB mutant by SDS-PAGE electrophoresis showed no differences in the band pattern between these two strains in range of 73 kDa. The enoate reductase expression may have been too low to visualize it using standard staining procedure. A more sensitive method may be needed such as western blot technique, but this depends on having an antibody against enoate reductase, which is not available. However bands at 25 and 34 kDa were less intensive in the mutant. Since these bands were too small to be encoded by the deleted gene, the expression of these proteins may have been connected to regulatory changes due to the disruption of the Stickland pathway.

Phenylnitropropene biotransformations using whole cells and protein extracts showed that enoate reductase fldZ::CTermB knock-out mutant, was not able to reduce *(E)*-1-nitro-2-phenylpropene, in contrast to the wild type DSM795. The experiment confirmed the hypothesis that enoate reductase was responsible for reduction of this substrate. On the other hand, reduction of *(E)*-1-phenyl-2-nitropropene was characterized by lower yield, when protein extracts were used. It is possible that this substrate was hydrogenated by two independent reductases, where enoate reductase is one of them and the second one is unknown.

It was confirmed that enoate reductase from *C. sporogenes* can reduce phenylnitropropenes in a stereospecific way and it is worth considering this enzyme as a potential new biocatalyst for industrial biotransformations. In the future the gene encoding enoate reductase will be identified using PCR primers designed for the enoate reductase gene in the closely related species *C. botulinum*. Afterwards, the enzyme will be overexpressed in *E. coli* and the scope for reduction of nitroalkenes and other

unsaturated compounds will be tested. Moreover, the fldZ::CTermB mutant will be used for purification of the enzyme responsible for the reduction of *(E)*-1-phenyl-2-nitropropene. The inactivation of enoate reductase will make the process simpler by reducing the number of protein fractions showing the nitropropene reductase activity. All these findings provide useful recommendations for future work as *C. sporogenes* contains unique enzyme activities and can give a big contribution in the field of industrial biotransformations.

7. Literature

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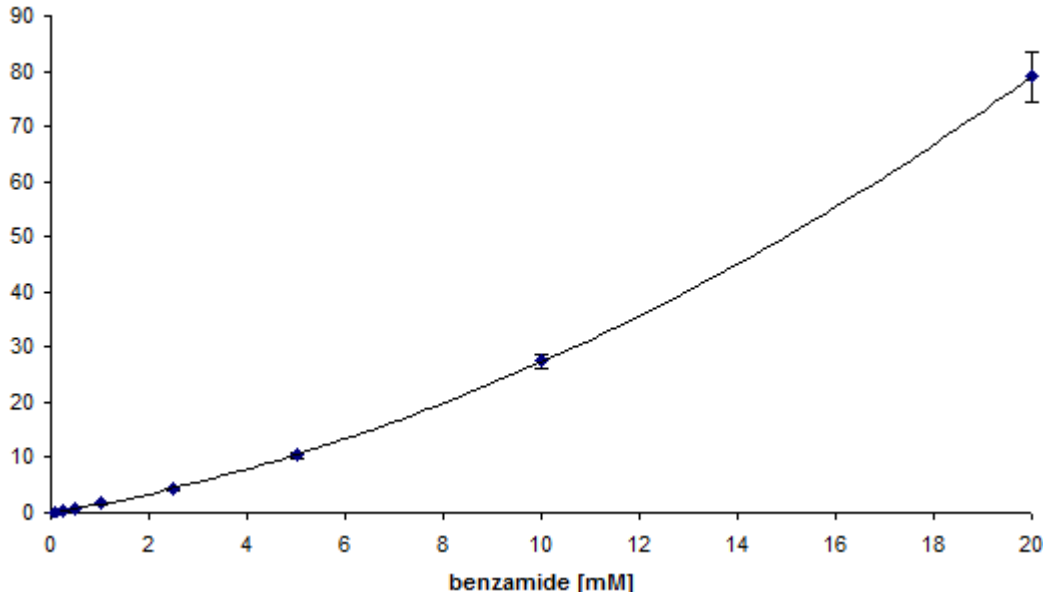
8. APPENDICES

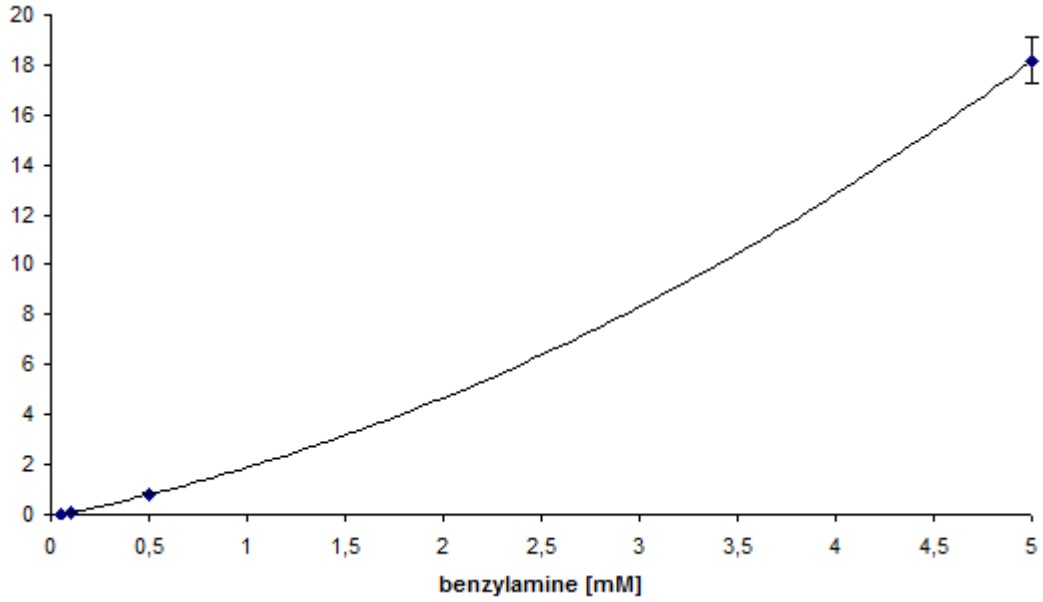
APPENDIX 1. Calibration curves for amide reduction assay

1. Calibration curves prepared with GC-MS (CP-Sil column)

1.1. Benzamide calibration curve against 0.5 mM cyclohexanecarboxamide (dissolved in DCM and diluted within the range concentrations to be tested experimentally).

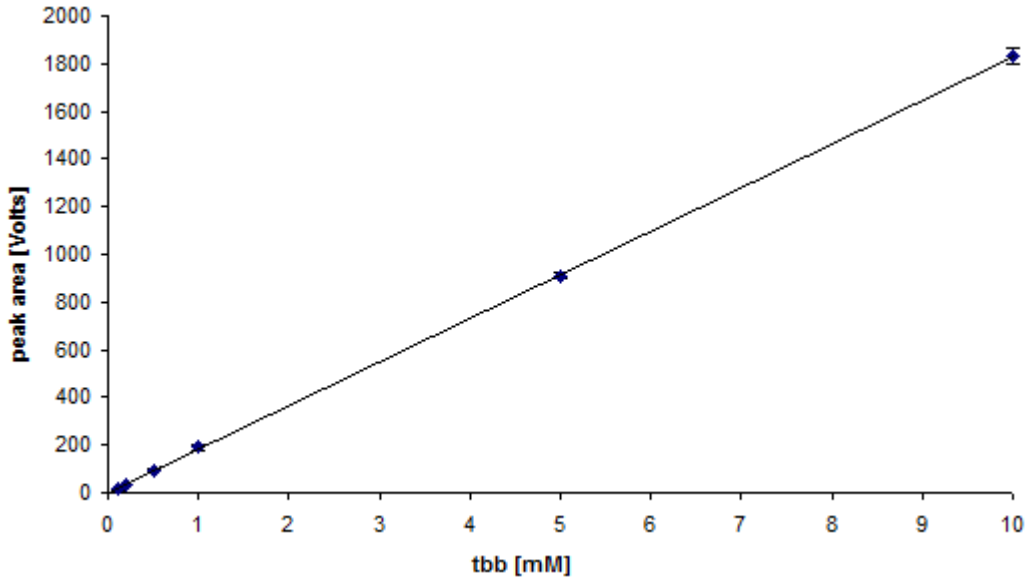


1.2. Benzylamine calibration curve against 0.5 mM cyclohexanecarboxamide (dissolved in DCM and diluted within the range concentrations to be tested experimentally).

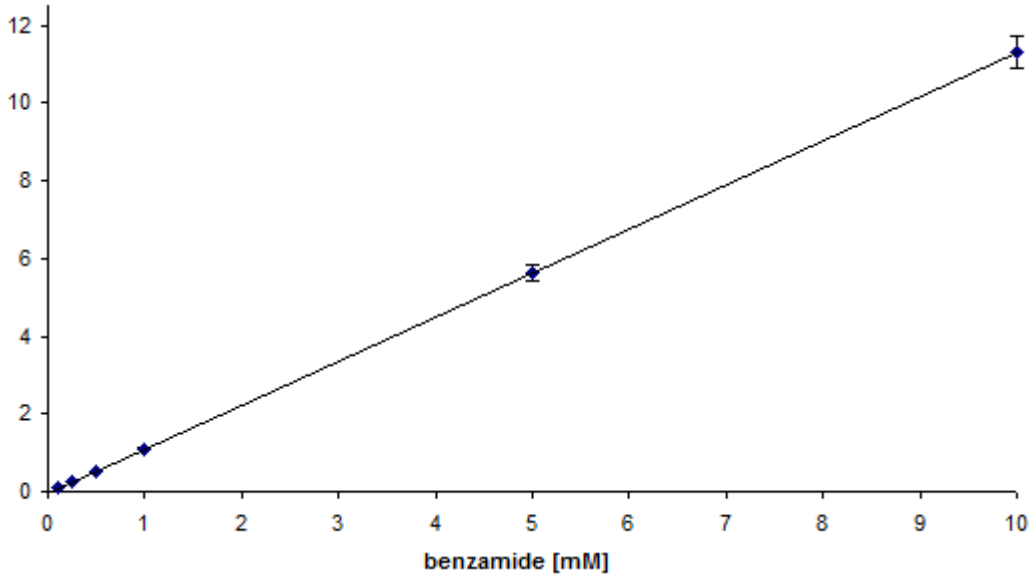


2. Calibration curves prepared with GC-FID (CAM column)

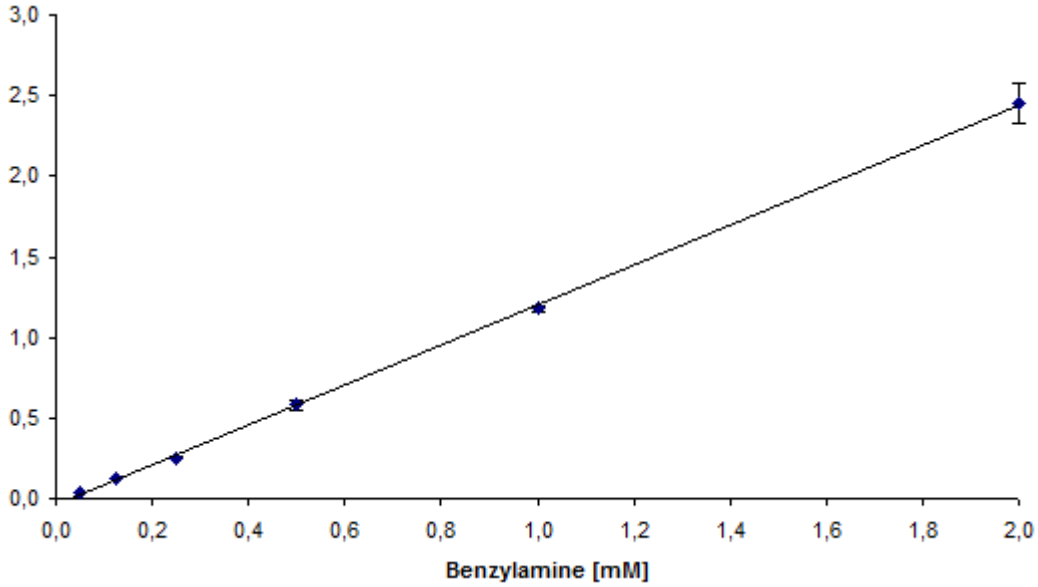
2.1. *tert*-butylbenzene calibration curve (dissolved in TBME and diluted within the range concentrations to be tested experimentally.



2.2. Benzamide calibration curve against 0.5 mM *tert*-butylbenzene (dissolved in ethanol, diluted with 50 mM potassium phosphate buffer within the range concentrations, basified to pH>10 and extracted with 0.5 mM *tert*-butylbenzene in TBME)

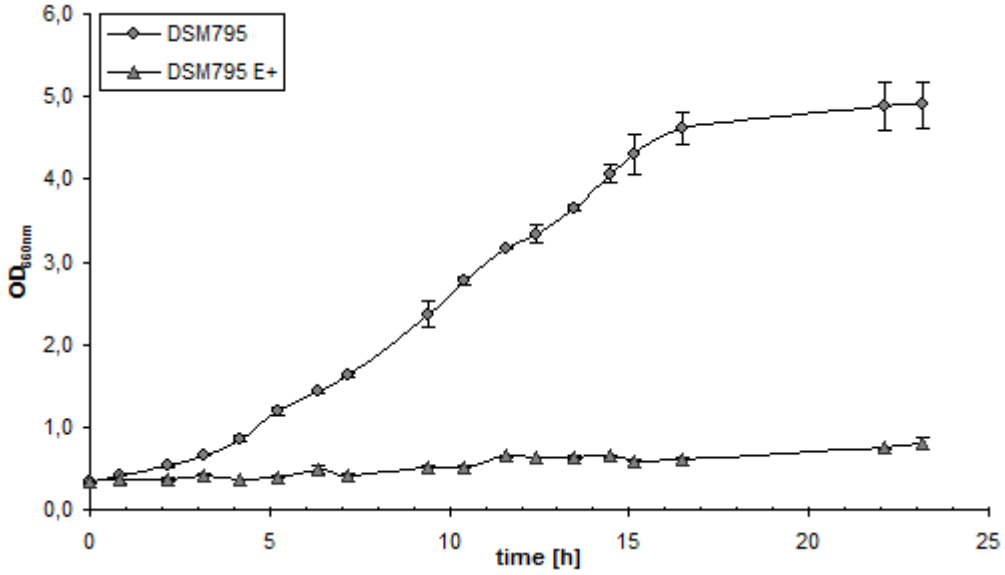


2.3. Benzylamine calibration curve against 0.5 mM *tert*-butylbenzene (dissolved in ethanol, diluted with 50 mM potassium phosphate buffer within the range concentrations, basified to pH>10 and extracted with 0.5 mM *tert*-butylbenzene in TBME)

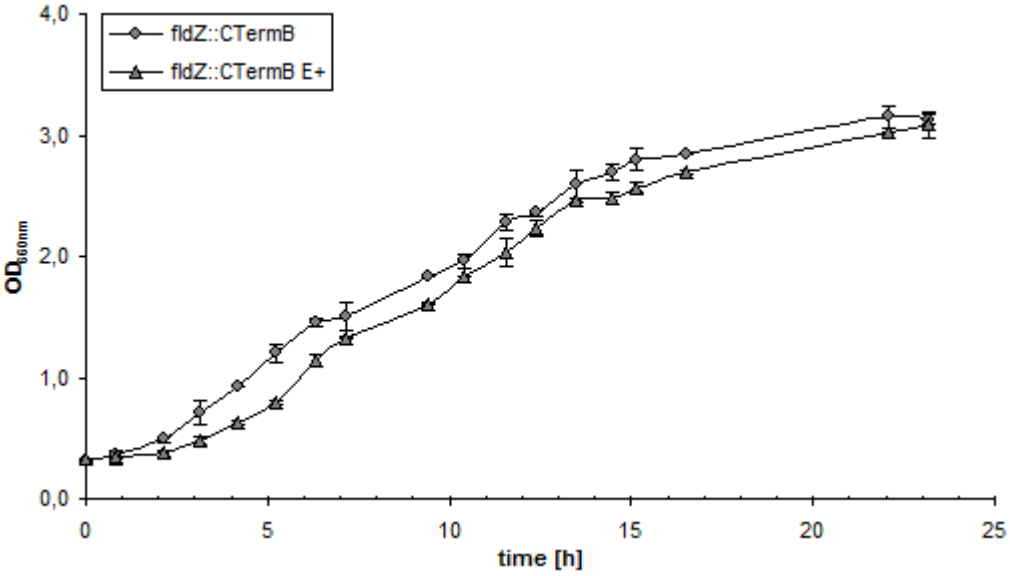


APPENDIX 2. Growth curves of DSM795 and fldZ::CTermB with and without 2.5 µg/mL erythromycin

1. Growth curve of DSM795 in Giesel medium supplemented with 27 mM glucose in the presence of erythromycin 2.5 µg/mL



2. Growth curve of fldZ::CTermB in Giesel medium supplemented with 27 mM glucose in the presence of erythromycin 2.5 µg/mL



APPENDIX 3. Results of growth curve analysis of DSM795 and fldZ::CTermB in Giesel medium supplemented with 27 mM glucose and 12.1 mM L-phenylalanine

DSM795:

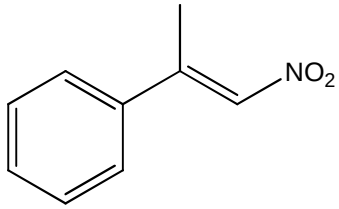
Time	OD660nm (average of three replicates)			Standard deviation		
	Giesel	Giesel + 27 mM glucose	Giesel + 12.1 mM Phe	Giesel	Giesel + 27 mM glucose	Giesel + 12.1 mM Phe
0.00	0.33	0.31	0.32	0.01	0.00	0.01
1.17	0.57	0.52	0.52	0.05	0.02	0.04
2.17	0.94	0.80	0.89	0.09	0.07	0.05
3.17	1.55	1.29	1.45	0.10	0.10	0.14
4.25	2.15	1.99	1.94	0.03	0.08	0.11
5.17	2.48	2.53	2.44	0.21	0.17	0.13
6.17	2.59	3.37	2.64	0.07	0.04	0.26
7.17	2.63	3.91	2.85	0.04	0.03	0.25
8.17	2.69	4.54	2.98	0.16	0.15	0.12
9.17	2.69	4.75	3.08	0.13	0.38	0.12
10.17	2.71	5.02	3.10	0.14	0.16	0.06
11.17	2.69	5.04	3.12	0.20	0.25	0.16
12.17	2.65	5.03	3.14	0.17	0.36	0.23

fldZ::CTermB:

Time	OD660nm (average of three replicates)			Standard deviation		
	Giesel	Giesel + 27 mM glucose	Giesel + 12.1 mM Phe	Giesel	Giesel + 27 mM glucose	Giesel + 12.1 mM Phe
0.00	0.42	0.30	0.31	0.01	0.02	0.01
1.17	0.54	0.51	0.52	0.05	0.01	0.04
2.17	0.99	1.01	0.91	0.09	0.03	0.02
3.17	1.42	1.60	1.31	0.01	0.11	0.11
4.25	1.54	2.06	1.79	0.09	0.02	0.15
5.17	1.59	2.42	2.11	0.04	0.11	0.17
6.17	1.64	2.57	2.44	0.08	0.20	0.05
7.17	1.66	2.72	2.59	0.06	0.20	0.21
8.17	1.68	2.74	2.68	0.06	0.19	0.25
9.17	1.70	2.76	2.66	0.05	0.12	0.25
10.17	1.65	2.77	2.68	0.15	0.09	0.04
11.17	1.62	2.75	2.65	0.02	0.21	0.15
12.17	1.55	2.78	2.63	0.12	0.24	0.02

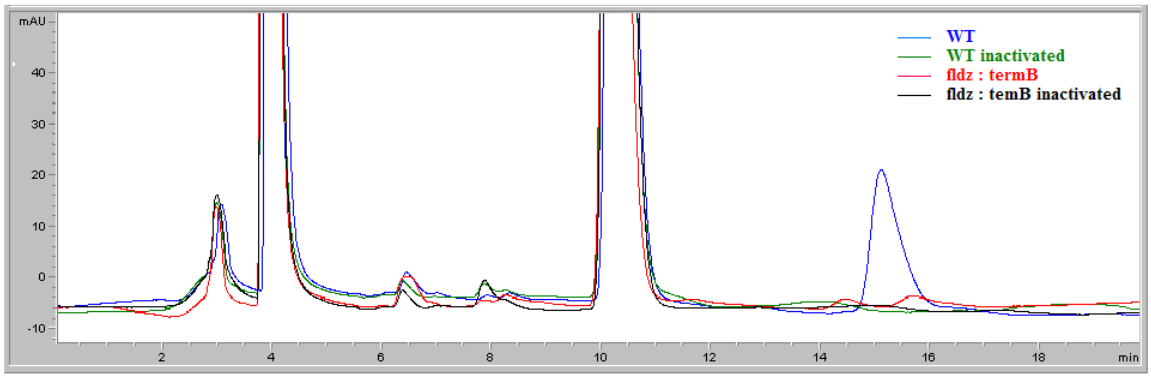
APPENDIX 4. Results of the whole call biocatalysts phenylnitropropene reduction assay

Substrate: *(E)*-1-nitro-2-phenyl-propene

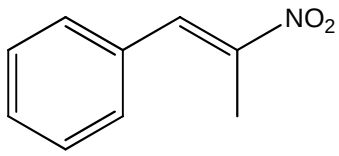


	Concentration [mM]			Standard Error of Mean		
	product	substrate	ee.	product	substrate	ee.
DSM795	0.33	1.52	100%	0.015	0.055	0.000
DSM795 - inactivated	0.00	1.86	-	0.000	0.048	-
fldZ::CTermB	0.00	1.31	-	0.000	0.027	-
fldZ::CTermB - inactivated	0.00	1.88	-	0.000	0.037	-

Retention times:
4.07 s – *tert*-butylbenzene
10.57 s – *(E)*-1-nitro-2-phenyl-propene
15.6 s – (R)-1-nitro-2-phenyl-propane

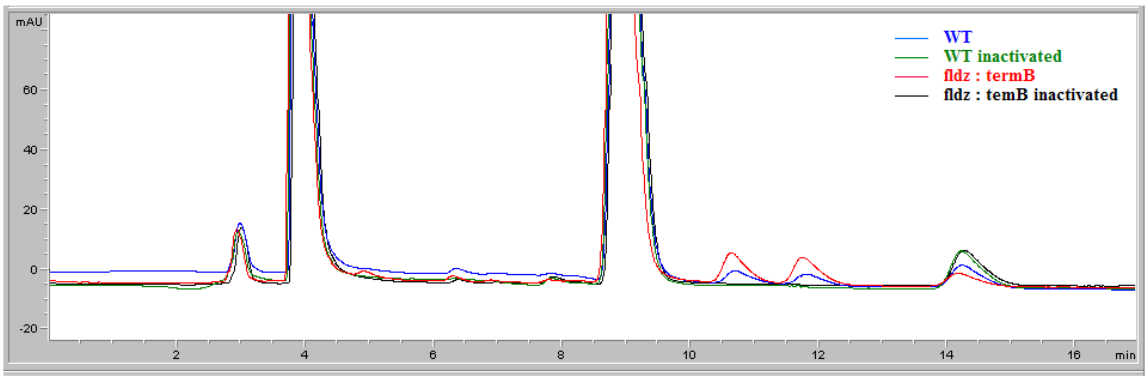


Substrate: *(E)*-1- phenyl-2-nitro-propene



	Concentration [mM]			Standard Error of Mean		
	product	substrate	ee.	product	substrate	ee.
DSM795	0.08	1.69	3%	0.008	0.055	1.6%
DSM795 - inactivated	0.00	1.79	-	0.000	0.002	-
fldZ::CTermB	0.12	1.49	1%	0.044	0.148	1.4%
fldZ::CTermB - inactivated	0.00	0.00	-	0.000	0.063	-

Retention times:
4.01 s – *tert*-butylbenzene
9.27 s – *(E)*-1-nitro-2-phenyl-propene
10.23 s – (S)-1-nitro-2-phenyl-propane
11.60 s – (R)-1-nitro-2-phenyl-propane

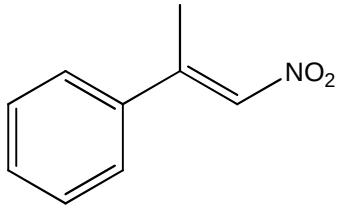


APPENDIX 5. Results of the spectrophotometric cinnamate reduction assay

	DSM795		fldZ::CTermB		DSM795 no substrate		fldZ::CTermB no substrate	
	Average	S.E.M.	Average	S.E.M.	Average	S.E.M.	Average	S.E.M.
1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
3	-0.0014	0.0000	0.0006	0.0001	0.0004	0.0000	0.0005	0.0000
5	-0.0050	0.0001	0.0001	0.0002	0.0004	0.0001	-0.0008	0.0000
7	-0.0080	0.0001	-0.0001	0.0003	0.0008	0.0001	-0.0013	0.0001
9	-0.0105	0.0000	-0.0005	0.0001	-0.0015	0.0000	-0.0004	0.0000
11	-0.0135	0.0002	0.0005	0.0003	0.0003	0.0001	0.0016	0.0001
13	-0.0161	0.0004	0.0013	0.0007	0.0001	0.0002	0.0036	0.0002
15	-0.0190	0.0002	0.0006	0.0004	-0.0006	0.0001	0.0022	0.0001
17	-0.0211	0.0002	0.0002	0.0005	-0.0009	0.0002	0.0025	0.0001
19	-0.0238	0.0003	0.0009	0.0005	0.0006	0.0002	0.0027	0.0001
21	-0.0269	0.0003	0.0007	0.0006	-0.0002	0.0002	0.0028	0.0001
23	-0.0293	0.0003	0.0010	0.0007	-0.0005	0.0002	0.0034	0.0002
25	-0.0321	0.0004	0.0010	0.0007	-0.0008	0.0002	0.0037	0.0002
27	-0.0347	0.0002	0.0009	0.0005	0.0000	0.0002	0.0024	0.0001
29	-0.0376	0.0003	0.0015	0.0007	0.0004	0.0002	0.0034	0.0002
31	-0.0402	0.0003	0.0006	0.0005	-0.0006	0.0002	0.0025	0.0001
33	-0.0428	0.0003	0.0008	0.0006	-0.0001	0.0002	0.0029	0.0001
35	-0.0457	0.0003	0.0004	0.0006	-0.0013	0.0002	0.0031	0.0002
37	-0.0485	0.0002	0.0001	0.0004	-0.0012	0.0001	0.0022	0.0001
39	-0.0514	0.0002	0.0001	0.0004	-0.0012	0.0001	0.0022	0.0001
41	-0.0540	0.0002	0.0007	0.0004	-0.0004	0.0001	0.0022	0.0001
43	-0.0567	0.0001	-0.0006	0.0001	-0.0016	0.0000	-0.0007	0.0000
45	-0.0596	0.0002	-0.0010	0.0004	-0.0007	0.0001	-0.0019	0.0001
47	-0.0623	0.0003	-0.0016	0.0006	-0.0009	0.0002	-0.0028	0.0001
49	-0.0644	0.0003	-0.0015	0.0005	-0.0015	0.0002	-0.0025	0.0001
51	-0.0677	0.0004	-0.0019	0.0007	-0.0011	0.0002	-0.0037	0.0002
53	-0.0705	0.0001	-0.0006	0.0001	-0.0018	0.0000	0.0007	0.0000
55	-0.0733	0.0002	0.0003	0.0004	-0.0006	0.0001	0.0018	0.0001
57	-0.0757	0.0001	-0.0003	0.0001	-0.0004	0.0000	0.0007	0.0000
59	-0.0786	0.0003	-0.0018	0.0006	-0.0020	0.0002	-0.0029	0.0001
61	-0.0817	0.0003	-0.0019	0.0006	-0.0014	0.0002	-0.0031	0.0002
63	-0.0842	0.0001	0.0000	0.0003	-0.0010	0.0001	0.0013	0.0001
65	-0.0877	0.0002	0.0002	0.0003	-0.0010	0.0001	0.0016	0.0001
67	-0.0904	0.0004	-0.0020	0.0008	-0.0015	0.0003	-0.0042	0.0002
69	-0.0936	0.0003	-0.0021	0.0007	-0.0021	0.0002	-0.0034	0.0002
71	-0.0963	0.0003	-0.0002	0.0005	-0.0024	0.0002	0.0026	0.0001
73	-0.0994	0.0003	-0.0020	0.0007	-0.0016	0.0002	-0.0034	0.0002
75	-0.1024	0.0003	-0.0020	0.0007	-0.0020	0.0002	-0.0034	0.0002
77	-0.1053	0.0000	-0.0015	0.0000	-0.0026	0.0000	0.0001	0.0000
79	-0.1084	0.0004	-0.0023	0.0008	-0.0024	0.0003	-0.0041	0.0002
81	-0.1112	0.0000	-0.0007	0.0000	-0.0014	0.0000	-0.0002	0.0000
83	-0.1144	0.0005	-0.0025	0.0010	-0.0020	0.0003	-0.0049	0.0002
85	-0.1173	0.0003	-0.0019	0.0007	-0.0012	0.0002	-0.0034	0.0002
87	-0.1202	0.0003	-0.0014	0.0006	-0.0009	0.0002	-0.0029	0.0001
89	-0.1232	0.0004	-0.0023	0.0008	-0.0020	0.0003	-0.0040	0.0002
91	-0.1265	0.0004	-0.0021	0.0008	-0.0018	0.0003	-0.0042	0.0002
93	-0.1298	0.0003	-0.0022	0.0007	-0.0024	0.0002	-0.0033	0.0002
95	-0.1327	0.0004	-0.0023	0.0008	-0.0023	0.0003	-0.0039	0.0002
97	-0.1360	0.0001	-0.0006	0.0002	-0.0016	0.0001	0.0008	0.0000
99	-0.1388	0.0003	-0.0020	0.0007	-0.0017	0.0002	-0.0034	0.0002
101	-0.1425	0.0001	-0.0013	0.0003	-0.0017	0.0001	-0.0013	0.0001

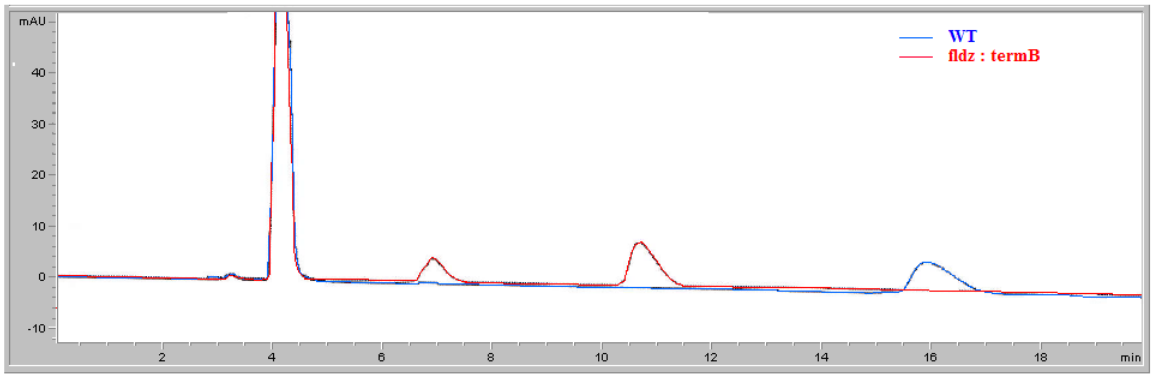
APPENDIX 6. Results of phenylnitropropene reduction assay using protein extracts

Substrate: *(E)*-1-nitro-2-phenyl-propene

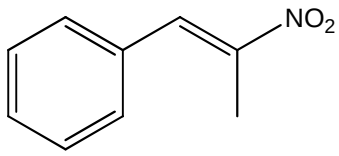


	Concentration [mM]			Standard Error of Mean		
	product	substrate	ee.	product	substrate	ee.
DSM795	1.66	0.00	100%	0.011	0.000	0.0
fldZ::CTermB	0.00	0.19	-	0.000	0.002	

Retention times:
3.97 s – *tert*-butylbenzene
9.43 s – *(E)*-1-nitro-2-phenyl-propene
14.34 s – (R)-1-nitro-2-phenyl-propane

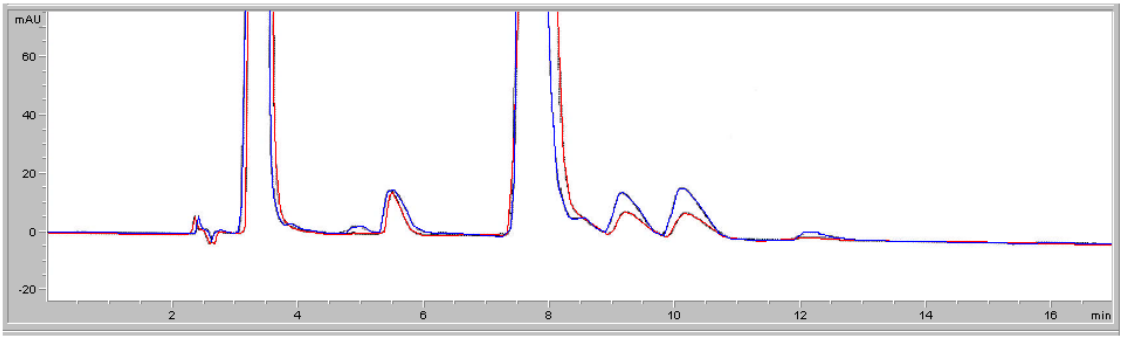


Substrate: *(E)*-1- phenyl-2-nitro-propene



	Concentration [mM]			Standard Error of Mean		
	product	substrate	ee.	product	substrate	ee.
DSM795	0.95	0.24	9.5%	0.009	0.000	0.5
fldZ::CTermB	0.47	0.53	4.0%	0.002	0.001	0.0

Retention times:
3.97 s – *tert*-butylbenzene
8.60 s – *(E)*-1-nitro-2-phenyl-propene
10.33 s – (S)-1-nitro-2-phenyl-propane
11.39 s – (R)-1-nitro-2-phenyl-propane



APPENDIX 7. List of Risk assessments and COSHH forms

Risk Assessments:

1. Growth and biotransformation using *C. sporogenes*
2. Preparing medium for anaerobic bacteria
3. Transport and use of gas cylinders and regulators
4. Use of anaerobic work stations
5. Use of autoclaves and sterilization equipment
6. Use of centrifuges and ultra-centrifuges
7. Use of electrophoresis equipment
8. Use of French press
9. Use of manifold with flammable gases
10. Use of solvents

COSHH forms:

1. Acetic acid
2. Acrylamide
3. Benzamide
4. Benzylamine
5. Bromophenol Blue
6. Butanol
7. Cyclohexanecycloamide
8. Dichloromethane
9. *(E)*-1-nitro-2-phenyl-propene and *(E)*-1-phenyl-2-nitro-propene
10. Ethanol
11. Hyrdochloric acid
12. Propanol
13. SDS
14. Sodium selenite
15. TEMED
16. *Tert*-butyl metyl ether
17. Tris
18. Virkon