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Reductions using *Clostridium sporogenes*

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ABSTRACT

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Reductions using *Clostridium sporogenes*

Clostridium sporogenes was previously shown to be an extraordinary source for unusual reductases. It can catalyze reduction of wide a range of substrates such as nitroalkenes, enoates and nitro compounds, and can be used to generate chiral products.

In preliminary studies, the ClosTron gene knock-out system for Clostridia was used to inactivate the *fldZ* gene assumed to encode the enzyme responsible for reduction of cinnamic acid in the reductive branch of L-phenylalanine fermentation *via* the Stickland reaction.

Biotransformations with the fldZ mutant showed that *C. sporogenes* possesses multiple enzymatic activities, reducing enoates, β,β- and α,β-disubstituted nitroalkenes with different yields and enantioselectivities. The fldZ reductase was found to be responsible for reduction of cinnamic acid, *(E)*-1-nitro-2-phenylpropene, *(E)*-2-nitro-1-phenylpropene and β-nitrostyrene. However, the mutant could still reduce *(E)*-2-nitro-1-phenylpropene, β-nitrostyrene and cinnamic acid confirming the presence of other C=C double bond reductases in *C. sporogenes*.

The analysis of the *C. sporogenes* genome sequence allowed identification of two hypothetical genes encoding proteins with homology to flavin-containing C=C double bond reductases, fldZ 2-enoate reductase and OYE-like reductase, which were subsequently cloned, overexpressed in *E. coli* under anaerobic conditions and tested for reduction of unsaturated compounds. The activity tests showed that fldZ possesses a narrow substrate range and can reduce only aromatic enoates such as cinnamic acid or *p*-coumaric acid. FldZ also reduced *(E)*-1-nitro-2-phenylpropene and *(E)*-2-nitro-1-phenylpropene with excellent and poor enantioselectivities (>99% and 16% respectively). On the other hand, the OYE-like reductase did not show activity towards unsaturated substrates in the activity assays and the substrate range of this reductase is unknown.

Growth experiments comparing wild type *C. sporogenes* and the mutant in complex and minimal media showed that the fldZ reductase in not involved in the L-phenylalanine fermentation. Further analysis of the *C. sporogenes* genome resulted in identification of a novel reductase that might be involved in reduction of cinnamoyl-CoA to 3-phenylpropionyl-CoA in the Stickland reaction.

Biocatalytic reduction of aromatic nitro compounds to amines can be used as alternative to chemo-reductive routes in preparation of pharmaceutical and agrochemical products. Protein extracts of *C. sporogenes* were found to reduce aromatic nitro compounds with different yields depending on the substrate structure and electron donor used in the reaction. The genome of *C. sporogenes* was screened and that allowed identification of six genes encoding hypothetical nitroreductases, which were subsequently overexpressed in *E. coli*. However, biotransformations using the recombinant nitroreductases did not show amine product formation.

A novel Nylon 6 biosynthesis pathway was designed starting from biorenewable feedstocks. The crucial step in this pathway, reductive cleavage of pipecolic acid to 6-aminocaproic acid was proposed to be catalysed by *C. sporogenes* D-proline reductase. Thus, the activity of this enzyme was tested towards L- and D-pipecolic acid. Biotransformations showed that pipecolic acid was not accepted as a substrate. In the future, the idea of using D-proline reductase for Nylon biosynthesis may be exploited by improving the reductase activity using protein engineering techniques.

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DEDICATION

I would like to dedicate this Doctoral thesis to my late aunt, Anna Mordaka.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C=C	carbon-carbon double bond
CoA	coenzyme A
dNTP	deoxyribonucleotide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ee.	enantiomeric excess
FAD	flavin adenine dinucleotide
FMN	riboflavin 5'-monophosphate
g	gravitational acceleration
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
LB	lysogeny broth
$NAD(P)^+$	β-nicotinamide adenine dinucleotide (2'-phosphate) oxidised
NAD(P)H	β-nicotinamide adenine dinucleotide (2'-phosphate) reduced
NCIMB	National Collection of Industrial, Food and Marine Bacteria
ORF	open reading frame
OYE	Old Yellow Enzyme
PCR	polymerase chain reaction
SDS	sodium dodecyl sulphate
spp.	*species pluralis*
THP	tris(hydroxypropyl)phosphine
Tris	tris(hydroxymethyl)aminomethane

1. Introduction

Whole microorganisms and isolated enzymes can be used as biocatalysts for modifications of chemical compounds providing major advantages over chemical catalysis. Biocatalysts are usually efficient, environmentally acceptable and show chemo-, regio- and enantioselectivity. As they catalyse a broad spectrum of chemical reactions, they are an attractive alternative to traditional chemical synthesis in the pharmaceutical, chemical and textile industries (Faber, 2011). Nowadays, there is a great need for discovery of new enzymes showing unique properties.

The majority of Clostridia are benign and possess a variety of biocatalytic abilities, which could be exploited for the biosynthesis of chemicals in a process alternative to traditional industrial routes. Certain members of Clostridia (*C. acetobutylicum*, *C. beijerinkii*, *C. thermocellum* and *C. phytofermentas*) are employed to produce biofuels such as butanol and ethanol from biomass derived substrates (Kuehne *et al.*, 2011). Also, attempts have been made to use Clostridia as an effective system for delivery of anti-cancer agents, as clostridial spores can selectively germinate in the hypoxic regions of solid tumours (Minton, 2003). However, Clostridia achieved significant notoriety and bad publicity for the members which cause severe diseases in humans and animals. Tetanospasmin and botulin, toxins produced by *C. tetani* and *C. botulinum* respectively, are among the most dangerous known and have been even considered as a potential bioweapon that could be used in terrorist attacks (Farrar *et al.*, 2000; Wein and Liu, 2005). *C. difficile* is responsible for antibiotic induced diarrhoea and is the main nosocomial pathogen responsible for hundreds of deaths in hospitals (Eggertson, 2004). *C. perfringens* is the third most common causative agent for enteritis necroticans food poisoning and causes infections, necrosis and gas gangrene (Warrel *et al.*, 2003).

At present, 114 genome sequences of *Clostridium* spp. have been deposited in the Genbank. Therefore, unique clostridial enzymes or whole metabolic pathways can be identified, cloned and expressed in non-pathogenic heterologous hosts, which can be easily and inexpensively grown in a laboratory or an industrial plant. This strategy, which is alternative to classical biotechnology methods, overcomes the disadvantages of using Clostridia, as working with anaerobic bacteria introduces additional challenges when compare to the usage of standard laboratory strains like *E. coli* or yeasts. Cultivation of oxygen sensitive strains requires dedicated workstations for maintaining anoxic environment. Moreover, handling of pathogenic bacteria is associated with a certain risk and requires additional safety measures. Therefore, genetically engineered (*C. botulinum*

with mutated gene encoding the toxin, Bradshaw *et al.*, 2010) or natural non-pathogenic strains (such as *C. difficile* lacking genes encoding toxin A and toxin B) are used (Fluit *et al.*, 1991). An alternative is to use non-pathogenic analogous species, such as *C. sporogenes,* for predicting growth models of *C. botulinum* in the food industry. For efficient growth Clostridia require also complex media containing rare trace elements and vitamins (Lovitt *et al.*, 1987). Finally the genetic methods to manipulate Clostridia are limited and in clear need of development. Recently, new tools were developed such as standardized modular system for *Clostridium-E. coli* shuttle plasmids (Heap *et al.*, 2009), the ClosTron gene knock out system for production of stable genomic mutants (Heap *et al*., 2010) and a system for integration of DNA into bacterial chromosomes without a counter-selection marker (Heap *et al.*, 2012). These methods were shown to be easily available and very efficient and can significantly improve the molecular biology techniques for Clostridia.

C. sporogenes is an anaerobic bacterium obtaining energy for growth from an unusual fermentation of amino acids called the Stickland reaction, in which one molecule of amino acid acts as electron donor and another acts as electron acceptor (Stickland, 1934). *C. sporogenes* can use a variety of amino acids as growth substrates (Barker, 1980). That makes it an excellent source of unique proteins, especially responsible for catalysing redox reactions.

Whole cells and crude protein extracts of *C. sporogenes* were successfully used to reduce activated alkenes such as enoates and nitroolefins (Bühler *et al.*, 1980; Fryszkowska *et al.*, 2008), amides (Dipeolu *et al.*, 2005), imines (Monticello and Costilow, 1981), aromatic and aliphatic nitro compounds (Angermaier and Simon, 1983a; Angermaier and Simon, 1983b; Dipeolu, 2008; Dipeolu *et al.*, 2010) and proline (Stickland, 1935; Lovitt *et al.*, 1986).

Hydrogenation of C=C double bonds in activated alkenes is highly desirable as it can introduce up to two new asymmetric carbons in the product. *C. sporogenes* was found to reduce α,β- and β,β-disubstituted nitroolefins with different yields and enantiomeric excesses depending on the substrate structure (Fryszkowska *et al.*, 2008). In this thesis the hypothesis that nitroolefins can be reduced by a clostridial 2-enoate reductase involved in the L-phenylalanine fermentation was tested by generation of genomic knock out mutants. Preliminary studies using the *C. sporogenes* fldZ enoate reductase mutant showed that this enzyme may be involved in reduction of *(E)*-1-nitro-2-phenylpropene with an excellent enantioselectivity (Mordaka, 2010). Attempts to purify the reductase responsible for reduction of *(E)*-2-nitro-1-phenylpropene showed that *C. sporogenes* may possess more

than one C=C double bond reductase (Karl Fisher, personal communication). Therefore, the scope for reduction of unsaturated nitroolefins and enoates by the *C. sporogenes* fldZ enoate reductase mutant was further tested. The physiological role of fldZ in fermentation of amino acids by *C. sporogenes* was also discussed. The fldZ reductase was identified, overexpressed in a heterologous host and characterized. Moreover, the analysis of the *C. sporogenes* genome revealed the presence of novel enzymes that can be responsible for reduction of C=C double bonds in activated alkenes and enoyl-CoA compounds.

Amines are used as building blocks for preparation of agrochemical and pharmaceutical products (Breuer, 2004). Therefore, *C. sporogenes* was also tested as biocatalysts for reduction of aromatic nitro compounds to amines, which was reported for the first time by Dipeolu (2008). The reaction was further optimized by using protein extracts and different electron donors. The analysis of the *C. sporogenes* genome allowed identification of six hypothetical nitroreductases, which were subsequently overexpressed and tested in biotransformations.

Finally, a novel Nylon 6 biosynthesis pathway starting from L-lysine was postulated. The crucial step in the pathway, conversion of L-pipecolic acid into 6-aminocaproic acid, was proposed to be carried out by proline racemase and D-proline reductase, enzymes involved in reduction of proline in amino acid fermentation by *C. sporogenes*. Thus, the substrate range of D-proline reductase was tested.

Overall, the purpose of this project was to use *C. sporogenes* as a source of novel enzymes showing unique properties such as substrate range and enantioselectivity that can be used in industrially relevant biotransformations.

2. Literature review

2.1. Biotransformations in organic chemistry

Biotransformation is a process where an organic compound is chemically altered by biological systems (called biocatalysts) such as whole organisms or isolated enzymes. The difference between biotransformation and biosynthesis is that the former process describes the natural capability of biological systems to change xenobiotic compounds that are not normally produced or expected to be present in the system. It relies on the fact that some enzymes can accept broad substrate ranges and show catalytic activities to analogues of the natural substrates or intermediates of the catalyzed reaction (Leuenberger, 1984).

Biocatalysis has a long tradition from the first experiences with manufacturing food and alcoholic drinks in ancient Mesopotamia, China and Japan. Nowadays, biotransformations are mainly used in food manufacturing, pharmaceutical, fine chemical, textile and starch industries (Bornscheuer and Buchholz, 2005). Biocatalysts are also used in bioremediation processes, as microbes are characterized by catabolic diversity allowing them to transform or accumulate a huge range of compounds (Diaz, 2008).

Biocatalysis has many advantages over chemical catalysis (Faber, 2011). Enzymes are usually very efficient and accelerate the rate of reactions by a factor of 10^8-10^{10}. Thus, they can be employed in much lower concentrations than chemical catalysts. Biocatalysts are environmentally acceptable and biodegradable, in contrast to heavy metals used in chemical catalysis. They usually act under mild conditions, neutral pH and low temperature. Enzymes are compatible with each other and several biocatalytic reactions can be carried out as a reaction cascade at the same time. That simplifies the reaction process, allows unstable intermediates to be avoided and shifts the reaction towards the products when single reactions are characterized by an unfavourable equilibrium. Enzymes can catalyze a broad spectrum of reactions and are not restricted to their natural roles, accepting also non-natural substrates. However, the biggest advantage of using enzymes comes from their selectivities. Biocatalysts show chemoselectivity (act on a single type of functional group in the substrate), regioselectivity (distinguish identical functional groups situated in different positions in the substrate) and enantioselectivity (prochiral substrates may be converted into chiral products or enzymes may convert only one enantiomer of the substrate).

However, using biocatalysts for catalysing chemical reactions is connected with certain limitations (Faber, 2011). Enzymes are characterized by narrow operation parameters and elevated temperatures, extreme pH or high salt concentrations may inhibit their activity. They display the highest catalytic activity in water. Biotransformations in

organic solvents or biphasic systems are still possible, but the reactions are usually less efficient. Moreover, enzymes require their natural cofactors such as NAD(P)H or ATP, which are usually unstable and expensive. However, cofactors in redox reactions can be regenerated by using a second redox reaction to allow them to re-enter the reaction cycle. Finally, enzymes are subject to inhibition phenomena, which require optimization of the reaction to avoid substrate- and product-inhibition.

Biotransformations can be carried out by whole cells or isolated enzymes (Faber, 2011). Using whole microbial cells as biocatalysts eliminates a need to recycle cofactors as inexpensive equivalents such as carbohydrates can be used to drive the reaction. Moreover, large quantities of the biocatalyst can be produced from cheap carbon sources, however this requires expensive equipment. Whole cell biotransformations give more by-products in the reactions. On the other hand, isolated enzymes are characterized by higher activities and a higher tolerance to organic solvents. They can be used in simple apparatus, but using the cofactor recycling system is necessary.

All of these features make biocatalysis an attractive tool in modern synthetic chemistry and causes a rapidly increasing need for discovery of new or more efficient biocatalysts.

2.2. Biocatalytic reductions in chiral synthesis

New types of enzymes are needed for currently inaccessible biotransformations. Oxidoreductases were successfully used for profitable biosynthesis of both chiral and achiral compounds. About 25% of proteins delivered from sequences deposited in the databases are responsible for catalysing redox reactions. A variety of substrates accepted by these enzymes makes them an excellent tool for synthesis purposes (Hall and Bommarius, 2011). Different strategies leading to formation of chiral products were applied to the industrially relevant biotransformations (Hall and Bommarius, 2011).

Biocatalytic reductions can be used for deracemization of racemic mixtures *via* stereoinversion or dynamic reductive kinetic resolution (Hall and Bommarius, 2011). Stereoinversion employs a sequence of enantioselective oxidation combined with asymmetric reduction. Deracemization of *rac*-2-decanol using stereoinversion approach was accomplished by a combined oxidation/reduction sequence using lyophilised cells of *Rhodococci* spp. for both steps. Racemic 2-decanol was transformed to (*S*)-2-decanol with excellent enantiomeric excess of 92% and yield of 82% in the combined one-pot oxidation/reduction sequence (Voss *et al.*, 2007). On the other hand, the alcohol dehydrogenase isozyme-10 (SsADH-10) from *Sulfolobus solfataricus* was used for dynamic

reductive kinetic resolution of 2-arylpropanal substrates. Treatment of the substrates with the enzyme in the presence of NADH led to efficient kinetic resolution with ee. exceeding 90%. Importantly, the SsADH-10 enzyme could be conveniently recycled by exploiting the differential solubility of the organic substrate/product and used in several reaction cycles without decrease of ee. (Friest *et al.*, 2010).

Chiral compounds can also be obtained by stereospecific biocatalytic reductions of prochiral compounds containing carbonyl groups or activated C=C double bonds according to Fig. 2.1. (Faber, 2011).

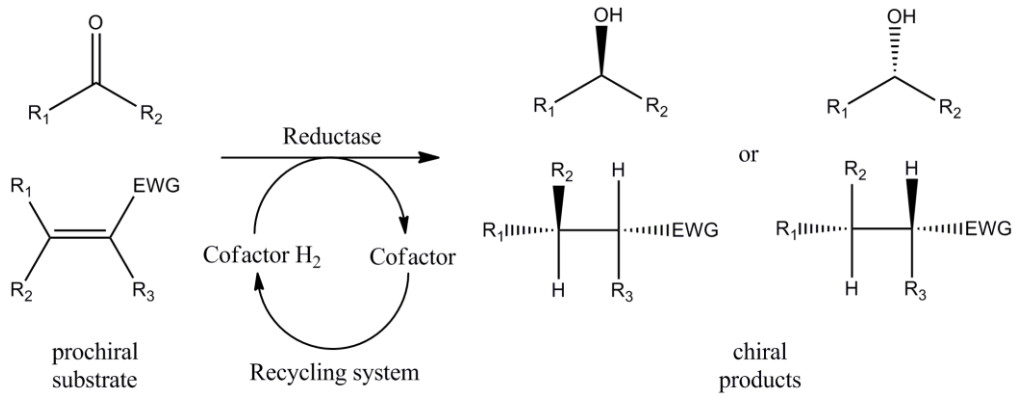


Fig. 2.1. Reductions of carbonyl groups or C=C double bonds in chiral synthesis (Faber, 2011).

Alcohol dehydrogenases (ADHs) were used for enantioselective reduction of various ketones to produce enantiopure secondary alcohols. Because these reductase-catalyzed reactions are dependent on NAD(P)H, an effective method for regeneration of the consumed cofactors was developed by using recycling systems comprising of glucose/ glucose dehydrogenases, glucose-6-phosphate/glucose-6-phosphate dehydrogenases or alcohol/alcohol dehydrogenases (Goldberg *et al.*, 2008). The reduction proceeds through hydride attack on either the *si*- or *re*- face of the substrate, while the enzyme transfers the hydride of the nicotinamide cofactor (Hall and Bommarius, 2011). The enantioselectivity of the reaction depends on the size of substituents on the carbonyl group. The selectivity of ADHs was successfully changed by protein engineering. The mutation of Cys-295 to alanine in *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (SADH) was found to cause a significant shift of enantioselectivity towards the *(S)*-configuration in the reduction of some ethynyl ketones to the corresponding chiral propargyl alcohols. Furthermore, C295A SADH was characterized with much higher activity towards *t*-butyl and some alpha-branched ketones than the wild-type SADH (Heiss *et al.*, 2001). On the other hand, mutation W110A changed the substrate range of the enzyme, which now uses *(S)*-1-phenyl-

2-propanol, *(S)*-4-phenyl-2-butanol and the corresponding ketones as substrates. Kinetic parameters on these substrates are in the same range as those of the wild type enzyme substrate 2-butanol, making W110A an excellent catalyst (Ziegelmann-Fjeld *et al.*, 2007).

Aldehyde reductase from *Sporobolomyces salmonicolor* was successfully used for reduction of ethyl 4,4,4-trifluoroacetoacetate to *(R)*-ethyl 4,4,4-trifluoro-3-hydroxy-butanoate, a building block for pharmaceuticals such as antidepressant Befloxatone (Fig. 2.2.).

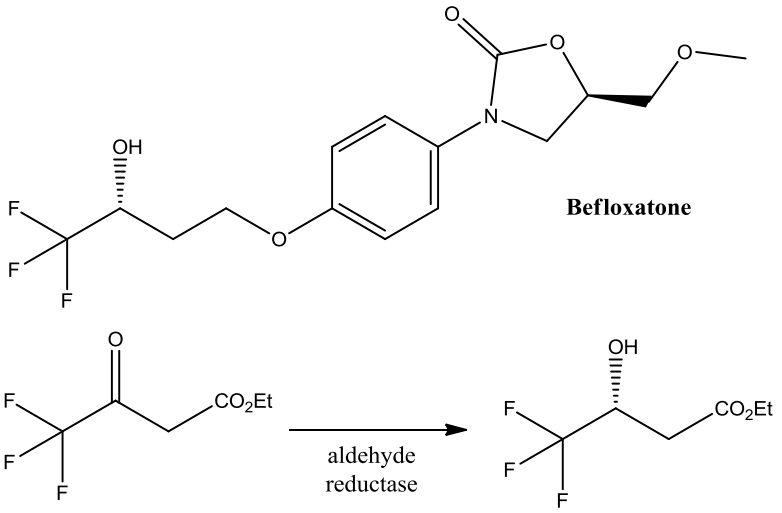


Fig. 2.2. Reduction of ethyl 4,4,4-trifluoroacetoacetate to *(R)*-ethyl 4,4,4-trifluoro-3-hydroxybutanoate by the aldehyde reductase from *Sporobolomyces salmonicolor* using glucose dehydrogenase cofactor regeneration.

The reaction was carried out in a water/butyl acetate biphasic system using *E. coli* whole cells expressing the aldehyde reductase and glucose dehydrogenase for NADH recycling. The biotransformation resulted in 50% yield and 100% enantiomeric excess of the product (Shaw *et al.*, 2003).

The reduction of two carbonyl groups of 3,5-dioxo-6-(benzyloxy)hexanoic acid ethyl ester by diketoreductase was carried out with whole cells and cell extracts of *Acinetobacter calcoaceticus* grown on glycerol. Reaction yield of 72% and optical purity of 99% were obtained when the reaction was carried out with glucose dehydrogenase cofactor recycling system (Patel *et al.*, 1993).

The second type of biocatalytic reaction leading to chiral products, hydrogenation of C=C double bonds in activated alkenes, is highly desirable as it can introduce up to two new asymmetric carbons in the product. The first C=C double bond reductase, Old Yellow Enzyme (OYE1), was isolated from brewers' bottom yeast *Saccharomyces pastorianus* and called 'yellow enzyme' due to yellow colour determined by the presence of flavin cofactor (Warburg and Christian, 1932). Enzymes belonging to the OYE-family were found to

catalyze the reduction of various compounds such as nitro esters, nitro aromatics or activated α,β-unsaturated compounds (Hall and Bommarius, 2011). The reaction proceeds through a Michael-type addition of [H_2] onto C=C double bond, following a ping-pong mechanism where hydride from the flavin attacks the C_β atom and a proton from the solvent is added onto the C_α atom (Fig. 2.3.; Stuermer *et al.*, 2007; Toogood *et al.*, 2010).

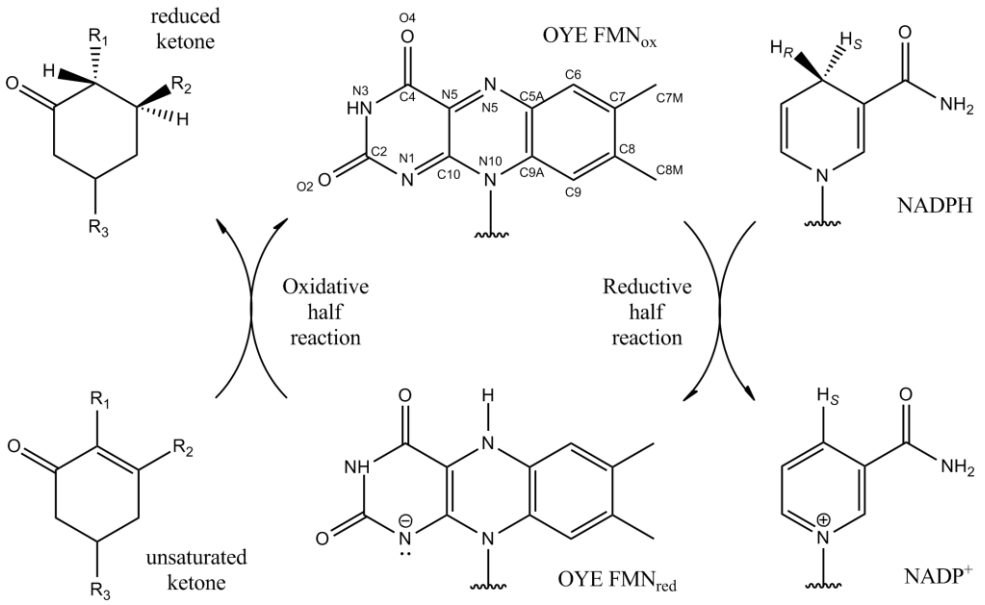


Fig. 2.3. Reaction catalyzed by OYE enzymes (taken from Toogood *et al.*, 2010).

The OYE-family reductases play diverse physiological roles. The general role of OYEs is the detoxification of electrophilic compounds such as acrolein reduction by OYE2 from *Saccharomyces cerevisiae* (Toogood *et al.*, 2010). Other roles are dependent on the species. Reductases from plants such as *Oryza sativa* and *Solanum lycopersicum* were found to be involved in biosynthesis of a plant hormone, jasmonic acid. OYE from *Aspergillus fumigatus* is responsible for biosynthesis of ergot alkaloid. The enzyme from *Azoarcus evansii* catalyses reduction in the aerobic metabolism of anthranilate, whereas reductase from *Trypanosoma cruzi* is involved in prostaglandin F2α synthesis (Toogood *et al*., 2010).

Reduction of activated C=C double bonds by OYE is an attractive tool in biocatalysis, as the enzymes can accept a wide range of activating groups (ketone, carboxylic acid, carboxylic ester, lactone, imide, nitrile, nitro, aldehyde and ynone) and the reaction often provides good enantiomeric excess of the product (Hall and Bommarius, 2011).

Nitroalkenes are important intermediates in organic synthesis as they can be easily converted to corresponding amines, aldehydes, carboxylic acids or denitrated compounds (Toogood *et al.*, 2008). Highly enantioselective reduction of C=C double bond in nitroalkenes was shown for a wide range of enzymes such as YqjM from *Bacillus subtilis*,

OPR1 and OPR3 from *Arabidopsis thaliana*, OYE2 and OYE3 from *Saccharomyces cerevisiae*, OYE1 from *Saccharomyces pastorianus*, NCR from *Zymomonas mobilis*, PETNR from *Enterobacter cloacae*, NEM reductase from *E. coli* or XenA from *Pseudomonas putida* (Toogood *et al.*, 2010).

Several OYEs were found to be stereocomplementary. 12-Oxophytodienoate reductase isoenzymes OPR1 and OPR3 from *Solanum lycopersicum* were found to possess a broad substrate spectrum for the asymmetric bioreduction of α,β-unsaturated enals, enones, dicarboxylic acids, nitro compounds and *N*-substituted maleimides. Stereocomplementary behavior of both isoenzymes was observed in the reduction of a nitroalkene that led to the formation of opposite stereoisomers in high enantiomeric excess (Fig. 2.4; Hall *et al.*, 2007).

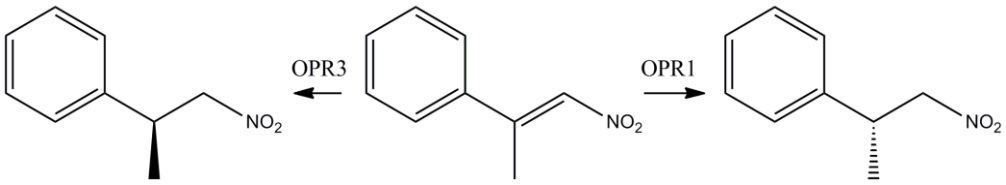


Fig. 2.4. Enzyme based stereocontrol with OPR1 and OPR3 reductases (Hall *et al.*, 2007).

Members of the OYEs were recently applied to the synthesis of non-racemic aryl-substituted α-methyldihydrocinnamaldehyde derivatives used as olfactory principles in perfumes. *(R)*-Enantiomers were obtained using the YqjM and 12-oxophytodienoic acid reductase isoenzyme OPR1 from *S. lycopersicum* with e.e. 53%. On the other hand *(S)*-aldehydes were produced up to 97% ee. using isoenzyme OPR3, nicotinamide 2-cyclohexene-1-one reductase NCR from *Zymomonas mobilis* and yeast OYE isoenzymes 1-3 (Stueckler *et al.*, 2010a). Another example of applied reduction using OYEs is asymmetric bioreduction of methyl 2-hydroxymethylacrylate and its *O*-allyl, *O*-benzyl and *O*-TBDMS derivatives to form (*R*)-enantiomer of methyl 3-hydroxy-2-methylpropionate. The reactions were characterized by high e.e. of the products (Stueckler *et al.*, 2010b).

A separate class of C=C double bond reductases are 2-enoate reductases from anaerobic bacteria such as *Clostridium* spp. (Simon *et al.*, 1985; Fryszkowska *et al.*, 2008). They catalyze reduction of α,β-disubstituted aldehydes, cyclic ketones, methyl ketones and aromatic nitroalkenes in a stereoselective manner and were applied to synthesis of optically pure compounds (Simon *et al.*, 1985; Fryszkowska *et al.*, 2008; Fryszkowska *et al.*, 2010). 2-Enoate reductases were proposed to be involved in the unique metabolic

pathways, in which *Clostridium* spp. can obtain carbon and energy for growth (Bühler *et al.*, 1980).

The restrictions and limitations of using reductases as biocatalysts in synthetic chemistry come from their specificity and enantioselectivity. Nowadays, enzymes with new properties can be created using protein engineering methods. Alternatively, new enzymatic activities can be obtained by identification of new proteins by metagenomic approaches or traditional screening of microbes from environmental samples.

2.3. The Stickland reaction in *Clostridium* spp.

A large number of reductases used in biocatalysis have been identified in strict or facultative anaerobic organisms. Anaerobic microorganisms in the absence of oxygen must use alternative electron acceptors to maintain metabolism. Therefore, they developed a variety of unique metabolic pathways leading to reduction of naturally occurring compounds.

Clostridium sporogenes is a Gram positive obligate anaerobic endospore-forming bacterium. It belongs to the clostridial cluster I (converts proline to 5-aminovaleric acid), subgroup B (utilizes arginine, phenylalanine, serine, tyrosine, tryptophan, methionine and glycine and produces 2-aminobutyric acid and 4-aminobutyric acid; Mead, 1971). *C. sporogenes* is a proteolytic organism unable to grow except in media containing proteins or amino acids. However, carbohydrates are fermented too, but they are not sufficient to maintain growth (Stickland, 1934). *C. sporogenes* can obtain energy from coupled deamination of pairs of amino acids (acting as donor and acceptor of hydrogen respectively) in the Stickland reaction. In the presence of *C. sporogenes* cells, certain amino acids reduced methylene blue, brilliant cresylblue and benzylviologen, thus acting as hydrogen donors. On the other hand, some amino acids could reoxidize reduced phenosafranine, benzylviologen and methyl viologen, acting as electron acceptors.

Reactions can occur between any electron donor and electron acceptor according to scheme presented in Fig. 2.5. The electron donor amino acid is oxidized to a volatile carboxylic acid one carbon atom shorter than the original amino acid. Thus, D-alanine is converted to acetate, carbon dioxide and ammonia. The electron acceptor amino acid is reduced to a volatile carboxylic acid the same length as the original amino acid. For example, glycine is converted to acetate and ammonia, whereas L-proline is reductively cleaved to 5-aminovaleric acid (Nisman, 1954).

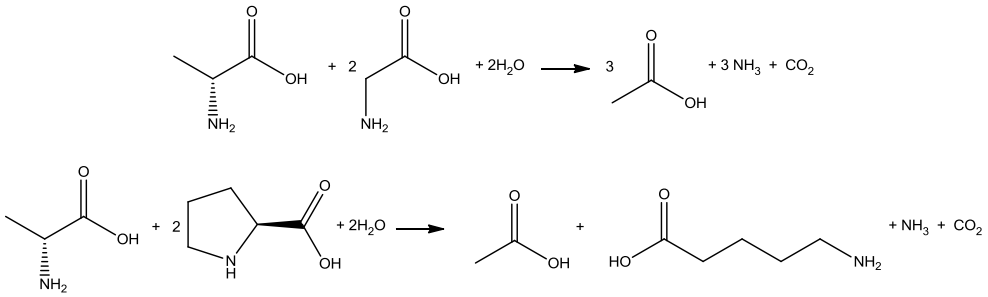


Fig. 2.5. Examples of couple deamination of alanine/glycine and alanine/proline in the Stickland reaction by *C. sporogenes* (Stickland, 1935a; Stickland, 1935b).

A list of compounds tested for being electron donors and acceptors in the Stickland reaction is presented in Table 2.1.

Table 2.1. List of compounds tested for donating and accepting electrons in *Clostridium* spp.

Substrate	Electron donor	Electron acceptor
α-aminovaleric acid	+ (3)	
β-alanine	− (2)	− (2)
D-alanine	+ (1,3)	− (4)
L-alanine	− (1)	
D-arginine	− (1)	− (1); + (2,4)
L-asparagine	+ (3)	− (4)
L-aspartic acid	+ (1)	− (1)
L-cysteine	+ (2)	− (2); + (4)
D-glucose	+ (3)	− (4)
D-glutamic acid	+ (1)	− (1)
glyceric aldehyde	+ (3)	+ (4)
glycine	− (1)	+ (1,4)
glycolic acid	− (2)	− (2,4)
L-histidine	+ (1,3)	− (1,4)
L-hydroxyproline	− (1)	+ (1,4)
L-isoleucine	+ (3)	− (4,6)
lactic acid	+ (3)	
L-leucine	+ (1,3,6)	− (4); + (6)
D-lysine	− (1); + (3)	− (1,4)
L-methionine		+ (5)
D,L-ornithine	− (2); + (3,6)	+ (2,4,6)
L-phenylalanine	+ (1,3)	− (4); + (7)
D-proline		+ (8)
L-proline	− (1)	+ (1,4)
pyruvic acid	+ (1,3)	
D,L-serine	+ (1,3)	− (1,4)
taurine	− (2)	− (2)
threonine		+ (5)
L-tryptophan	+ (1,3)	+ (4)
L-tyrosine	+ (1,3)	− (1); + (4)
D-valine	+ (1,3)	− (4,6)

1. Stickland (1934); 2. Woods (1936); 3. Nisman (1954); 4. Barker (1981); 5. Mean (1971); 6. Elsden and Hilton (1978); 7. Bader *et al.* (1982).

Proteins responsible for reduction of proline and glycine in proteolytic *Clostridium sticklandii* have been identified and characterized. Both enzymes contain selenium and are processed post-translationally to form a catalytic active pyruvoyl group (Garcia and Stadtman, 1992; Bednarski *et al.*, 2001).

Some amino acids can act as both electron donors and electron acceptors in the Stickland reaction (Fig. 2.6.). L-Phenylalanine was found to be fermented to ammonia, carbon dioxide, phenylacetic acid and 3-phenylpropionic acid (Elsden and Hilton, (1978). The metabolic pathway in which *C. sporogenes* oxidizes and reduces L-phenylalanine was proposed by Bühler *et al.* (1980). In the first step, which is common for both oxidative and reductive branches, an amino group is transferred from L-phenylalanine to 2-oxoglutarate resulting in the formation of phenylpyruvic acid. Then, in the oxidative branch phenypyruvic acid is converted to phenlacetyl-CoA, which is subsequently used to form phenylacetate. This reaction is coupled to ATP conservation *via* the substrate level phosphorylation. In the reductive branch of the pathway, phenylpyruvic acid is reduced to *(R)*-phenyllactate, which is converted to CoA derivative and subsequently reduced to *(E)*-cinnamic acid. In the last step of the pathway, cinnamic acid is reduced to 3-phenylpropionic acid by the enoate reductase (Bühler *et al.*, 1980; Dickert *et al.*, 2000).

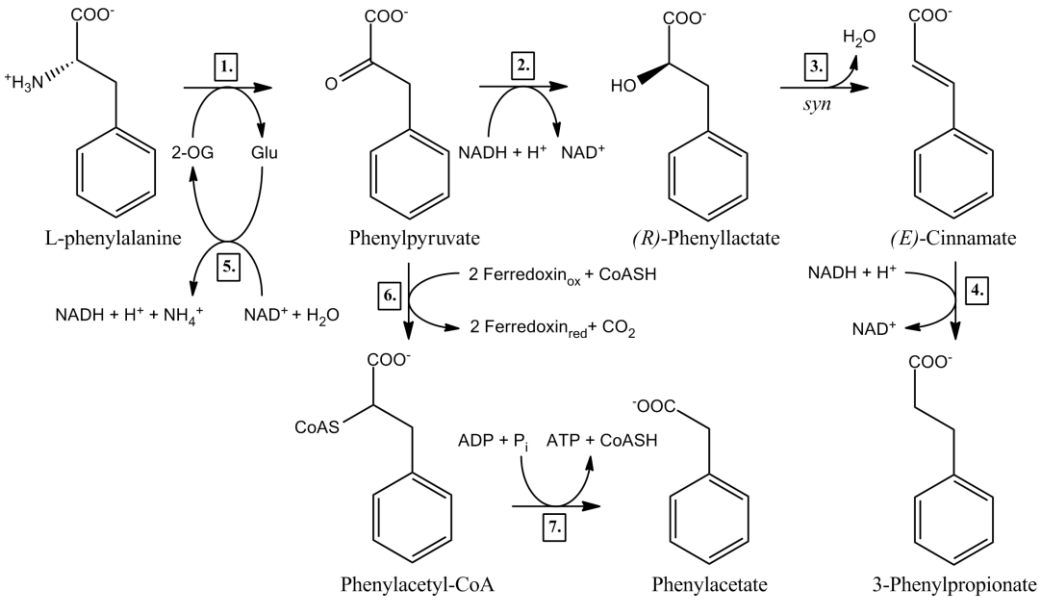


Fig. 2.6. Fermentation of L-phenylalanine by *C. sporogenes* (taken from Dickert *et al*., 2000). Enzymes: **1.** aromatic amino acid aminotransferase (EC 2.6.1.57); **2**. phenyllactate dehydrogenase (EC 1.1.1.-); **3**. phenyllactate dehydratase; **4.** cinnamate reductase (EC 1.3.1.31); **5.** glutamate dehydrogenase (EC 1.4.1.2); Glu, glutamate; 2-OG, 2-oxoglutarate; **6.** phenylpyruvate:ferredoxin 2-oxidoreductase (CoA-acetylating) (EC 1.2.7.-); **7.** phosphate phenylacetyltransferase (EC 2.3.1.-) and phenylacetate kinase (EC 2.7.2.-).

The phenyllactate dehydrogenase (fldH) responsible for reduction of phenylpyruvic acid to *(R)*-phenyllactic acid was successfully purified and identified in *C. sporogenes* (Dickert *et al.*, 2000). The enzyme was found to consist of two subunits (38 kDa and 42 kDa) and accepted electrons from NADH.

The phenyllactate dehydratase, which catalyses the reversible syn-dehydration of *(R)*-phenyllactate to *(E)*-cinnamate, was purified from *Clostridium sporogenes* grown anaerobically on L-phenylalanine (Dickert *et al.*, 2000). The dehydration proceeded in two steps, a CoA-transfer from cinnamoyl-CoA to phenyllactate followed by the dehydration of phenyllactyl-CoA. The purification yielded in an enzyme complex consisting of three subunits, fldA (46 kDa), fldB (43 kDa) and fldC (40 kDa).

FldA was found to possess activity of cinnamoyl-CoA:phenyllactate CoA-transferase, which was not sensitive to oxygen (Dickert *et al.*, 2000). The N-terminus of fldA (39 amino acids) showed homology to CaiB (39% sequence identity), which is involved in carnitine metabolism in *E. coli*. Both enzymes are CoA-transferases exhibiting high substrate specificity and they do not form enzyme CoA-ester intermediates.

The FldBC complex was found to be responsible for phenyllactate dehydratase activity and the reaction was inhibited in the presence of oxygen. The dehydratase contains [4Fe-4S] cluster (Dickert *et al.*, 2000). Both subunits of phenyllactyl-CoA dehydratase (FldBC) show significant sequence similarities to both subunits of 2-hydroxyglutaryl-CoA dehydratase (HgdAB) from *Acidaminococcus fermentans*. Phenyllactate dehydratase required initiation by ATP, $MgCl_2$ and a reducing agent such as dithionite. The initiation was mediated by an extremely oxygen-sensitive initiator protein (FldI), which was present in the cell-free extract (Dickert *et al.*, 2002). FldI was identified, overexpressed, purified from *E. coli* and characterized as a homodimeric protein containing one [4Fe-4S] cluster. Oxidized FldI showed significant ATPase activity, which was suggested to be essential for unidirectional electron transfer. FldI showed significant homology to the component A of 2-hydroxyglutaryl-CoA dehydratase from *A. fermentans*, which could replace FldI in the activation of fldBC phenyllactate dehydratase.

All four genes encoding proteins were identified and shown to be clustered in the order fldAIBC, which showed over 95% sequence identity of nucleotide and protein levels with a gene cluster detected in the genome of the closely related *Clostridium botulinum* Hall strain A. and with a cluster encoding hadAIBC proteins in the genome of *Clostridium difficile* (Dickert *et al.*, 2002).

The last step of the pathway, reduction of *(E)*-cinnamic acid to 3-phenylpropionic acid, was proposed to be catalysed by the 2-enoate reductase (Bühler *et al.*, 1980). The reaction is coupled to ATP formation (Bader and Simon, 1983). ATP formation was observed in *C. sporogenes* whole cells incubated with cinnamic acid under atmosphere of hydrogen. ATP generation was strongly inhibited in the presence of *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP), which is known to uncouple proton transfer. Moreover, ATP formation was only observed with intact, freshly harvested cells (Bader and Simon, 1983). When protein extract were used, cinnamic acid was reduced at the expense of NADH, however ATP formation was not restored, which suggested that ATP was generated *via* electron transfer phosphorylation.

It was also shown that *C. sporogenes* reduces L-tryptophan, L-tyrosine and L-leucine to indole-3-propanoic acid, 3-(*p*-hydroxyphenyl)propanoic acid and 4-methylvaleric respectively (Jellet *et al.*, 1980; Barker, 1981). On the other hand L-valine and L-isoleucine were oxidized to isobutyric acid and 2-methylbutyric acid, but not reduced (Barker, 1981). The capability of whole cells to form ATP during the hydrogenation of enoates derived from amino acids acting as electron acceptors and donors was compared with the substrate specificity of the enoate reductase. Only substrates of the enoate reductase increased the generation of ATP showing that the enoate reductase may determine the substrate range of the Stickland reaction (Bader and Simon, 1983).

The variety of compounds that are accepted in the Stickland reaction makes proteolytic Clostridia an excellent source of unusual oxidoreductases, which may be used in industrial biotransformations.

2.4. Clostridial 2-enoate reductases

Clostridial 2-enoate reductases are extremely useful in chiral synthesis as they catalyse the stereospecific hydrogenation of activated C=C double bonds (Fig. 2.7.).

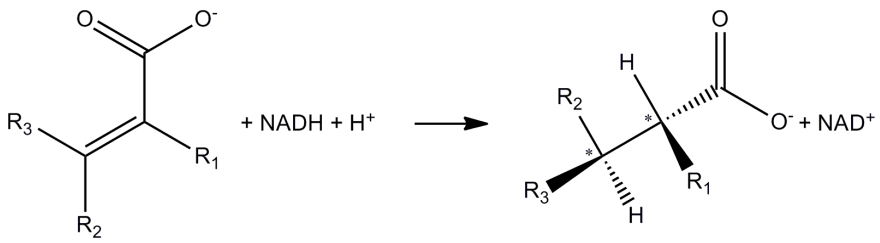
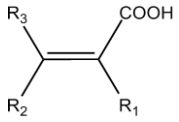
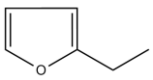
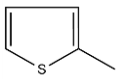


Fig. 2.7. Stereospecific hydrogenation of enoates by 2-enoate reductase.

The activity of the enoate reductase activity in Clostridia was first described by Simon and co-workers in 1974. *Clostridium kluyveri* was found to reduce numerous α,β-unsaturated carboxylic acids with hydrogen gas used as the electron donor (Table 2.2.). The procedure enabled to obtain on a preparative scale a wide variety of chiral compounds (Simon *et al.*, 1974).

Table 2.2. List of 2-enoates hydrogenated by *C. kluyveri* (from Simon *et al.*, 1974).

Acid				**Relative rate of reduction**
	R_1	**R_2**	**R_3**	
tiglic	CH_3	CH_3	H	1.0
angelic acid	CH_3	H	CH_3	0.2
acrylic acid	H	H	H	3.2
methacrylic acid	CH_3	H	H	2.0
dimethylacrylic acid	H	CH_3	CH_3	0.2
E-2-pentenoic acid	H	C_2H_5	H	1.0
E-2-hexenoic acid	H	*n*-C_3H_7	H	0.9
sorbic acid	H	*n*-C_3H_5	H	1.0
ethyl hydrogen fumarate	H	$C_2H_5O_2C$	H	0.6
E-3-furfurylacrylic acid	H		H	1.0
E-3-(2-thienyl)acrylic acid	H		H	1.3
cinnamic acid	H	C_6H_5	H	0.9
E-α-methylcinnamic acid	CH_3	C_6H_5	H	0.7
Z-*p*-chloro-α-methoxycinnamic acid	OCH_3	C_6H_4Cl	H	0.8
Z-*p*-bromo-α-methoxycinnamic acid	OCH_3	C_6H_4Br	H	0.8
1-cyclohexanecarboxylic acid	$-(CH_2)_4-$		H	0.1

2-Enoate reductases were found in various saccharolytic and proteolytic Clostridia such as *C. kluyveri* and *C. tyrobutyricum, C. sporogenes, C. thermoaceticum* and in *Peptostreptococcus anaerobius* (Tischer *et al.*, 1979; Bader *et al.*, 1980; Giesel *et al.*, 1981; Rohdich *et al.*, 2001).

Structural properties of enoate reductase were determined for enzymes from *C. kluyveri* and *C. tyrobutyricum* (Tischer *et al*., 1979; Kuno *et al.*, 1985). The enzyme has a molecular weight of 940,000 ± 20,000 Da and is a dodecamer (tetramer of trimers) of a single type of 73,000 ± 2,000 Da subunit. The purified enzymes showed the presence of FMN, FAD and iron and acid labile sulphur.

The reduction of C=C double bonds proceeds through a Bi Bi ping pong mechanism and is not reversible (Bühler and Simon, 1982). The reaction is extremely oxygen sensitive and relatively stable against hydrogen peroxide. Experiments using different types of inhibitors showed that three binding domains are present in the reductase. The first domain is responsible for binding of NADH and can be blocked with dicoumarol or morin. The second domain binds enoates and can be blocked with fumarate, whereas the third one is an electron transfer subunit that can bind reduced methylviologen (Bühler and Simon, 1982).

Although the substrate specificity of reductases from saccharolytic *C. tyrobutyricum* and *C. kluyveri* is broad, the stereospecificity of reaction remains very high. Enoate reductases from proteolytic strains such as *C. sporogenes* show much narrower substrate range and accept only aromatic enoates such as cinnamic acid (Bühler *et al.*, 1980). The enoate reductases accept electrons from NADH and reduced methyl viologen showing that they are able to accept both electron pairs as well as single electrons (Simon, 1991). They are able to deliver electron pairs and single electrons to NAD^+, viologens, dichlorophenol indophenol, hexacyano ferrate-III and oxygen.

Regarding the substrate range, some general rules have been found to apply (Simon *et al.*, 1985). R_1 cannot be too large and R_2 can be a phenyl ring (Fig. 2.7.). Halogens are accepted on the α-carbon, but are reductively eliminated from the β-position. If R_2 and R_3 are different and E/Z isomers are used as substrates, different enantiomers are produced if the β-carbon becomes chiral. Therefore, pure isomers of substrates should be used when enantiopure product is needed. The C=C double bond can also be a part of a ring as shown for reduction of cyclohexene-1-carboxylate.

Enoate reductase from *C. tyrobutyricum* and *C. kluyveri* were also found to catalyze reduction of α,β-unsaturated aldehydes and alcohols (Simon, 1991). However, these reactions are not enantioselective as the enoate reductase causes the racemization of the products.

The physiological role of 2-enoate reductases is reduction of enoates at the expense of NADH generated from oxidation of amino acids or carbohydrates. An additional function may be conservation of energy, as ATP formation is coupled to the reduction of cinnamic acid by *C. sporogenes*. The physiological role of enoate reductase in saccharolytic Clostridia is not known (Bühler *et al.*, 1980; Bader and Simon, 1983).

The genes encoding enoate reductases were identified in *C. tyrobutyricum* and *Moorella thermoacetica* (Rohdich *et al.*, 2001). Sequence comparison showed that enoate

reductases are similar to the Old Yellow Enzyme from yeasts and to a family of flavoproteins comprising 2,4-dienoyl-coenzyme A reductase from *E. coli*. The recombinant enoate reductase from *M. thermoacetica* was overexpressed in *E. coli* grown under anaerobic conditions. The protein produced was soluble and active and showed enzymatic activity towards *(E)*-methylbutenoate using NADH as the electron donor. The expression of the enoate reductase from *C. tyrobutyricum* resulted in insoluble and inactive product.

Due to the broad substrate range and high stereoselectivity of the reaction, enoate reductases were successfully used for preparation of chiral carboxylates (Simon *et al.*, 1985). High yields and good enantiomeric excess of the products were achieved when whole cells of Clostridia were used as biocatalysts with hydrogen gas as the electron donor. The efficiency of the biotransformations was improved by using catalytic concentrations of methylviologen, which was reduced by endogenous clostridial hydrogenase at the expense of hydrogen gas and served as electron donor in the biotransformations of enoates (Simon *et al.*, 1985). Reduced methylviologen could also be recycled by using electrochemical cells together with enoate reductases immobilized on cellulose filters or modified carbon electrodes (Thanos and Simon, 1987).

Although the enoate reductase from *C. sporogenes* has never been purified and identified, it was successfully applied to industrially relevant biotransformations. Crude protein extracts of *C. sporogenes* containing the enoate reductase were used for highly enantioselective reduction of C=C double bonds in β,β- and α,β-disubstituted nitroalkenes (Fig. 2.8.; Fryszkowska *et al.*, 2008).

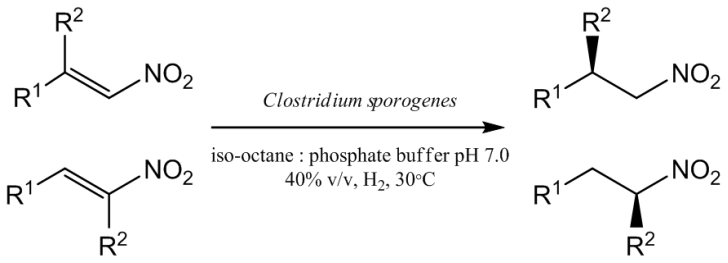


Fig. 2.8. Enantioselective reduction of C=C double bonds in β,β- and α,β-disubstituted nitroalkenes by cell-free extracts of *C. sporogenes* (taken from Fryszkowska *et al.*, 2008).

Aryl derivatives of β,β-disubstituted nitroalkenes such as *(E)*-1-nitro-2-phenylpropene were reduced in 35-86% yield with more than 97% ee for *(R)*-enantiomers. In contrast, α,β-disubstituted nitroalkenes such as *(E)*-2-nitro-1-phenylpropene were poor substrates, yielding *(S)*-enantiomer products in low yield (10-20%), and the ee varying from 30 to 70% depending on NADH concentration.

2-Enoate reductases from anaerobic bacteria such as *C. sporogenes*, *Acetobacterium woodii* and *Ruminococcus productus* were successfully used for the synthesis of chiral γ-amino acids (Fig. 2.9.; Fryszkowska *et al.*, 2010).

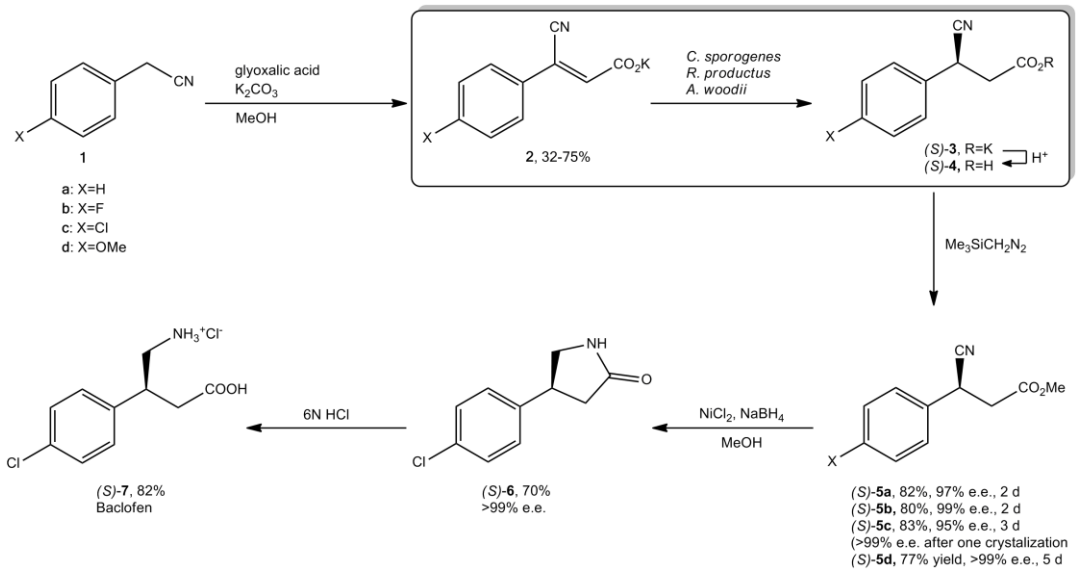


Fig. 2.9 Chemoenzymatic asymmetric synthesis of β-aryl-γ-amino acids using enoate reductases from anaerobic bacteria (taken from Fryszkowska *et al.*, 2010).

A new asymmetric methodology was proposed for preparation of γ-amino butyric acid (GABA) derivatives, which can be used to treat a range of central nervous system diseases. The key step in the new method was the use of crude extracts containing the enoate reductase for asymmetric synthesis of *(S)*-β-aryl-β-cyanopropanoic acid. The usefulness of the new approach was demonstrated by synthesis of the *(S)*-enantiomer of baclofen with 99% ee and 43% overall yield (Fryszkowska *et al.*, 2010).

The enoate reductases from *Clostridium* spp. were found to be an extremely useful tool in the synthetic chemistry. However, they cannot be fully exploited in the industrial biotransformations without making them more accessible to chemists. Thus, enoate reductases should be expressed in heterologous hosts such as *E. coli* for easier processing. Development of new efficient methods for recombinant protein expression under anaerobic conditions is needed.

2.5. Nitroreductases

New routes for synthesis of achiral as well as chiral amines are increasingly in demand, as these compounds are widely used as building blocks for preparation of pharmaceuticals and agrochemical products (Roldán *et al.*, 2008; Dipeolu, 2008).

Biocatalytic routes for amine synthesis are often based on activities of hydrolases and transaminases (Koszelewski *et al.*, 2010). The most frequently used method for generation of chiral amines is the kinetic resolution of racemic materials by enantioselective hydrolysis. Another approach employs transaminases for asymmetric amination of ketones or kinetic resolution starting from racemic amines. Using chemical catalysts, chiral and achiral amines are synthesized by a direct reduction of amides, azides, imines or nitro compounds with metal catalysts (Clayden *et al.*, 2000), which are associated with high toxicity. Therefore, new biocatalytic methods are needed for generation of amines and reductive biosynthesis of amines using nitroreductases would create an attractive alternative to existing methods.

Only a few natural nitroaromatic compounds are present in nature such as chloramphenicol, nitropyoluteorin, oxypyrrolnitrin and phidolopin (Roldán *et al.*, 2008). Most nitro compounds are xenobiotic chemicals and are released to the environment as a consequence of industrial processes. Thus, nitroreductases are involved in the bioremediation process of soil, water and air. Another aspect of using nitroreductases is the anticancer therapy, in which the pro-drug is enzymatically reduced to the highly cytotoxic hydroxylamine derivative to sensitize the tumour cells (Minton, 2003; Roldán *et al.*, 2008).

Nitroreductases are NAD(P)-dependent flavoenzymes catalysing reduction of nitro groups in a variety of polynitrated aromatic compounds (Roldán *et al.*, 2008). The reduction of nitro group can be carried out through one- or two-electron mechanism (Fig. 2.9.). The bacterial nitroreductases type I are oxygen-insensitive enzymes catalysing the sequential reduction of nitro groups through the addition of electron pairs to generate nitroso, hydroxylamino and amino products. They have been found in a variety of organisms including bacteria, archea and eukaryote. They are homodimers of molecular weight about 50-60 kDa and consisting of 24-30 kDa subunits. The reaction proceeds through a Bi Bi ping pong mechanism. Nitroreductases are strongly inhibited by dicoumarol, *p*-hydroxymercuribenzoate, *p*-iodosobenzoic acid, sodium azide and Cu^{2+} ions (Kinouchi and Ohnishi, 1983; Roldán *et al.*, 2008).

Most bacteria contain several types of type I nitroreductases showing different specificities towards nitro substrates and accepting different electron donors (Kinouchi and Ohnishi, 1983). Two groups of nitroreductases are distinguished depending on homology to nitroreductases NfsA and NfsB from *E. coli*. Nitroreductases group A show homology to NfsA nitroreductase and are usually NADPH-dependent, whereas nitroreductases group B are similar to NfsB and accept electrons from NADH. Main substrates for type I

nitroreductases are nitrofurazone, *p*-nitrobenzoate, nitrofuran and other nitro compounds as well as quinones (Roldán *et al.*, 2008).

In contrast, bacterial nitroreductases type II catalyze the reduction of nitro group by the addition of one electron, forming a nitro anion radical, which is subsequently oxidised by molecular oxygen in futile cycle (Fig. 2.10.).

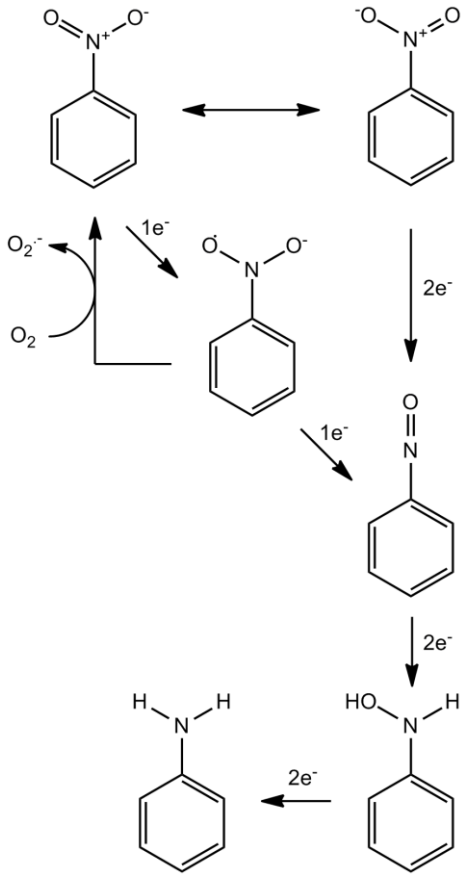


Fig. 2.10. Reduction of nitro compounds by oxygen-sensitive and insensitive nitroreductases (taken from Roldán *et al.*, 2008).

Nitro compounds can also be denitrated by OYE family reductases. PETN reductase from *Enterobacter cloacae*, NemA reductase from *E. coli* and xenobiotic reductases from *Pseudomonas putida* were found to reduce the nitro groups of polynitroaromatic compounds such as pentaerythritol tetranitrate, glycerol trinitrate and trinitrotoluene (Roldán et al., 2008).

Most nitroreductases do not catalyze the complete reduction of nitro group to amines and hydroxylamine products are formed (Roldán *et al.*, 2008). There are few reports of successful biocatalytic reduction of aromatic nitro compounds to amines. Whole cells of *Peptostreptococcus productus* U-1 grown in a fructose-limited chemostat with caffeate

were used to reduce *(E)*-2-nitro-1-phenylpropene and *(E)*-2-nitro-1-phenylbutene in a two-liquid phase reaction system (Fig. 2.11.; Korbekandi *et al.*, 2008).

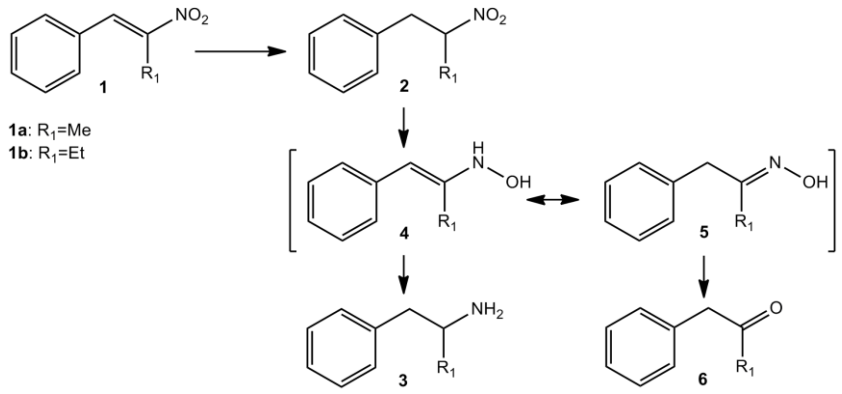


Fig. 2.11. Proposed reaction scheme for nitroalkene reduction using *P. productus* (taken from Korbekandi *et al.*, 2008).

The aminoalkanes were formed in 47% and 7.5% yields respectively, by reduction of both the aliphatic nitro group and the C=C double bond. Direct reduction of nitroalkanes was also demonstrated using synthetic racemic nitrophenylpropane as a substrate, which was reduced to amine derivative in 45% yield.

The biocatalytic activity of nitroreductase from *Salmonella typhimurium* (NRSal) was investigated for the reduction of nitroalkenes and nitroaromatics (Yanto *et al.*, 2010). The recombinant protein was purified to homogeneity and NRSal showed broad substrate acceptance for various nitro compounds such as 1-nitrocyclohexene and aliphatic nitroalkenes as well as nitrobenzene. Nitrobenzene was reduced to form nitrosobenzene first and phenylhydroxylamine next. Azoxybenzene was the side product and was formed by spontaneous condensation of intermediates. However, the yield for amine product, aniline, was very poor (6%).

Aromatic nitro compounds were also successfully reduced by *C. sporogenes*. The NADH-dependent reduction of the nitro group of *p*-nitrobenzoate was catalysed using protein crude extracts. Two different enzymatic activities were found to reduce aromatic nitro groups. At first the nitro radical anion was formed as a result of transfer of one electron. Subsequently, the intermediate compound was hydrogenated further, but extremely slowly (Angermaier and Simon, 1983b). Nitrobenzene was also reduced to aniline using whole cells with yields of 8% in aqueous ethanol (4% v/v) and 45% in a biphasic heptane/aqueous system (Dipeolu, 2008). The reaction was improved by using water miscible ionic liquids as the co-solvent. [EMim][EtSO4] was toxic to *C. sporogenes* and

decreased the growth rate by 58%, but allowed reduction of nitrobenzene with higher yield up to 79% (Dipeolu et al., 2009).

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

2.6. Reduction of proline by *Clostridium* spp.

Biocatalysis in not limited to preparation of fine chemicals only. Fossil fuel shortage and environmental issues make a great need for engineered pathways in production of bulk chemicals as well. Biosynthesis of xenobiotic compounds that are not naturally synthesized in living organisms is extremely challenging. Therefore, identification of new enzymes with previously unknown activities is highly demanded. Proteolytic Clostridia possess a unique reductase responsible for the cleavage of proline ring to form 5-aminovaleric acid. This enzymatic activity may possibly be exploited for biosynthesis of bifunctional compounds that are used in the polymer industry.

L-Proline and L-4-hydroxyproline were found to oxidise leuco dyes such as benzyl viologen and neutral red, when suspension of *C. sporogenes* were used as biocatalyst (Stickland, 1934). Thus, these two amino acids were proposed to act as hydrogen acceptors in the chemical reaction by which *C. sporogenes* obtains energy for growth in media containing proteins or amino acids. This hypothesis was confirmed by the observation that L-proline could accept electrons from amino acids such as D-alanine, D-valine and L-leucine, which acted as electron donors in the reaction (Stickland, 1934). The product of L-proline reduction was 5-aminovaleric acid and the reaction took place by a simple ring opening without deamination (Stickland, 1935a). The reduction of proline was found to be coupled to a vectorial transmembrane proton translocation allowing for ATP synthesis *via* electron transfer phosphorylation (Lovitt *et al*., 1986).

Another proteolytic strain, *Clostridium sticklandii* (previously *Clostridium* HF) was found to reduce proline at the expense of L-ornithine (Stadtman, 1954). Both L- and D-proline were accepted as substrates and converted into 5-aminovaleric acid when crude exctracts and partially purified enzyme fractions were used (Stadtman, 1956). The biotransformation depended on the presence of dithiols, magnesium ions and reduced nicotinamide adenine dinucleotide in the reaction mixture. The proline reduction system was found to comprise two components, a proline racemase and a D-proline reductase

(Stadtman and Elliott, 1957). The racemase component rapidly converted either optical isomer of proline to the racemic mixture, but exhibited no reductase activity. The latter component catalyzed the reduction of D-proline to 5-aminovaleric acid and had no activity on L-proline. The reduction was coupled to the oxidation of 1,3-dimercaptopropanol, which could not be replaced by molecular hydrogen or NADH.

The *C. sticklandii* D-proline reductase was purified to homogeneity (Seto and Stadtman, 1976). Initial experiments showed the enzyme was a membrane-bound protein and was released by treatment with detergents. The molecular weight was determined to be approximately 300 kDa containing 10 subunits of about 31 kDa (Seto and Stadtman, 1976). However, further studies showed that the reductase was a soluble protein located in the cytoplasm. It was a decamer having a molecular mass of about 870 kDa consisting of 23, 26 and 45 kDa subunits (Kabisch *et al.*, 1999). The specific activity of the 870 kDa reductase was 100 times higher than the activity of previously purified 300 kDa protein, showing that the 23, 26 and 45 kDa subunits were essential components of active D-proline reductase. The 26 kDa polypeptide contained selenocysteine, which correlated with the presence of selenium in the enzyme, determined by inductively coupled plasma mass spectrometry (Kabisch *et al.*, 1999).

The reductase accepted NADH as electron donor, but not NADPH (Seto and Stadtman, 1976). The electrons from NADH to the reductase were transferred by two proteins involving FAD-containing NADH dehydrogenase and a 250 kDa iron-containing protein (Schwartz and Müller, 1979). However, dithiothreitol and other dithiols could also be used as artificial reducing agents in the reactions (Seto and Stadtman, 1976).

Studies of the mechanism of D-proline reductase action showed that a carbonyl group of pyruvate was required for the catalytic activity (Hodgins and Abeles, 1969). The pyruvate was found to be located on a polypeptide connected with the N-terminus of the reductase *via* an ester bond (Seto, 1978; Seto, 1980). The carbonyl group formed an adduct with the nitrogen of the substrate and nucleophilic attack at the α-carbon of proline was carried out by an electron-donating selenol anion of selenocysteine (Fig. 2.12.). That resulted in the ring cleavage after hydrolytic release of the product from the pyruvate adduct (Hodgins and Abeles, 1969). Subsequently the selenide/sulphide group was reduced by the electron donor (Kabisch *et al.*, 1999).

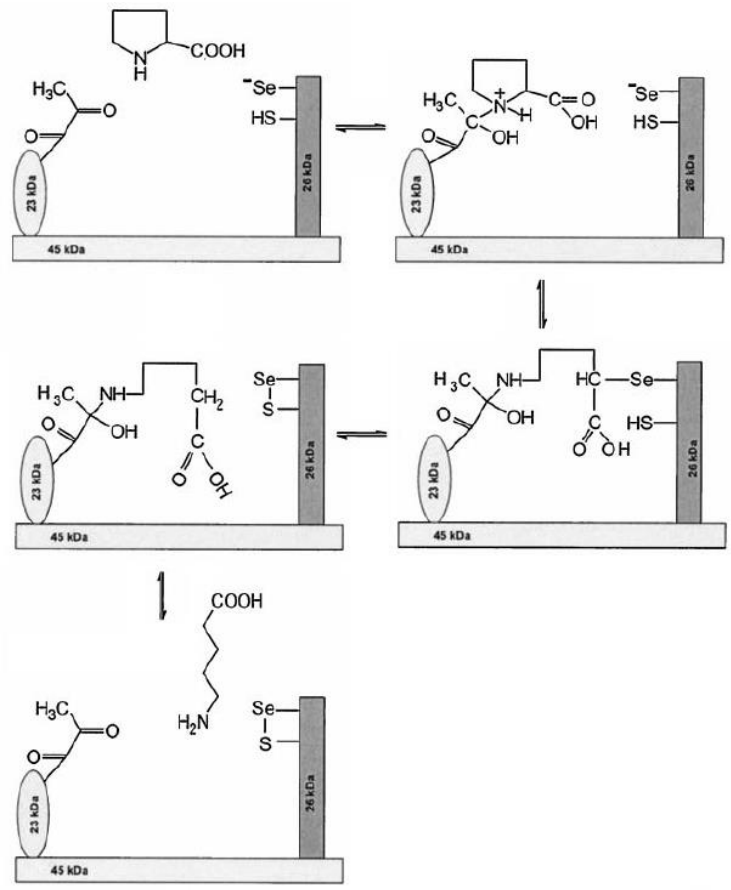


Fig. 2.12. Proposed reaction mechanism of D-proline reductase (taken from Kabisch et al., 1999).

The genes encoding D-proline reductase subunits were identified in *C. sticklandii* (Kabisch *et al*., 1999). The *prdA* gene encoded a proprotein consisting of 630 amino acids that was posttranslationally cleaved to form 23 and 45 kDa subunits. The product of the *prdB* gene encoding 242 amino acids had 26 kDa and contained a TGA codon for selenocysteine.

The proline reductase was also identified in the proteolytic nosocomial pathogen *Clostridium difficile* (Jackson *et al.*, 2006). Genes *prdA* and *prdB* predicted to encode subunits of the enzyme were located within the *prd* operon and showed organization similar to that found in *C. sticklandii*. Located downstream was an open reading frame predicted to encode the proline racemase (*prdF*). The product of *prdR* gene located upstream of *prdA* was found to activate transcription of the proline reductase system genes in the presence of proline and negatively regulated the expression of genes encoding glycine reductase (Bouillaut *et al.*, 2013).

The purified reductase utilized D-proline as a substrate, but in the presence of L-proline no formation of 5-aminovaleric acid was observed (Jackson *et al.*, 2006). It was also shown that L- and D-4-hydroxyproline could replace proline as the electron acceptor in the

fermentation and the presence of these amino acids induced the expression of D-proline reductase. However, they were not utilized as substrates by the purified proline reductase. In contrast to previous reports, the proline reductase from *C. difficile* did not require divalent cations such as Mg^{2+} and remained active in the presence of chelating agents. That suggested that *C. difficile* proline reductase may reduce proline by a different mechanism than the enzyme from *C. sticklandii* (Jackson *et al.*, 2006).

The enzymatic activities described in this literature review show that anaerobic bacteria, such as *C. sporogenes*, possess unusual metabolic pathways and unique enzymes that may be used as biocatalysts for industrially relevant reactions. Therefore, further characterization of reductases responsible for hydrogenation of C=C double bonds, aromatic nitro reduction and proline reduction is highly desirable and may possibly make a great contribution in the field of biocatalysis.

3. Objectives of investigation

The main aim of this study was to test *C. sporogenes* as a whole cell biocatalyst and source of enzymes for industrially relevant reductions of C=C double bonds, aromatic nitro groups and novel intermediates in Nylon production.

Biocatalytic reduction of nitroalkenes is a promising reaction in chiral synthesis, as it gives up to two new asymmetric carbons in the structure and the product can be easily converted into corresponding amines, aldehydes, carboxylic acids or denitrated compounds (Fryszkowska *et al.*, 2008). *C. sporogenes* was found to hydrogenate the C=C double bonds in α,β- and β,β-disubstituted nitroalkenes, but purification of enzyme catalysing this type of reduction was unsuccessful (Fryszkowska *et al*., 2008). Preliminary studies on the *C. sporogenes* fldZ enoate reductase knock out mutant showed that this enzyme may be involved in reduction of *(E)*-1-nitro-2-phenylpropene and *(E)*-2-nitro-1-phenylpropene (Mordaka, 2010). However, additional biotransformations and attempts to purify the nitroalkene reductase suggested that *C. sporogenes* may possess more than one enzyme reducing C=C double bonds. Thus, the aims of this part of the project were:

- Genetic analysis of the fldZ mutant (testing the strain stability, determination of number of intron integration sites in the genome);
- Testing wild type *C. sporogenes* and the fldZ mutant for reduction of unsaturated carboxylic acids, nitroalkenes and nitro compounds;
- Identification of genes encoding C=C double bond reductases in *C. sporogenes* DSM795 by analysis of the genome sequences available in the Genbank;
- Overexpression of *C. sporogenes* C=C double bond reductases in a heterologous host, purification and characterization of the enzymes.

The fldZ mutant showed unexpected physiological behaviour when grown in media supplemented with carbohydrates and amino acids. Therefore, a side objective of the project was:

- Determination of physiological function of the fldZ enoate reductase in *C. sporogenes*.

Whole cells of *C. sporogenes* were also found to reduce aromatic and aliphatic nitro groups to amines (Angermaier and Simon, 1983a; Angermaier and Simon, 1983b; Dipeolu,

2008; Dipeolu *et al.*, 2009). However, the enzymes responsible have not been purified and identified. The aims of the second part of the project were:

- Testing protein extracts of wild type *C. sporogenes* for nitroreductase activity, optimization of reaction conditions in respect to electron donors;
- Identification of genes encoding hypothetical nitroreductases in *C. sporogenes* DSM795 by analysis of genomic sequences;
- Overexpression of nitroreductases in a heterologous host and testing their activity towards a library of aromatic nitro compounds.

Traditional metabolic pathways are engineered to develop microorganisms for efficient production of chemicals, fuels and materials from renewable feedstocks. Currently Nylon 6 is synthesised by ring-opening polymerization of caprolactam, which is produced at industrial scale starting from fossil fuels. Thus, identification of new methods for sustainable synthesis of Nylon 6 is highly demanded. The aims of the third part of the project were:

- Analysis of metabolic pathway databases, screening for new routes leading to production of Nylon 6 monomers;
- Development of a method for detection of 6-aminocaproic acid;
- Testing *C. sporogenes* D-proline reductase activity towards pipecolic acid.

4. Materials and methods

4.1. Materials

4.1.1. Reagents

Acids, bases and salts: acetic acid (Fisher), ammonium chloride (Sigma-Aldrich), ammonium molybdate (BDH), ammonium persulphate (Fisher), ammonium sulphate (Sigma-Aldrich), boric acid (Sigma-Aldrich), calcium chloride (Sigma-Aldrich), dipotassium hydrogen orthophosphate (Fisher), hydrochloric acid (Sigma-Aldrich), imidazole (Sigma-Aldrich), iron sulphate heptahydrate (Rectapur), magnesium chloride (BDH), magnesium sulphate (Fisher), magnesium sulphate heptahydrate (Sigma-Aldrich), manganese sulphate tetrahydrate (Analar), potassium hydroxide (Fisher), potassium phosphate monobasic (Sigma-Aldrich), sodium dodecyl sulphate (Fisher), sodium fumarate (Sigma-Aldrich), sodium hydrogen carbonate (Sigma-Aldrich), sodium hydroxide (Fisher), sodium phosphate dibasic dodecahydrate (Sigma-Aldrich), sodium selenite pentahydrate (Acros), Tris base (Melford);

Amino acids: D-alanine (Sigma-Aldrich), glycine (Fisher, Sigma-Aldrich), L-alanine (Sigma-Aldrich), L-arginine (Sigma-Aldrich), L-cysteine (Sigma-Aldrich), L-cysteine HCl (Sigma-Aldrich), L-histidine (Sigma-Aldrich), L-isoleucine (Sigma-Aldrich), L-leucine (Sigma-Aldrich), L-methionine (Sigma-Aldrich), L-phenylalanine (Sigma-Aldrich), L-tryptophan (Sigma-Aldrich), L-tyrosine (Sigma-Aldrich), L-valine (Sigma-Aldrich);

Antibiotics: carbenicillin (Sigma-Aldrich), chloramphenicol (Sigma-Aldrich), erythromycin (Sigma-Aldrich), kanamycin sulphate (Novagen);

Biotransformation substrates: cinnamic acid (Aldrich), DL-pipecolic acid (Sigma-Aldrich), D-pipecolic acid (Tokyo Chemical Industry), L-pipecolic acid (Tokyo Chemical Industry), NADH (Sigma-Aldrich), NADPH (Sigma-Aldrich);

Derivatization agents: *o*-phthalaldehyde (Sigma-Aldrich), trimethylsilyldiazomethane (Sigma-Aldrich);

Electrophoresis reagents and molecular-weight size markers: 1kb DNA Ladder (New England Biolabs); 1kb Plus Ladder (Invitrogen), acrylamide:N,N'-methylenebisacrylamide (Sigma-Aldrich), Agarose (Fisher), bromophenol blue (Sigma-Aldrich), EZBlue stain (Sigma-Aldrich), Instant Blue Stain (Novexin), Loading Dye Buffer 6x (Fermentas), Mini-PROTEAN® TGX™

Precast Gels (Bio-Rad), N,N,N',N'-tetramethylethylenediamine (Sigma-Aldrich), PageRuler^{TM}Prestained Protein Ladder (Fermentas), Spectra Protein Ladder (Fermentas);

Enzymes: Antarctic Phosphatase (New England Biolabs), Benzonase® Nuclease (Sigma-Aldrich), *Bgl*II FD (Fermentas), cytochrome *c* (Sigma-Aldrich), *Eco*RI FD (Fermentas), High Activity Bovine Thrombin (Novagen), *Hind*III FD (Fermentas), JumpStart REDTaq DNA Polymerase (Sigma-Aldrich), KOD Polymerase (Novagen), *Nde*I FD (Fermentas), *Not*I FD (Fermentas), Proteinase K solution (QIAGEN), RNase A (QIAGEN), T4 ligase (Fermentas), *Xba*I FD (Fermentas);

Gases: CO_2 (BOC), H_2 (BOC), He ultra-high purity (BOC), N_2 (BOC);

Kits: BugBuster® Protein Extraction Reagent (Novagen), Chelex 100 (Sigma-Aldrich), CloneJET™ PCR Cloning Kit (Fermentas), cOmpleteMiniEDTA-free protease inhibitor (Roche), dNTP mix (Roche), Dynabeads® His-Tag Isolation (Invitrogen), FailSafe™ PCR System (Epicentre), His-Bind Purification Kit (Novagen), Ni-NTA Spin Kit (QIAGEN), Pierce Protein Refolding Kit (Fisher), Plasmid Midi Kit (QIAGEN), QIAprep Spin Miniprep Kit (QIAGEN), QIAquick Gel Extraction Kit (QIAGEN), QIAquick PCR Purification Kit (QIAGEN), TransformAid™ Bacterial Transformation Kit (Fermentas);

Media components: agar (Melford), casamino acids (Sigma-Aldrich), cooked meat medium (Lab M Limited), D-glucose (Fisher), glycerol (Fluka, Sigma-Aldrich), LB granulated medium (Melford), peptone (Foremedium, Melford), resazurin (Aldrich), tryptone (Foremedium, Melford), yeast extract (Melford);

Reducing agents: 2-β-mercaptoethanol (Sigma-Aldrich), dithiothreitol (Sigma-Aldrich), glutathione (Sigma-Aldrich), sodium thioglycollate (Fluka), tris(hydroxypropyl)phosphine (Novagen);

Solvents and detergents: [EMim][EtSO4] (Sigma-Aldrich), 3,4-dinitrotoluene (Sigma-Aldrich), butan-2-ol (Sigma-Aldrich), ethanol absolute (Fisher), *iso*-octane (Sigma-Aldrich), phenol:chloroform:isoamyl alcohol (Sigma-Aldrich), propan-2-ol (Sigma-Aldrich), *tert*-butylbenzene (Acros), *tert*-butylmetylether (Sigma-Aldrich), Triton X-100 (Sigma-Aldrich), Tween®-20 (Sigma-Aldrich);

Vitamins and cofactors: biotin (Sigma-Aldrich), cyanocobalamin (Sigma-Aldrich), FAD (Sigma-Aldrich), FMN (Sigma-Aldrich), folic acid (Sigma-Aldrich), lipoic acid (Sigma-Aldrich),

nicotinic acid (Sigma-Aldrich), *p*-aminobenzoic acid (Sigma-Aldrich), pantothenic acid (Sigma-Aldrich), pyridoxyamine HCl (Sigma-Aldrich), riboflavin HCl (Sigma-Aldrich), thiamine HCl (Sigma-Aldrich).

4.1.2. Substrates and products for biotransformations

Substrates and racemic products for phenylnitropropene biotransformations ((*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. Other substrates and products of biotransformations were purchased from Sigma-Aldrich with the highest purity grades available.

4.1.3. Bacterial strains

Clostridium sporogenes DSM795 and *Escherichia coli* DSM5911 (K-12, W3110) cultures were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). *E. coli* JM107 strain was obtained from Fermentas. *E. coli* BL21(DE3)pLysS competent cells were obtained from Merck. *C. sporogenes* NCIMB10696, *E. coli* TOP10 and *E. coli* CA434 were obtained from the Clostridia Research Group, Centre for Biomolecular Sciences, University of Nottingham.

Clostridium sporogenes genomic knockout mutant (*fldZ*::CT*erm*B) was prepared using the Clostron technique (see Appendix 8.1.) by Dr. Benjamin Blount and Prof. Nigel Minton (Clostridia Research Group, Centre for Biomolecular Sciences, University of Nottingham).

4.1.4. Oligonucleotides and synthetic genes

Synthetic oligonucleotides (Tab. 4.1) were synthesised by Eurofins Genetic Service.

Tab. 4.1. List of synthetic oligonucleotides.

Name	Sequence
CLOSPO_02780f	5'-GAATTAGAGCTTCCATTAGGTCTACACAC-3'
CLOSPO_02780r	5'-AGATTGCGGTGGTACTATTATCTCTTATAC-3'
EBSUni	5'-CGAAATTAGAAACTTGCGTTCAGTAAAC-3'
ENR-F1	5'-GACAGAAAGAGTCCATGCATATGG-3'
ENR-R1	5'-TCCATTCCTGCAACACCGCCACC-3'
ErmRAM-F	5'-ACGCGTTATATTGATAAAAATAATAATAGTGGG-3'
ErmRAM-R	5'-ACGCGTGCGACTCATAGAATTATTTCCTCCCG-3'
pJET1.2 Forward	5'-CGACTCACTATAGGGAGAGCGGC-3'
pJET1.2 Reverse	5'-AAGAACATCGATTTTCCATGGCAG-3'
PM001	5'-AACCATGGCTATGAAGGATAAGTATAAGGTACTTTATGAC-3'
PM002	5'-AAGCGGCCGCAATATTTTTAGCTACTTCATAT-3'
PM003	5'-GGATGTACTACAAGTGCTATTCAC-3'
PM004	5'-CCTACAATAGATGGTATACTTACTCTACC-3'

PM006	5'-CTATTATTACAGCAGGAAGAATG-3'
PM008	5'-AGTGCTGGATTTAGYCTAC-3'
PM011	5'-GAAAGTTCAACATTAAATCAAAYTATGC-3'
PM012	5'-CAACCTCTGTTTCTCTTATTCC-3'
PM015	5'-ATCGTCATGATGTTTTTCACAGAACAACATGA-3'
PM016	5'-AAAGCGGCCGCCTTTAAAAGATTTGATG-3'
PM017	5'-AAACATATGAAAGACAAATACAAAGTGCTGTATGAC-3'
PM018	5'-AAAGCGGCCGCAATATTCTTCGCTACTTCATAGGCTGAC-3'
PM019	5'-AAAGCGGCCGCTCAAATATTCTTCGCTACTTCATAGGCTGAC-3'
PM020	5'-CGTACATATGAAAAGTTTATTTGATAAAACCTGC-3'
PM021	5'-AGTGCGGCCGCCATATTAAATATGCA-3'
PM022	5'-AAAGCGGCCGCTCACATATTAAATATGCA-3'
PM023	5'-TAGATACTATAATACAAATAAGTGCGTACTTGTAA-3'
PM024	5'-GGACTTATTATACAACTGATGGTAAACCT-3'
PM025	5'-CTAGTATATCATGTTTACTTGACAAAAGT-3'
PM026	5'-TTCAGCTGCTCTTACAATCATTGA-3'
PM027	5'-CTACTAAAAAGTGCATACTTCTTTTTCTG-3'
PM028	5'-AATGATTATARAAAACAGTTTGGGAAATATC-3'
PM029	5'-TTATGTTAGATTTATTAAAACAAAGAAGAAG-3'
PM030	5'-CTAAGTATATACCGAGTATAATTGCA-3'
PM031	5'-AGAACACACATACTAATGAAAAAGGAGTG-3'
PM032	5'-CATAATGGAAGTATCATTCTATTACATGCAGT-3'
PM033	5'-GGGAATTATAAATGAATGCTATATTAAAG-3'
PM034	5'-CTTGACTAATATACCTTATATCTGATC-3'
PM035	5'-TCATTGAACAAATTATATGTTAGTTAGAGGA-3'
PM036	5'-CGTAATTTCCTTATCTTATGTAAGTTATTACATTTT-3'
SalI-R1	5′-ATTACTGTGACTGGTTTGCACCACCCTCTTCG-3′
T7 promoter	5'-TAATACGACTCACTATAGGG-3'
T7 terminator	5'-GCTAGTTATTGCTCAGCGG-3'

Synthetic genes were synthesised by Eurofins Genetic Service (*fldZ* gene) and Biomatik USA, LLC (*CsNTR1*, *CsNTR3*, *CsNTR4*, *CsNTR5*, *CsNTR6* and *CsNTR7* genes).

4.2. Methods

4.2.1. Maintenance media

4.2.1.1. Maintenance of *E. coli* JM107, K-12 and BL21(DE3)pLysS

The LB medium was composed of tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L) and agar (20 g/L) in final volume 200 mL in 0.5 L Duran bottles. The medium was autoclaved (12L Classic Media Portable Autoclave, Prestige Medical) and left in the water bath to cool down to 50°C (GLS Aqua Plus, Grant). The medium for BL21(DE3)pLysS strain was supplemented with glucose (1% final concentration, filter sterilized using a syringe filter, pore size 0.2 µm, Sartorius) and chloramphenicol (272 µL, 25 mg/mL solution in ethanol). Agar plates were poured when the medium was still warm and fluid.

4.2.1.2. 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 (0.2 mL of 1% w/v stock solution) and agar (20 g/L) with final volume 200 mL in 0.5 L narrow neck Pyrex bottles. The solution was sparged with oxygen-free N_2 for at least 1 h. The suba seals cap was 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 and agar plates were poured in the anaerobic cabinet (MARK3 Anaerobic Workstation or MACS, Don Whitley Scientific Limited) when the medium was still warm and fluid.

4.2.1.3. Maintenance of *C. sporogenes fldZ*::CT*erm*B

The TYG medium was composed of tryptone (30 g/L), yeast extract (20 g/L), sodium thioglycollate (1 g/L), resazurin (0.1 mL of 1% w/v stock solution) and agar (20 g/L) with final volume 100 mL in a 0.5 L narrow neck Pyrex bottle. The solution was sparged with oxygen-free N_2 for at least 1 h. The suba seals cap was 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°C. In the anaerobic cabinet, erythromycin was dissolved in ethanol, sterilized by filtration and added to final concentration 2.5 μg/mL. Agar plates were poured when the medium was still warm and fluid.

4.2.2. Growth media (culture media)

4.2.2.1. LB medium

LB medium was composed of LB granulated medium (25 g) with final volume of 1 L in Duran bottle and sterilized by autoclaving. For BL21(DE3)pLysS strain, medium was composed of LB granulated medium (25 g) in 0.95 L of dH_2O. The medium was sterilized, let to cool down to the room temperature and supplemented with 20% D-glucose solution (50 mL, filter sterilized).

4.2.2.2. TB medium (terrific broth medium)

Terrific broth was composed of tryptone (12 g), yeast extract (24 g) and glycerol (4 mL) with final volume 900 mL in 1 L Duran bottle. The medium was autoclaved, cooled down to room temperature and supplemented with phosphate buffer/glucose solution (100 mL, filter sterilized, containing glucose, 100 g/L; KH_2PO_4, 23.1 g/L; K_2HPO_4, 125.4 g/L).

4.2.2.3. Nouguchi medium

Nouguchi medium (Nouguchi *et al.*, 1997) was composed of LB granulated medium (25 g) with final volume of 900 mL in Duran bottle and sterilized by autoclaving. After cooling down, the medium was supplemented with glucose/magnesium/nitrate solution (100 mL, filter sterilized, containing glucose, 1g/L; $MgSO_4 \cdot 7H_2O$, 2.465 g/L; $NaNO_3$, 5.1 g/L).

4.2.2.4. M9 mineral medium

M9 mineral medium was composed of NH_4Cl (0.8 g), NaCl (0.5 g), $Na_2HPO_4 \cdot 12H_2O$ (15.08 g) and KH_2PO_4 (3 g) with the final volume of 910 mL in 1 L Duran bottle. The medium was autoclaved, cooled down to room temperature and supplemented with trace element solution (50 mL, filter sterilized; containing $MgSO_4 \cdot 7H_2O$, 4 g/L; $CaCl_2$, 2 g/L; thiamine HCl, 22.4 mg/L; $FeCl_3 \cdot 6H_2O$, 0.2 g/L; $ZnSO_4 \cdot 7H_2O$, 36 mg/L; $CuCl_2 \cdot 2H_2O$, 24 mg/L; $MnSO_4 \cdot 4H_2O$, 31.6 mg/L; $CoCl_2 \cdot 6H_2O$, 36 mg/L) and glucose/carbonate solution (40 mL, filter sterilized, containing glucose, 112.5 g/L; $NaHCO_3$, 250 g/L).

4.2.2.5. GF medium

GF medium (Spencer *et al.*, 1973) was composed of KH_2PO_4 (5.44 g), K_2HPO_4 (10.5 g), $(NH_4)_2SO_4$ (1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $MnSO_4 \cdot 4H_2O$ (5 mg), $FeSO_4 \cdot 7H_2O$ (0.125 mg), $CaCl_2$ (0.5 mg) with the final volume of 900 mL in 1 L Duran bottle. The medium was autoclaved, cooled down and supplemented with glycerol (15 mL, 80% solution, autoclaved) and fumarate/amino acid solution (85 mL, containing sodium fumarate, 75.26 g/L; casamino acids, 5.88 g/L).

4.2.2.6. Sodium formate medium

Sodium formate medium (Ingledew and Poole, 1984) was composed of LB granulated medium (25 g) with final volume of 900 mL in Duran bottle and sterilized by autoclaving. After cooling down, the medium was supplemented with glucose/fumarate solution (100 mL, filter sterilized, containing glucose, 200 g/L; $NaHCO_3$, 10 g/L; sodium formate, 34 g/L).

4.2.2.7. Preparation of *C. sporogenes* growth medium (Giesel *et al.*, 1981)

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; $Fe_2SO_4 \cdot 7\ H_2O$, 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 kept 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 using a syringe 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.

4.2.2.8. Preparation of *C. sporogenes* minimal medium (Lovitt *et al.*, 1987)

The solution (1 L) was composed of basal medium (containing KH_2PO_4, 2 g/L; K_2HPO_4, 2 g/L; $MgCl_2{\cdot}6H_2O$, 0.2 g/L; $(NH_4)_2SO_4$, 5.0 g/L), 10% $NaHCO_3$, (25 mL, added after sterilization); 0.01% w/v resazurin, general vitamin solution (1 mL; biotin, 2 g/L; folic acid, 2 g/L; pyridoxyamine HCl, 10 g/L; thiamine HCl, 5 g/L; riboflavin HCl, 5 g/L; nicotinic acid, 5 g/L; pantothenic acid, 5 g/L; cyanocobalamin, 1 g/L; *p*-aminobenzoic acid, 5 g/L; lipoic acid, 5 g/L), amino acid solution (100 mL; glicyne, 30 mM; valine, 20 mM; isoleucine, 20 mM; arginine, 20 mM; leucine, 10 mM; histidine, 10 mM; methionine, 10 mM; phenylalanine, 10 mM; tryptophan, 10 mM; tyrosine, 1.25 mM) trace element solution (10 mL; nitrilotriacetic acid, 12.8 g/L; $Fe_2SO_4{\cdot}7H_2O$, 0.1 g/L; $MnCl_2{\cdot}4H_2O$, 0.1 g/L; $CoCl_2{\cdot}2H_2O$, 0.17 g/L; $CaCl_2{\cdot}2H_2O$, 0.1 g/L; $ZnCl_2$, 0.1 g/L; $CuCl_2$, 0.01 g/L; H_3BO_4, 0.01 g/L; $NaMoO_4{\cdot}2H_2O$, 0.01 g/L; NaCl, 1 g/L; $NaSeO_3$, 0.017 g/L; $NiSO_4{\cdot}6H_2O$, 0.026 g/L; $NaWO_4{\cdot}2H_2O$, 0.1 g/L) and reducing reagent (L-cysteine, 5%, from 5 to 10 mL as judged by the discolouration of resazurin). All solutions were degassed by sparging with N_2 for at least 1 h. Basal medium, reducing agent solution and amino acid solution were prepared freshly every time. Trace element solution was stored in a fridge (+4°C) whereas vitamin solution was frozen at -20°C. Basal medium and trace element solution were sterilized by autoclaving in bottles sealed with the suba seals caps held in place with copper wire. Other solutions were sterilized by filtration using a syringe filter in the anaerobic work station. All solutions were mixed together in the anaerobic work station. The medium was pre-reduced by overnight incubation in the anaerobic cabinet.

4.2.2.9. TYG medium

The TYG medium was composed of tryptone (30 g/L), yeast extract (20 g/L) and resazurin (0.2 mL of 1% w/v stock solution) with final volume 200 mL in a 0.5 L Duran bottle. The medium was sterilized by autoclaving using a desk top autoclave and immediately transferred to the anaerobic cabinet. The medium was left to cool down and be reduced in the cabinet overnight. Then the medium was dispensed to sterile 20 or 50 mL polypropylene sample tubes (Falcon). For cultivation of *C. sporogenes* knock out mutants, erythromycin was dissolved in ethanol, sterilized by filtration and added to the medium to the final concentration 2.5 μg/mL.

4.2.3. Preparation of large scale *C. sporogenes* cultures

In the anaerobic work station (Mark 3 or MACS, Don Whitley Scientific Limited), agar medium in Petri dishes was inoculated with *C. sporogenes* taken from a single colony of the working 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 growing medium and inoculated from the single bacteria colony grown in a Petri dish. The culture was incubated in an anaerobic work station with shaking (200 rpm, POS-300 Orbital Shaking Platform, Grant Bio) at 30°C overnight. Then the pre-culture (20 mL) was used to inoculate the main culture (500 mL). The culture was cultivated until it reached the desired phase of growth based on the OD measurements (UVmini 1240, Shimadzu). Then cells were harvested under anaerobic conditions and used for whole cell biotransformations or preparation of protein crude extracts.

4.2.4. Growth of *C. sporogenes* in 96-well plates

Growth rates and biomass production of triplicate *C. sporogenes* cultures were measured in 96-well plates. The inoculum was prepared in the Lovitt medium (10 mL) in a glass universal bottle (27 mL) supplemented with the desired electron donors (27 mM D-glucose, D- or L-alanine) and acceptors (45 mM glycine, L-proline, L-phenylalanine, L-tryptophan, L-valine, L-leucine or L-isoleucine). The medium was inoculated with a single colony of *C. sporogenes* from an agar plate using an inoculation loop. The pre-culture was incubated at 30°C overnight with shaking at 200 rpm. The stationary phase pre-culture (500 μL) was used to inoculate fresh Lovitt medium (10 mL). The main culture was incubated at 30°C for 1 h with shaking (200 rpm). After the adaptation phase, the aliquots of the culture (240 μL) were added to wells of a sterile flat bottom 96-well plate (Corning). The cultures

were sealed with the Breathe Easy film (R.B. Radley and Co.) and transferred into a Nephelostar plate reader (BMG Labtech Ltd.). Anaerobic conditions were maintained by placing the plate reader in an AtmosBag™ (Sigma) and flushing with nitrogen throughout the experiment. The cultures were incubated at 30°C for 48 h with orbital shaking (shaking width 4 mm). The light scattering in the wells was measured every 20 min (2 s per well, period delay 0.5 s). The gain was set at 40, with the laser beam focus at 2.5 mm. The optical densities of the cultures at 660 nm were calculated using calibration curves prepared using conventional OD measurements (UVmini 1240, Shimadzu). Optimization of the NEPHELOstar settings and the calibration curve is presented in Appendix 8.2.

4.2.5. Harvesting of *C. sporogenes*

In the anaerobic cabinet the cell culture was transferred into centrifuge pots (Bottle Assembly, Polypropylene, Beckman Coulter, 500 mL) and sealed. The bottle was taken out of the cabinet and centrifuged at 4500 x g for 10 minutes using a high performance centrifuge (Avanti J26-XP, J-LITE® JLA-10.500 fixed angle rotor, Beckman Coulter). After centrifugation the pots were transferred into the anaerobic cabinet, where the supernatant was discarded and the cells were resuspended in degassed phosphate buffer (50 mM, pH 7) to give an OD of 200 (87 mg of dry-weight/mL; calibration curve presented in Appendix 8.3.). The centrifugation and washing procedures were repeated two more times to remove the residual medium before the cells were used for biotransformations or preparation of protein crude extracts.

4.2.6. Preparation of *C. sporogenes* membrane-free protein extracts

Harvested and washed cells were re-suspended anaerobically in degassed 100 mM phosphate buffer pH 7.0 containing dithiothreitol (0.1 g/L), flavin adenine dinucleotide (10 µM) and protease inhibitor (cOmpleteMiniEDTA-free protease inhibitor cocktail tablets, according to the producer's instructions) at the approximate ratio of 1 g dry-weight cells to 2.0 mL of buffer. Cells were lysed by passage a cell disruptor (Constant Systems Ltd.) at pressure of 40,000 psi. The protein extract (about 5-6 mL) was collected under a flow of nitrogen gas in a 30 mL universal bottle. The bottle was immediately transferred into an ice bath, sealed using a suba seal cap and the headspace of the bottle was flushed with nitrogen for at least 15 minutes. The bottle was transferred to the anaerobic workstation where the extract was poured into 50 mL polypropylene screw-on-cap centrifuge bottles (polypropylene, Beckman Coulter). The extracts were centrifuged at 75000 x g for 30 min at 4°C (Avanti J26-XP, JA-25.50 fixed angle rotor, Beckman Coulter) and the high speed

supernatant was separated from the cell debris and insoluble protein fraction using a pipette. The quality of the extracts was determined by enzyme assays, biotransformations and SDS-PAGE.

4.2.7. Protein quantification

The DC Protein Assay (Bio-Rad) was used to determine protein concentration in the samples. The working Reagent A' was composed of Reagent S (20 µL) and Reagent A (1 mL). Protein extracts were diluted 10 and 50 times. A sample (100 µL) was pipetted into a 15 mL test tube. Reagent A' (500 µL) was added and the sample was vortexed. Then Reagent B (4 mL) was added into each tube and vortexed immediately. After incubation (15 min) absorbance at 750 nm was read using a spectrophotometer (Agilent 8453 UV-Vis). Protein concentration was calculated based on the freshly prepared calibration curve of bovine serum albumin protein standard (Appendix 8.4.).

4.2.8. Assays and analytical methods

4.2.8.1 Assay and analytical method for phenylnitroalkene 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 anaerobic *iso*-octane (4.8 mL) mixed with anaerobic 50 mM potassium phosphate buffer (7.2 mL) containing *C. sporogenes* cells (final OD 1.0) or protein crude extract (1 mL, 20 mg) and *tert*-butylbenzene (25 µL) as an internal standard. Whole cell reactions were started by injection of hydrogen gas using a gas manifold (2 min). To the reactions with protein crude extracts, NADH or NADPH (15 µmoles) was added. Reactions were shaken in the anaerobic cabinet at 200 rpm at 30°C for 72 h. Samples of the organic phase, which separated readily, were analyzed using HPLC (Agilent). The samples (15 µL) were auto-injected to a Chiralcel OJ column (diameter 4.6 mm × 250 mm) and compounds were separated with hexane/isopropyl alcohol (9:1) and detected using UV spectrophotometer (by wavelength 254 nm). Retention times of the internal standard, substrates and products were compared to the retention times of analytical standards.

4.2.8.2. Assay and analytical method for cinnamic acid reduction

The biotransformation was performed in 30 mL screw top vials with silicon-PTFE caps. Each assay contained substrate (2 mM final concentration) in anaerobic 50 mM potassium phosphate buffer (5 mL) containing *C. sporogenes* cells (OD 1.0) or protein extract (1 mL, 20 mg). For biotransformations using protein extracts the reaction contained

also NADH or NADPH (3 mM final concentration). When whole cells were used the reactions was started by injection of hydrogen gas using the gas manifold (2 min). Reactions were shaken in the anaerobic cabinet at 200 rpm at 30°C for 72 h. After incubation, samples (2 mL) were centrifuged (13000 rpm, 1 min, Eppendorf MiniSpin). The supernatants were transferred into fresh 15 mL centrifuge tubes (Falcon), acidified with 5 M HCl (0.2 mL) and extracted with diethyl ether (2.5 mL) containing an internal standard (*tert*-butylbenzene, 5 µL). After centrifugation (4000 rpm, 15 min, Eppendorf 5810) the ethereal phase was dried by passing through a 1 mL pipette tip containing anhydrous $MgSO_4$. To 0.5 mL of dried sample methanol (0.1 mL) was added, followed by a few drops (approx. 30 µL) of (trimethylsilyl)diazomethane solution (2.0 M in diethyl ether), until the mixture had a persistent yellow colour. Acetic acid (15 µL) was added to quench the reaction after 30 min. Samples were analysed using GC-MS (Agilent) on HP5-MS column (Agilent). The temperature program was as follows: carrier gas: ultra-pure helium; injector and detector temperature at 300°C; split ratio 100:1; start at 45°C, hold 5 min, then 20°C/min to 300°C, hold 10 min. Retention times of the internal standard, substrates and products were compared to the retention times of analytical standards (Appendix 8.5).

4.2.8.3. Assay and analytical method for nitrobenzene reduction

The biotransformation of nitrobenzene was performed in 30 mL screw top vials with silicon-PTFE caps. Each assay contained substrate (2 mM final concentration) in 4% [EMim][$EtSO_4$] diluted in 50 mM potassium phosphate buffer containing *C. sporogenes* cells (OD 1.0) or protein extract (20 mg). Reactions were started by injection of hydrogen gas using the gas manifold (2 min). Reactions were shaken in the anaerobic cabinet at 200 rpm at 30°C for 72 h. Samples were analyzed using GC-MS (Agilent) on HP5-MS column (method as above).

4.2.8.4. Assay and analytical method for aromatic nitro reduction

The biotransformation was performed in 2 mL polypropylene microcentrifuge tubes. Each assay contained the substrate (3 mM final concentration) in 0.6 mL of anaerobic heptane, 1.2 mL anaerobic 50 mM potassium phosphate buffer pH 7.0 containing protein crude extract (0.1 mL, 2 mg) and NAD(P)H (2 mM). Reactions were shaken in the anaerobic cabinet at 200 rpm at 30°C for 24 h. Then limonene (15 µL; 121.7 mM in heptane) and 3,4-diaminotoluene (60 µL; 60 mM in 50 mM potassium phosphate buffer pH 7.0) were added as internal standards. The samples were vortexed for 30 s and centrifuged

at 13000 for 5 min. The heptane and aqueous fractions were analysed separately by GC-MS using HP5-MS column (method as above).

4.2.8.5. Assay and analytical method for proline reduction (Kabisch *et al.*, 1999)

The biotransformation was performed in a polypropylene microcentrifuge tube (2 mL). The reaction mixture (0.5 mL) contained potassium phosphate buffer (100 mM pH 8.0), $MgCl_2$ (10 mM), dithioerythritol or NADH (20 mM), proline or pipecolic acid (10 mM), and enzyme. The reaction was incubated at 30°C with shaking (200 rpm) and terminated after 22 h by addition of 5% $HClO_4$ (0.3 mL). To remove precipitation, the reaction was centrifuged at 13000 rpm for 5 min and the supernatant (5 µL) of was transferred into 96-well plate and the fluorescence solution was added (195 µL, see below). The fluorescence was determined at 470 nm using excitation at 340 nm (FLUOstar OPTIMA, BMG Labtech). The fluorescence solution was prepared by adding an *o*-phthalaldehyde solution (1 ml; 80 mg/mL in ethanol) and 2-mercaptoethanol (0.2 mL) to 0.4 M HBO_3 buffer (100 mL, pH 9.7, adjusted with KOH). The product concentration was determined by comparison of the fluorescence with the calibration curves prepared for 5-aminovaleric acid and 6-aminocaproic acid (see Results 7.3.2.).

4.2.8.6. Spectrophotometric cinnamic acid reduction assay

Cinnamic acid reduction activity of *C. sporogenes* and *E. coli* crude extracts was determined using a spectrophotometric assay. The reaction was set up in anaerobic conditions in gas-tight quartz cuvettes (Hellma-Analytics) containing protein extract (20 µL), 10 mM cinnamic acid dissolved in water (100 µL, pH adjusted to 7.0), 2.5 mM NADH (50 µL) and 50 mM potassium buffer pH 7.0 (830 µL). The reduction of the substrate was observed by measuring the decrease in absorbance at 340 nm due to consumption of NADH. The reaction was continued for 5 min reading the absorbance every 2 s. Enzyme activity was calculated using the initial rate of decrease and the extinction coefficient NADH as 6,220 $M^{-1} \cdot cm^{-1}$.

4.2.9. Nucleic acid extraction and purification

4.2.9.1. Genomic DNA extraction

Overnight culture grown in TYG medium (10 mL) was centrifuged at 6000 rpm for 10 min (Agilent 5810) and the supernatant was discarded. The pellet was re-suspended in PBS-lysozyme lysis buffer (180 µL; containing NaCl, 8 g/L; KCl, 0.2 g/L; Na_2HPO_4, 1.44 g/L; KH_2PO_4, 0.24 g/L; lysozyme, 10g/L) and incubated at 37°C for 30 min gently agitated

occasionally. RNase A solution (4 µL; 10 mg/mL) was added and the sample was incubated at 37°C for 15 min. Proteinase K solution (25 µL; >600 mAU/ml), dH_2O (85 µL) and sodium dodecyl sulphate solution (110 µL; 10% w/v) were added and the sample was mixed by inversion and incubated at 65°C for 30 min gently agitated occasionally. After incubation phenol:chloroform:isoamyl alcohol solution (400 µL; 25:24:1, saturated with 10 mM Tris HCl, pH 8.0; 1 mM EDTA) was added and mixed thoroughly by inversion. The sample was transferred to a phase-lock tube (phase-lock gel heavy 2.0 mL, Eppendorf) and centrifuged at 13000 rpm for 3 min. The top layer was transferred into a fresh phase-lock tube and the phenol:chloroform:isoamyl alcohol extraction was repeated further two times. The top layer was transferred into a 1.5 mL microcentrifuge tube containing 3 M sodium acetate solution pH 5.2 (40 µL) and ice cold absolute ethanol (800 µL). The mixture was mixed gently but thoroughly and incubated at -80°C for 30 min. The sample was centrifuged at 13000 rpm for 15 min and the supernatant was poured off quickly to avoid re-suspension of the pellet. The sample was washed by adding ethanol solution (1 mL; 70% v/v) and centrifuged at full speed for 3 min. The supernatant was poured off quickly and the DNA pellet was air dried for 45 min at room temperature. The pellet was re-dissolved in 50 µL of EB buffer (QIAGEN).

4.2.9.2. Small scale plasmid purification (QIAprep Spin Miniprep Kit, QIAGEN)

LB medium (5 mL) was prepared in round bottom polypropylene tube (14 mL, Falcon), supplemented with desired antibiotic(s) (carbenicillin, final concentration 50 µg/mL; chloramphenicol in ethanol, 34 µg /mL; kanamycin sulphate, 30 µg/mL) and inoculated with a single bacterial colony of *E. coli* from a Petri dish culture. The cultures were incubated at 37°C overnight with shaking (200 rpm). Then samples were harvested by centrifugation at 4000 rpm for 20 min (Eppendorf 5810R). When low copy plasmids were purified from *E. coli* JM107 or TOP10, chloramphenicol was added to the cultures (final concentration 170 µg/mL) 1 h before harvesting the cells in order to increase the plasmid yields. The pellets were re-suspended in P1 buffer (250 µL). Then P2 lysis buffer (250 µL) was added and samples were mixed thoroughly by inverting the tubes 4-6 times and incubated for 2 min at room temperature. The lysis reaction was stopped with chilled N3 buffer (350 µL). The samples were centrifuged at 13000 rpm for 10 min in a table-top microcentrifuge (Eppendorf MiniSpin). The supernatants were transferred to the QIAprep spin columns by pipetting. The samples were centrifuged for 1 min and the flow-throughs were discarded. To remove trace nuclease activity in JM107 strains, the spin columns were washed by adding PB buffer (0.5 mL), centrifuged for 1 min and the flow-throughs were

discarded. The columns were washed by adding PE buffer (0.75 mL) and centrifuged for 1 min. The flow-throughs were discarded and columns were centrifuged for an additional 1 min to remove residual wash buffer. Then QIAprep columns were placed in clean microcentrifuge tubes (1.5 mL) and plasmids were eluted by adding EB buffer (30-50 µL), letting them stand for 1 min and centrifugation for 1 min at 13000 rpm.

4.2.9.3. Large scale plasmid purification (Plasmid Midi Kit, QIAGEN)

LB medium (100 mL) in Erlenmeyer flask (250 mL) was supplemented with desired antibiotic(s) (carbenicillin, final concentration 50 µg/mL; kanamycin sulphate, 30 µg/mL) and inoculated with a single bacterial colony of *E. coli* taken from a Petri dish culture. The culture was incubated at 37°C overnight with shaking (200 rpm). The culture was transferred into polypropylene centrifuge tubes (50 mL, Falcon). The cells were harvested by centrifugation at 6000 rpm for 10 min (Eppendorf 5810R). The pellets were re-suspended in P1 buffer (4 mL) and vortexed. Then P2 lysis buffer (4 mL) was added and samples were mixed thoroughly by inverting the tubes 4-6 times and incubated for 5 min at room temperature. The lysis reaction was stopped with chilled P3 buffer (4 mL). The samples were mixed immediately and thoroughly by vigorously inverting 4-6 times and incubated on ice for 15 min. The samples were centrifuged at 18000 x g for 30 min at 4°C (Eppendorf 5810R) and the supernatants containing plasmid DNA were transferred promptly to fresh centrifuge tubes (50 mL). The supernatants were centrifuged again at 18000 x g for 15 min at 4°C and removed promptly. QIAGEN-tips 100 were equilibrated by applying Buffer QBT (4 mL) and the columns were emptied by gravity flow. The supernatants were applied to the QIAGEN-tips and allowed to enter the resin by gravity flow. The QIAGEN-tips were washed with Buffer QC (2 x 10 mL) and plasmids were eluted with Buffer QF (5 mL) to a fresh polypropylene tube (15 mL). The plasmids were precipitated by adding room temperature ethanol (3.5 mL), mixing and centrifugation at 18000 x g for 30 min at 4°C. The supernatants were decanted carefully and the pellets were washed with room temperature 70% ethanol (2 mL). The samples were centrifuged again at 18000 x g for 10 min and the supernatants were carefully removed without disturbing the pellets. The pellets were air-dried for 10-30 min and the DNA was re-dissolved in EB buffer (200 µL). The concentrations of the plasmid DNA were determined by spectrophotometric analysis (Nanodrop ND1000).

4.2.9.4. PCR product purification (QIAquick PCR Purification Kit)

PB Buffer (250 µL) was added to the PCR reaction mixture (50 µL). It was checked that the colour of the mixture was yellow. If the colour of the mixture was orange or violet, 3 M sodium acetate pH 5.0 was added (10 µL). A QIAquick spin column was placed in a 2 ml collection tube. The DNA was bound by applying the sample to the QIAquick column and centrifugation for 1 min at 13000 rpm (Eppendorf MiniSpin). The flow-through was discarded and the QIAquick column was placed back into the same tube. The column was washed by adding PE Buffer (0.75 mL) and centrifugation for 1 min. The flow-through was discarded. The QIAquick column was placed back in the same tube and centrifuged for an additional 1 min. Then the QIAquick column was placed in a clean microcentrifuge tube (1.5 mL). DNA was eluted by adding EB Buffer (50 µL) to the centre of the QIAquick membrane and the column was centrifuged for 1 min at 13000 rpm.

4.2.9.5. DNA gel extraction (QIAquick Gel Extraction Kit)

The DNA fragment was excised from the agarose gel under UV light with a clean, sharp scalpel. The exposure of the agarose gel to the UV radiation was shortened to the minimum to avoid creation of mutations in the DNA. The gel slice was weighed in a colourless microcentrifuge tube (1.5 mL). Three volumes of Buffer QG were added to 1 volume of gel (100 mg ~ 100 µL). When the gel slice was heavier than 400 mg it was split into separate fragments to avoid the spin column overloading. The sample was incubated at 50°C for 10 min and mixed by vortexing every 2-3 min to help dissolve the gel. After the gel slice was dissolved completely, it was checked the colour of the mixture is yellow. If the colour of the mixture was orange or violet, 3 M sodium acetate pH 5.0 was added (10 µL). One gel volume of isopropanol was added to the sample and mixed. A QIAquick spin column was placed in a collection tube (2 mL). The DNA was bound by applying the sample to the QIAquick column and centrifugation for 1 min at 1300 rpm (Eppendorf MiniSpin). The flow-through was discarded and the QIAquick column was placed back into the same tube. To remove traces of agarose, the spin column was washed by adding QG buffer (0.5 mL), centrifuged for 1 min and the flow-through was discarded. The column was washed by adding PE Buffer (0.75 mL) and centrifugation for 1 min. The flow-through was discarded. The QIAquick column was placed back in the same tube and centrifuged for an additional 1 min. Then the QIAquick column was placed in a clean microcentrifuge tube (1.5 mL). DNA was eluted by adding EB Buffer (30 µL) to the centre of the QIAquick membrane. The column was let to stand for 1 min and centrifuged for 1 min.

4.2.10. Southern blot hybridisation

The hybridisation probe was amplified by PCR using the PCR-based intron as the template (Clostridia Research Group, Nottingham) and primers EBSUni and SalI-R1 (Table 4.1.). Three separate reactions were resolved on an agarose gel and the size of the products was checked. The samples were merged, gel purified and eluted in a final volume of 20 μL dH_2O. The probe for the ladder was composed of λ DNA/*Hin*dIII marker (1 μL) and dH_2O (14 μL). The DNA was denatured by heating at 100°C for 10 min and then quickly chilled on ice. DIG-High Prime (4 μL) was added and the reaction was incubated at 37°C overnight. The reaction was stopped by adding 0.2 M EDTA pH 8.0 (2 μL) and heating at 65°C for 10 min.

Genomic DNA (1-5 μg) was digested with restriction enzymes. The reaction mixture (25 μL) was composed of genomic DNA (2 μL), 10x bovine serum albumin solution (2.5 μL), 10x restriction buffer (2.5 μL), restriction enzyme (2 μL, *Hin*dIII or *Eco*RI; New England Biolabs) and dH_2O (16 μL). The reaction was incubated at 37°C overnight. The control plasmid (pMTL007-E2) was digested in 15 μL and the reaction was composed of plasmid DNA (2 μL), 10x BSA (1.5 μL), 10x restriction buffer (1.5 μL) and restriction enzyme (0.5 μL). The reaction was incubated at 37°C for 4 h.

The reaction mixtures were mixed with the loading dye and run out on a 0.8% agarose gel with λ DNA/*Hin*dIII marker (5 μL). The gel was run at 120 V for 1.5-2 h. A picture of the gel was taken to be sure that the digestion was successful (even DNA smear in the wells containing digested genomic DNA).

The DNA was transferred onto nitrocellulose membrane. An empty gel tray was placed upside down in a plastic box. The wick was soaked in 0.4 M NaOH and laid over the base. The air bubbles were removed by rolling a serological pipette over the surface. The agarose gel was placed on the top of the wick. Hydrobond H+ membrane (GE Healthcare) was cut exactly the same size as the gel using a scalpel blade holding the membrane with forceps. The membrane was pre-wetted in 0.4 M NaOH and laid over the gel in one movement. Five blotting pads (Sigma) were cut to the same size as the membrane. One of the pads was pre-wetted in 0.4 M NaOH and laid over the membrane. The air bubbles were removed again. Four remaining dry blotting pads were laid over the top. The plastic box was filled with 0.4 M NaOH to ensure that the wick was not dried out. A weight (about 500 g) was placed on top and the apparatus was left for 2-3 h while the transfer occurred.

The transfer rig was disassembled down to the membrane. The gel lanes were marked on the membrane using a pencil. The membrane was placed inside cling film and the DNA was cross-linked to the membrane under UV light (2 min each side). The membrane was

briefly washed in 2x SSC buffer (containing 0.3 M NaCl, 30 mM Tris-Na-citrate buffer) to remove excess of NaOH. The membrane was placed into a hybridisation tube (DNA side in, pencil side out). DIG-Easy Hyb (Pre-hyb) buffer (8-10 mL) was added and the tube was incubated at 42°C for at least 1 h. The probes were made up in polypropylene centrifuge tubes (50 mL) into 10 mL DIG-Easy Hyb and boiled for 10 min, then placed into ice immediately. DIG-Easy Hyb (Pre-hyb) buffer was replaced with probe mix and the tube was incubated at 42°C overnight.

The membrane was washed twice with the low stringency washing buffer (100 mL; 2x SSC, 0.1% SDS) at room temperature for 5 min and twice with the high stringency washing buffer (100 mL; 0.5x SSC, 0.1% SDS) at 68°C for 15 min. The membrane was equilibrated in 1x maleic acid buffer (100 mL, 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) + 0.3% Tween®-20 and incubated at room temperature for 1 min. 1x Blocking buffer (Roche EasyHyb kit) was made up in 1x maleic acid buffer. The membrane was blocked in 1x blocking buffer (25 mL) at room temperature for 30 min. The anti-DIG Ab probe was diluted 1:10,000 in 1x blocking solution, added to the tube and incubated at room temperature for 30 min. The membrane was washed with 1x maleic acid buffer + 0.3% Tween®20 (100 mL) at room temperature for 15 min. The membrane was equilibrated in 25 mL of the detection buffer composed of Tris-HCl pH 9.5 (100 mM) and NaCl (100 mM) at room temperature for 2 min.

The blot was developed using the Single filter method. CSDP ready-to-use enzyme substrate (1.5 mL) was dispensed into a tube. The membrane was taken out of the hybridisation tube and laid DNA side up on an acetate sheet. The enzyme substrate was pipetted directly onto the membrane and the second acetate sheet was laid over the top of the membrane. The edges of the acetate sheets were sealed with tape and the membrane was incubated at 37°C for 10 min. The membrane was placed in a cassette, covered with a sheet of photo film (GE Healthcare) under red light and left for 15 min. The film was dropped into developer and fixative solution (500 mL).

4.2.11. Polymerase chain reaction

4.2.11.1. PCR using genomic DNA templates (FailSafe™ PCR System)

Polymerase chain reactions (final volume 50 µL) were prepared with the following components: *C. sporogenes* genomic DNA (10 ng), FailSafe PCR 2xPreMix (25 µL), FailSafe PCR Enzyme Mix (0.5 µL; 1.25 units), 50 µM forward and reverse primers (1 µL of each). The hot start protocol was performed. The reactions were assembled on ice and added directly to a thermal cycler pre-heated to 98°C. Reactions were carried out with the following

cycling conditions (35 cycles): initial denaturation, 98°C for 3 min; denaturation, 95°C for 30 s; annealing, from 48 to 62°C (2-5°C below the T_m of primers) for 30-50 s; elongation, 72°C, 1-2min (1 min for every kb of expected product) and final elongation, 72°C for 10 min. After amplification, the reactions were analysed by DNA gel electrophoresis and frozen at -20°C.

4.2.11.2. Introduction of restriction sites by PCR (KOD Polymerase)

Polymerase chain reactions (final volume 50 µL) were prepared with the following components: plasmid DNA (1 µL; 0.01-1 ng), 10x Buffer #1 for KOD DNA Polymerase (5 µL, Novagen), 5 mM dNTP mix (2 µL), 25 mM $MgCl_2$ (2 µL), primers forward and reverse (10 µM, 2 µL of each), DMSO (1 µL) and KOD DNA Polymerase (0.4 µL; 1 U). Reactions were carried out with the following cycling conditions (25 cycles): initial denaturation, 98°C for 3 min; denaturation, 98°C for 15 s; annealing, from 55 to 64°C (1-2°C below the T_m of primers) for 10 s; elongation, 72°C, 20-40 s and final elongation, 72°C for 5 min. After amplification, the reaction mixtures were analysed by DNA gel electrophoresis and frozen at -20°C.

4.2.11.3. Colony PCR (JumpStart REDTaq DNA Polymerase)

Colony PCR was used to quickly screen for plasmid inserts directly from *E. coli* (JM107, BL21(DE3)pLysS or TOP10) colonies. The reactions were assembled on ice in thin wall PCR tubes (0.5 mL) and were composed of 10x JumpStart REDTaq PCR Buffer (5 µL), 2 mM dNTP mix (5 µL), 10 µM forward primer (1.25 µL; T7 promoter or pJET1.2 forward), 10 µM reverse primer (1.25 µL; T7 terminator or pJET1.2 reverse), JumpStart REDTaq DNA Polymerase (2.5 µL, SIGMA) and dH_2O (35 µL). A small amount of *E. coli* colony was transferred from an agar plate using a sterile 100 µL pipette tip. A trace of the colony was transferred on a fresh LB agar plate supplemented with the relevant antibiotics and the rest was added to the PCR mix. Reactions were carried out with the following cycling conditions (35 cycles): initial denaturation, 94°C for 3 min; denaturation, 94°C for 30 s; annealing, 50°C for 30s; elongation, 72°C, 1.5-2.5 min (1 min for every kb of expected product) and final elongation, 72°C for 5 min. The reaction mixtures analysed by DNA electrophoresis.

4.2.12. Plasmid DNA restriction digestion

Plasmid DNA was digested using the FastDigest restriction enzymes *Bgl*II, *Nco*I, *Nde*I, *Not*I and *Xba*I (Fermentas). The reaction (50 µL) was composed of the plasmid DNA (0.5-5 µg), 10x Green FD Reaction Buffer (5 µL) and the enzyme(s) (1-2 µL of each). The reaction was incubated at 37°C for 1-6 hours or overnight. When the digestion reaction was incubated overnight, the reaction mixture was covered with mineral oil (50 µL, SIGMA) to prevent evaporation. When the reaction was complete, the enzymes were inactivated at

65-80°C for 5-10 min depending on the enzyme properties. The products of the restriction digestion were analysed using DNA agarose gel electrophoresis.

4.2.13. DNA vector dephosphorylation

The dephosphorylation reaction was composed of plasmid DNA (28 µL), 10x Antarctic Phosphatase Reaction Buffer (5 µL, New England Biolabs), Antarctic Phosphatase enzyme (1.5 µL; 7.5 U) and dH_2O (up to 50 µL). The sample was incubated at 37°C for 1 h. The enzyme was inactivated at 65°C for 5 min. DNA was precipitated by addition of 3 M sodium acetate (5 µL; pH 5.2, adjusted with acetic acid) and absolute ethanol (110 µL) and incubated at -20°C overnight. Then the sample was centrifuged at 13000 rpm (Eppendorf MiniSpin) for 20 min and the supernatant was removed. The pellet was washed with 70% ethanol (1 mL) and centrifuged again at 13000 rpm for 10 min. The supernatant was removed. The pellet was air-dried and re-suspended in EB buffer (30 µL; QIAGEN).

4.2.14. DNA ligation

4.2.14.1. Ligation of PCR products using CloneJET™ PCR Cloning Kit

Three independent PCR reaction mixtures were resolved on a 1% agarose gel and the size of the products was checked. The products were purified from primers, nucleotides, polymerases and salts using the QIAquick PCR Purification Kit (QIAGEN). When unspecific products occurred in the reaction, the products were additionally purified by gel electrophoresis.

To remove 3'-dA overhangs generated by enzyme mixtures containing *Taq* DNA polymerase, the blunting reaction was set up in microcentrifuge tube (1.5 mL). The reaction mixture was composed of the PCR product (1-2 µL), 2x Reaction Buffer (10 µL), DNA Blunting Enzyme (1 µL) and dH_2O (up to 18 µL). The sample was vortexed, incubated at 70°C for 5 min and chilled briefly on ice. Then pJET1.2/blunt Cloning Vector (1 µL; 50 ng) and T4 DNA Ligase (1 µL; 5 U) were added to the reaction mixture. The sample was vortexed briefly and incubated at room temperature for 30 min. The ligation mixture was used directly for the bacterial transformation.

4.2.14.2. Ligation into expression vectors

A cloning vector (pJET1.2, pUC19 or pBMH) carrying the gene insert was digested using restriction enzymes (*Nde*I FD and *Not*I FD, *Nco*I FD and *Not*I FD or *Xba*I and *Not*I). The gene insert was separated from the linearized vector by gel electrophoresis purification.

The expression vectors (pET20b(+) and pET28a(+)) were digested with the corresponding enzymes. The linear vector DNA was purified by gel electrophoresis and dephosphorylated to reduce its self-ligation.

The ligation mixture was composed of 10x T4 ligase buffer (3 µL), the dephosphorylated vector (50 ng), the gene insert (20-75 ng; vector/insert molar ratio 3:1), T4 ligase (1 µL, 5 units) and dH_2O (up to 30 µL). The reaction was incubated at room temperature for 1 h. Then the enzyme was inactivated at 70°C for 10 min. The reaction mixture (2.5 µL) was immediately used to transform *E. coli* JM107 or TOP10.

4.2.15. *E. coli* transformation

4.2.15.1. Transformation of JM107 and TOP10 strains

LB agar plate was seeded from a single bacterial colony of *E. coli* (JM107 or TOP10) using an inoculating loop and incubated overnight at 37°C. For preparation of competent cells, freshly streaked bacterial colonies were used (not older than 1-2 days).

C-medium (2 mL) in a round bottom polypropylene tube (14 mL; Falcon) was inoculated with a single bacterial colony from the LB plate and incubated overnight at 37°C. The stock culture was kept at 4°C for one week and used for preparation of competent cells.

On the day of transformation, C-medium (1.5 mL) and LB agar plates with desired antibiotics (carbenicillin or kanamycin sulphate) were pre-warmed at 37°C for at least 20 min. The T-solution (500 µL) was composed of T-solution A (250 µL) and T-solution B (250 µL) and kept on ice. The overnight bacterial stock culture in C-medium (150 µL) was added to pre-warmed C-medium and incubated at 37°C for 20 min with shaking (200 rpm). Then the cells were poured into a microcentrifuge tube (1.5 mL) and harvested by centrifugation (1 min; 13000 rpm). The pellet was re-suspended in chilled T-solution (300 µL) and incubated on ice for 5 min. The sample was centrifuged for 1 min at 13000 rpm and the supernatant was discarded. The pellet was re-suspended in chilled T-solution (120 µL) and incubated of ice for another 5 min. The ligation mixture (2.5 µL) was added to a new microcentrifuge tube and chilled on ice for 2 min. The prepared cells (50 µL) were added to the tube containing DNA, mixed and incubated on ice for 5 min. Then the cells (30-50 µL) were plated immediately on pre-warmed LB antibiotic agar plates using a sterile spreader and incubated overnight at 37°C.

4.2.15.2. Heat-shock transformation of BL21(DE3)pLysS

E. coli BL21(DE3)pLysS competent cells (Novagen) were removed from the -80°C freezer and thawed on ice for 2-5 min. The tube was gently finger-flicked 1-2 times to evenly resuspend the cells. The required number of 1.5 mL microcentrifuge tubes was placed on ice to pre-chill. Aliquots of cells (20 µL) were pipetted into pre-chilled tubes. Purified pET20b(+) plasmid (1 µL; 10 ng) was added directly to the cells and the tube was stirred gently. The tube was incubated on ice for 5 min and then heated for exactly 30 s in a 42°C water bath. The tubes were placed on ice for 2 min and room temperature SOC medium (80 µL, Novagen) was added to the tube. The cells were incubated at 37°C while shaking at 250 rpm for 60 min prior to plating on LB agar plates supplemented with antibiotics (carbenicillin, final concentration: 50 µg/mL; chloramphenicol: 34 µg/mL). Positive transformants were maintained on LB agar plates at 4°C and as 8% glycerol stocks at -80°C.

4.2.16. Electrophoresis

4.2.16.1. DNA electrophoresis in agarose gel

TAE buffer (1 L) was composed of Tris base (4.84 g), glacial acetic acid (1.14 mL) and 0.5 M EDTA pH 8.0 (2 mL). Agarose powder (1 g) and TAE buffer (100 mL) were mixed together in an Erlenmeyer flask (250 mL). The agarose was melted in a microwave until the solution became clear. The solution was let to cool down to about 50°C, swirling the flask occasionally to cool evenly. The gel casting tray (Alpha Laboratories) was assembled and the comb was placed. Ethidium bromide solution (7 µL; 1% w/v) was added to the melted agarose. The gel was poured into the casting tray and let cool until it was solid. The comb was carefully pulled out and the gel was placed in the electrophoresis chamber filled with the TAE buffer.

The samples (10µL each) were mixed with the 6x Loading Dye Buffer (2 µL; Fermentas). Each sample (10-12 µL) and the relevant DNA ladder (5 µL) were carefully pipetted into separate wells in the gel. The lid was placed on the gel box and the electrodes were connected to the power supply. The power supply was turned on to about 60-70 V and the electrophoresis was run until the blue dye approached the end of the gel. The power was turned off and the gel was observed under UV light. Pictures of the gel were taken using the Gel Imagining System (U:Genius3, Syngene).

4.2.16.2. SDS-PAGE electrophoresis

The separating buffer (100 mL) contained Tris base (18.15 g) and SDS (0.4 g) and the pH was adjusted to 8.8. The separating gel was composed of 40% acrylamide:N,N'-methylenebisacrylamide (37.5:1) water solution (8 mL, Sigma), dH_2O (6.8 mL), separating buffer (5 mL), 10% ammonium persulfate (100 µL) and TEMED (10 µL). The stacking buffer (100 mL) contained Tris base (6.04 g) and SDS (0.4 g) 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), dH_2O (6.1 mL), separating buffer (2.5 mL), 10% ammonium persulfate (50 µL) and TEMED (5 µL). The running buffer (1 L) contained glycine (14.4 g), Tris base (3 g) and SDS (1 g). The loading buffer contained 2.6% SDS, 1.3% 2-β-mercaptoethanol, 6% glycerol, 0.2% bromophenol blue, 0.05 M Tris HCl, 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 covered with water saturated butan-2-ol. After 45 min the 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. 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 95°C for 5 min and loaded onto the gel. PageRulerTM Prestained Protein Ladder (Fermentas) 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 overnight with Instant Blue Stain (Novexin) or EZBlue Stain (Sigma).

Alternatively Mini-PROTEAN TGX Precast Gels (Bio-Rad) were used together with 4x Laemmli Sample Buffer (Bio-Rad) supplemented with β-mercaptoethanol (final concentration 5%) and 10x Tris/glycine/SDS running buffer (Bio-Rad).

4.2.17. Protein overexpression in *E. coli* under anaerobic conditions

E. coli BL21(DE3)pLysS strain carrying pET20b(+) plasmid encoding the desired enzyme was maintained as an 8% glycerol cryo-stock and kept at -80°C. LB medium (10 mL) supplemented with carbenicillin (50 µg/mL) and chloramphenicol (34 µg/mL) was inoculated using a 10 µL loop with bacterial strain directly from the cryo-stock. The culture was incubated at 37°C overnight with shaking at 200 rpm. The culture (100-500 µL) was used to inoculate LB medium (20 mL) with antibiotics in a sterile 50 mL centrifuge tube

(Falcon). The culture was incubated at 37°C for about 6-7 h with shaking (200 rpm) until the OD_{600} of the culture reached 0.9-1. The culture was kept at 4°C overnight.

The next day the cells were centrifuged at 6000 rpm for 10 min at 4°C (Eppendorf 5810R). The supernatant was discarded and the pellet was transferred into the anaerobic cabinet. The cells were used to inoculate anaerobic TB medium (100 mL) supplemented with antibiotics, glucose (final concentration 1%) and riboflavin (1 μg/mL) in a Duran screw cap bottle (100 mL) containing a magnetic flea. The culture was mixed using a magnetic stirrer at 100 rpm. When the culture reached OD_{600} 0.4-0.5, the expression of the protein was induced by adding sterile 1 M isopropyl β-D-1-thiogalactopyranoside (40 μL). Samples of the culture (2 mL) were harvested before induction and at intervals after induction in order to determine the time point giving the higher protein production yield. The cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C.

The anaerobic lysis buffer (10 mL) was composed of 100 mM potassium phosphate buffer pH 7.0, flavin adenine dinucleotide (10 μM), riboflavin-5'-phosphate (10 μM), Benzonase® Nuclease (1 μL; ≥250 U), 10x BugBuster® Protein Extraction Reagent (1 mL) and cOmplete MiniEDTA-free protease inhibitor (1 tablet). Depending on the further purification steps, the lysis buffer was also supplemented with dithiothreitol (0.1 mg/mL) or tris(hydroxypropyl)phosphine (1 mM). The lysis buffer was sparged with nitrogen for at least 1 h.

The pellet was resuspended in the lysis buffer (250 μL of the buffer for every 1 mL of the culture of OD_{600} 1). The sample was incubated at room temperature for 10-15 min with shaking at 250 rpm and additional vortexing every 2-3 min. The tube was centrifuged at 18000 x g for 20 min at 4°C (Eppendorf 5810R). The supernatant (cell-free crude extract) was transferred into a fresh tube. The pellet was re-suspended in the original volume of the lysis buffer and represented the insoluble protein fraction. Both protein fractions were analysed using SDS-PAGE technique (20 μL of each sample was loaded onto the gel). The soluble protein fraction was used in enzymatic assays and biotransformations.

The culture for protein overexpression was scaled up to 1 L by increasing the volumes of the cultures and scaling up the protein preparation. In the 1 L scale experiments, the protein extracts (final volume 7 mL) were prepared using the cell disruptor (Constant Systems Ltd.) as described elsewhere (4.2.6.).

4.2.18. His-Tag purification

4.2.18.1. His-Bind Purification Kit (Novagen)

8x Binding Buffer pH 7.9 contained NaCl (4 M), Tris HCl (160 mM) and imidazole (40 mM). 8x Wash Buffer pH 7.9 contained NaCl (4 M), imidazole (480 mM) and Tris -HCl (160 mM). 4x Elute Buffer pH 7.9 contained imidazole (4 M), NaCl (2 M) and Tris HCl (80 mM). 4x Strip Buffer pH 7.9 contained NaCl (2 M), EDTA (400 mM) and Tris HCl (80 mM). 8x Charge Buffer contained $NiSO_4$ (400 mM). Buffers were supplemented with FMN (final concentration 10 µM), FAD (final concentration 10 µM) and sparged with nitrogen for at least 1 h.

Small polypropylene columns (Chromatography Columns, Novagen) held 2.5 mL of the settled resin and were used to purify up to 20 mg target protein. The bottle of His·Bind® Resin was gently mixed by inversion until completely suspended. The desired volume of slurry (1.5-2 mL) was transferred to the column using a wide mouth pipet. The column was transferred to the anaerobic work station where the resin was allowed to pack under gravity flow. When the level of the storage buffer dropped to the top of the column bed, the resin was charged and equilibrated by a sequence of washes, 3 resin volumes of sterile degassed dH_2O, 5 volumes of 1x Charge Buffer and 3 volumes of 1x Binding Buffer.

1x Binding Buffer was allowed to drain to the top of the column bed. The protein extract (7 mL) was loaded into the column and allowed to drain under gravity flow. The flow throughs were collected for further analysis. The column was washed with 10 volumes of 1x Binding Buffer and 6 volumes of 1x Wash Buffer. The bound protein was eluted with 6 volumes of 1x Elute Buffer. The column was washed with 6 volumes of 1x Strip Buffer to remove residual proteins by stripping Ni^{2+} from the column. The purified protein and the flow throughs were analysed using SDS-PAGE and used in enzyme activity assays.

4.2.18.2. Ni-NTA Spin Kit (QIAGEN)

The Lysis Buffer (NPI-10) contained 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0. The Wash Buffer (NPI-20) contained 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, pH 8.0. The Elution Buffer (NPI-500) contained 50 mM NaH_2PO_4, 300 mM NaCl and 500 mM imidazole, pH 8.0. The buffers were also supplemented with FMN (final concentration 10 µM), FAD (final concentration 10 µM), sparged with nitrogen and equilibrated in the anaerobic work station overnight. All the buffers were also supplemented with different concentrations of dithioerythritol used as the reducing agent.

The pellet derived from 5 mL cell culture volume was resuspended in 630 µL Lysis Buffer (NPI-10). Lysozyme Stock Solution (70 µL; 10 mg/mL) and Benzonase® Nuclease (15 U) were added and the sample was incubated at 4°C for 30 min. The lysate was centrifuged at 12000 x g for 30 min at 4°C and the supernatant was collected. The Ni-NTA spin column was equilibrated with NPI-10 Buffer (600 µL) and centrifuged at 2900 rpm for 2 min (Eppendorf MiniSpin). The cleared lysate containing the 6xHis-tagged protein was loaded onto the pre-equilibrated Ni-NTA spin column and the column was centrifuged at 1600 rpm for 5 min. The Ni-NTA spin column was washed twice with NPI-20 Buffer (600 µL) and centrifuged at 2900 rpm for 2 min. The protein was eluted twice with NPI-500 Buffer (300 µL) and centrifuged at 2900 rpm for 2 min. The eluate was collected and used in the enzyme activity assays.

4.2.18.3. Dynabeads® His-Tag Isolation (Invitrogen)

2x Binding/Wash Buffer was composed of sodium phosphate, pH 8.0 (100 mM), NaCl (600 mM) and Tween®-20 (0.02%). His Elution Buffer contained imidazole (300 mM), sodium phosphate pH 8.0 (50 mM), NaCl (300 mM) and Tween®-20 (0.01%). Buffers were also supplemented with FMN (final concentration 10 µM), FAD (final concentration 10 µM) and sparged with nitrogen for at least 1 h.

The sample containing the 6-His-tagged protein was prepared in 1x Binding/Wash Buffer (700 µL). The Dynabeads® were thoroughly resuspended in the vial by vortexing for 30 s. The Dynabeads® (50 µL; 2 mg) were transferred to a microcentrifuge tube (1.5 mL). The tube was placed on a magnet (DynaMag™-2, Invitrogen) for 2 min. The beads were collected on the wall of the tube and the supernatant was discarded. The protein extract was added to the beads. The tube was mixed well and incubated on a shaker at room temperature for 10 min. The tube was placed on the magnet for 2 min and the supernatant was discarded. The beads were washed 4 times with 1x Binding/Wash Buffer (300 µL) by placing the tube on the magnet for 2 min and discarding the supernatants. The beads were resuspended thoroughly between each washing step. To elute the protein, His-Elution Buffer (100 µL) was added and the tube was incubated on a shaker at room temperature for 5 min. The tube was placed on the magnet for 2 min and the supernatant containing the eluted histidine-tagged protein was transferred to a clean tube. The purified protein was analysed using SDS-PAGE and used in enzyme activity assays.

4.2.19. His-tag excision and protein refolding

The 6-His-tagged protein was purified in the anaerobic cabinet using His·Bind Purification Kit (see section 4.2.18.1) and eluted in 1x Elution Buffer pH 7.9 (6 mL). To remove the salts from the buffer and make the protein more concentrated, the sample was poured into Vivaspin Concentrator 6 (Vivaproducts) with 10,000 molecular weight cut off membrane. The assembled concentrator was inserted into a centrifuge (Eppendorf 5810R) and centrifuged at 4000 x g in a swing bucket rotor (A-4-62, Eppendorf) at room temperature until the volume of the sample decreased to about 1 mL (for about 10-15 min). In the cabinet, anaerobic 100 mM potassium phosphate buffer (5 mL) was added and the sample was gently mixed by pipetting. The concentrator was centrifuged again until the volume of the sample decreased to about 1 mL.

The 6-His-tag was cut off from the overexpressed protein by thrombin digestion. The reaction mixture was composed of purified and concentrated protein (450 µL) and High Activity Bovine Thrombin (50µL, 10 U; lyophilized powder resuspended in 200 mM NaCl, 50 mM sodium citrate, 1 ml 0.1% PEG, pH 6.5). The reaction was incubated at 37°C for 3 h.

The protein was refolded using Pierce Protein Refolding Kit (Fermentas). Nine Base Refolding Buffers and seven additional buffer additives were used to create a matrix covering a range of strong and weak denaturant conditions for the suppression of protein aggregation. The stock buffers were composed of Tris base (55 mM), NaCl (21 mM), KCl (0.88 mM), FMN (11 µM), FAD (11 µM), pH 8.2. Each buffer stock also contained the indicated denaturant concentrations (up to 1.1 M guanidine and up to 0.88 M arginine). The additives such as dithioerythritol (5 mM), reduced glutathione (up to 2 mM) and oxidized glutathione (up to 0.4 mM) were used as secondary matrix factors. The buffers were sparged with nitrogen for at least 1 h.

In the anaerobic workstation the Base Refolding Buffers (900 µL) were dispensed in nine 1.5 mL microcentrifuge tubes. The additives were added and the volumes were adjusted to 950 µL. The purified and concentrated protein sample (50 µL) was slowly added to each of the nine tubes and mixed. The samples were incubated for 6-24 h and the refolding efficiency was tested in the enzyme activity assay.

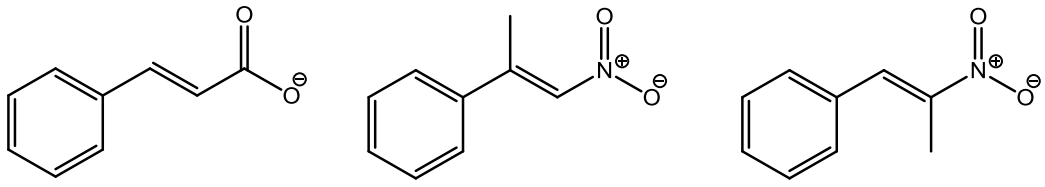
5. Results

5.1. Reduction of C=C double bonds by *Clostridium sporogenes* DSM795

Clostridium sporogenes was shown to catalyze hydrogenation of C=C double bonds in cinnamic acid (reported for the first time by Bühler in 1980), a range of α,β- and β,β-disubstituted nitroolefins (Fryszkowska *et al.*, 2008), pyrimidines, such as uracil (Hilton *et al.*, 1975) and unsaturated fatty acids (Verhulst *et al.*, 1985).

The purification and identification of the enzyme responsible for reduction of nitroolefins, *(E)*-1-nitro-2-phenylpropene and *(E)*-2-nitro-1-phenylpropene, was unsuccessful, although it was found that *C. sporogenes* possessed at least two enzymes that could reduce *(E)*-2-nitro-1-phenylpropene at the expense of NADH showing different enantioselectivities (Fryszkowska *et al.*, 2008; Dr. Karl Fisher, Manchester Interdisciplinary Biocentre, unpublished data, for details see Appendix 8.7.).

The similarities in structure between cinnamate and nitroolefins tested (Fig. 5.1.) led to a hypothesis that one of these enzymes could be the reductase responsible for hydrogenation of cinnamic acid in the L-phenylalanine fermentation by the Stickland reaction. The reduction of cinnamic acid to 3-phenylpropionic acid was found to be catalyzed by NADH-dependent 2-enoate reductase (Bühler *et al.*, 1980), belonging to a family of clostridial oxidoreductases containing a flavin adenine dinucleotide and an iron-sulphur centre (Tischer *et al*., 1979).



(E)-cinnamate *(E)*-1-nitro-2-phenylpropene *(E)*-2-nitro-1-phenylpropene

Fig. 5.1. Structures of *(E)*-cinnamate, *(E)*-1-nitro-2-phenylpropene and *(E)*-2-nitro-1-phenylpropene.

The nucleotide sequence of the gene encoding fldZ reductase remained unknown at that time, since the amino acid primary structure of the reductase was not determined and the sequence of *C. sporogenes* genome was not available in the genomic libraries. Moreover, previous attempts to overexpress clostridial 2-enoate reductases from *C. tyrobutyricum* and *C. thermoaceticum* in a heterologous host have met with the limited success due to their complex cofactor requirement and oxygen sensitivity.

Thus, to test the hypothesis that 2-enoate reductase is responsible for reduction of nitroolefins, an approach based on production of genomic knock out mutants was proposed. *C. sporogenes* 2-enoate reductase knock out mutant strain (called *C. sporogenes*

fldZ::CT*erm*B) was prepared by Dr. Benjamin Blount from the Clostridia Research Group using the ClosTron gene knock out system based on the sequence of the *fldZ* gene encoding 2-enoate reductase from closely related strain, *C. botulinum* Hall A ATCC3502 (for details see Appendix 8.1.). Preliminary experiments, showed that the crude extracts of the *fldZ* mutant, contrary to those of wild type, did not show activity towards cinnamic acid in the spectrophotometric reduction assay (published in Mordaka, 2010). The mutants were not able to reduce *(E)*-1-nitro-2-phenylpropene, whereas reduction of *(E)*-2-nitro-1-phenylpropene was still possible, but with a lower yield when compared to the wild type strain. The growth of *fldZ*::CT*erm*B was significantly decreased in complex media when glucose and L-phenylalanine were used as energy substrates suggesting that fldZ may be involved in the process of obtaining carbon and energy for growth. These experiments corroborated the hypothesis that *C. sporogenes* possessed multiple enzymes reducing C=C double bond in nitroolefins and that the fldZ reductase was one of them. Therefore, further experiments testing the scope for reduction of enoates and nitroalkenes using *C. sporogenes* C=C double bond reductases and the contribution of fldZ in the process of amino acid fermentation are presented here.

5.1.1. Stability and the Southern blot analysis of *fldZ*::CT*erm*B mutant

The ClosTron system used for the creation of *fldZ*::CT*erm*B is based on group II intron retrohoming process and the mutants produced with this method were extremely stable (Heap *et al*., 2007). However, to validate the *fldZ::*CT*erm*B strain stability and prevent cross-contaminations between different *C. sporogenes* strains, a robust and simple method for the wild type and the *fldZ* mutant identification was used based on detection of L1.LtrB intron with polymerase chain reaction. After every culture, cells (1 mL) were harvested and genomic DNA was extracted. DNA was used as a template in PCR reaction using primers ENR-F1 and EBSUni. The reaction products were separated on 1% agarose gel (Fig. 5.2.).

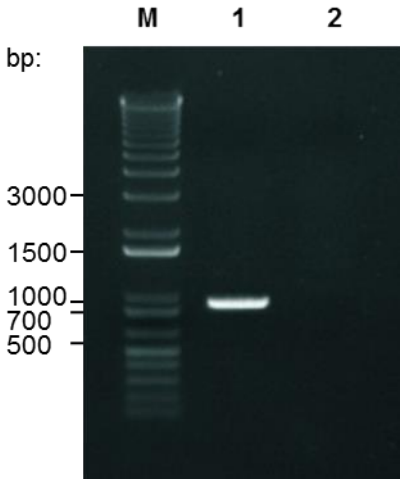


Fig. 5.2. *Erm* intron PCR screen. Reaction mixtures (10 µL) were run on 1% agarose gel with 1 kb Plus Ladder (Invitrogen; **M**). Templates: **1** – *fldZ*::CT*erm*B, **2** – *C. sporogenes* DSM795. Expected product for *fldZ*::CT*erm*B was about 870 bp.

The presence of the L1.LtrB intron integrated in the *fldZ* gene resulted in a PCR product of a size about 870 bp. No product was observed when *C. sporogenes* DSM795 genomic DNA was used as the reaction template, confirming the strain identities. This test was carried out after every experiment when *fldZ*::CT*erm*B was used. It also confirmed that genomic mutant knock out strains created with the ClosTron are extremely stable and the L1.LtrB intron remains integrated in the bacterial chromosome for many generations (more than fifty successful re-cultures).

The ClosTron system can be targeted to insertionaly inactivate any gene based on the possible integration sites for L1.LtrB-derived introns designed by the Perutka algorithm (Perutka *et al.*, 2004). However, considering the preliminary experiments showing multiple enzymatic activities reducing C=C double bonds in *C. sporogenes* and in the absence of the *C. sporogenes* genome sequence, additional genetic analysis of *fldZ*::CT*erm*B was performed. To determine the number of L1.LtrB integration events and thus, the number of inactivated genes, the Southern blot technique was used (Fig. 5.3.).

Genomic DNA was extracted from the wild type strain and the mutant and digested using two different restriction enzymes, *Eco*RI and *Hin*dIII. DNA fragments were separated using electrophoresis in 1% agarose gel, denatured in alkaline solution and transferred onto a cellulose membrane. The membrane was hybridised with a DIG-labelled *erm* probe overnight and the pattern of hybridization was visualized by autoradiography.

Fig. 5.3. Southern blot analysis of *fldZ*::CT*erm*B mutant using *erm* DIG labelled probe. Lanes: **1** – *fldZ*::CT*erm*B digested with *Hin*dIII, **2** – *fldZ*::CT*erm*B digested with *Eco*RI, **3** – DSM795 digested with *Hin*dIII, **4** – DSM795 digested with *Eco*RI, **5** – pMTL007 plasmid containing *erm* gene (positive control).

The probe hybridized to the *erm* plasmid used as a positive control in the experiment as well as to single bands in samples containing *fldZ*::CT*erm*B genomic DNA digested with *Eco*RI and *Hin*dIII restrictases. As expected, no bands were visible in samples containing genomic DNA of the wild type strain DSM795. The Southern blot showed that the *fldZ*::CT*erm*B mutant possessed only one intron integration site and thus only one gene in the *C. sporogenes* chromosome was knocked out.

5.1.2. Role of the fldZ reductase in amino acid fermentation

2-Enoate reductase was proposed to be involved in the L-phenylalanine fermentation *via* the Stickland reaction (Bühler *et al.*, 1980). In the last step of the reductive branch, cinnamic acid is reduced to 3-phenylpropionic acid and this reaction is coupled to ATP formation by electron transfer phosphorylation (Bader and Simon, 1983). The *fldZ* mutant showed decreased biomass production when L-phenylalanine was used as the energy substrate in the complex Giesel medium. However, a much more drastic

decrease in the growth was observed for medium supplemented with D-glucose (Mordaka, 2010). That was surprising since the fldZ activity was not postulated to be associated with metabolism of carbohydrates. To further exploit this subject, a series of growth experiments and metabolic footprinting analysis were performed for the *fldZ*::CT*erm*B strain.

5.1.2.1. L-Phenylalanine fermentation in a complex medium

C. sporogenes is a proteolytic organism unable to grow except in media containing proteins and amino acids. Carbohydrates are also fermented, but they are neither sufficient nor essential for growth (Stickland, 1934), although *C. sporogenes* has a high rate of glucose fermentation and its cell free extract contains all glycolytic enzymes catalysing glucose degradation to pyruvate (Golovchenko *et al.*, 1983). Thus, the growth of *C. sporogenes* in the complex Giesel medium relies on a mixed fermentation of amino acids and proteins (present in peptone and yeast extract) and carbohydrates (from yeast extract). To test the role of the fldZ reductase it was better to minimise the effect of glucose dissimilation by elimination of the yeast extract from the medium. However, apart from carbohydrates and proteins, yeast extract contains also essential water soluble vitamins, growth factors and purine and pyrimidine bases and cannot be completely excluded from the complex medium. Thus, for the growth experiments the yeast extract concentration in Giesel medium was decreased from 0.5 to 0.1% w/v. Growth of DSM795 and *fldZ*::CT*erm*B was determined in the modified Giesel medium supplemented with 12.1 mM L-phenylalanine. Moreover, the formation of 3-phenylpropionic acid during the growth was also determined by gas chromatography (Fig. 5.4.).

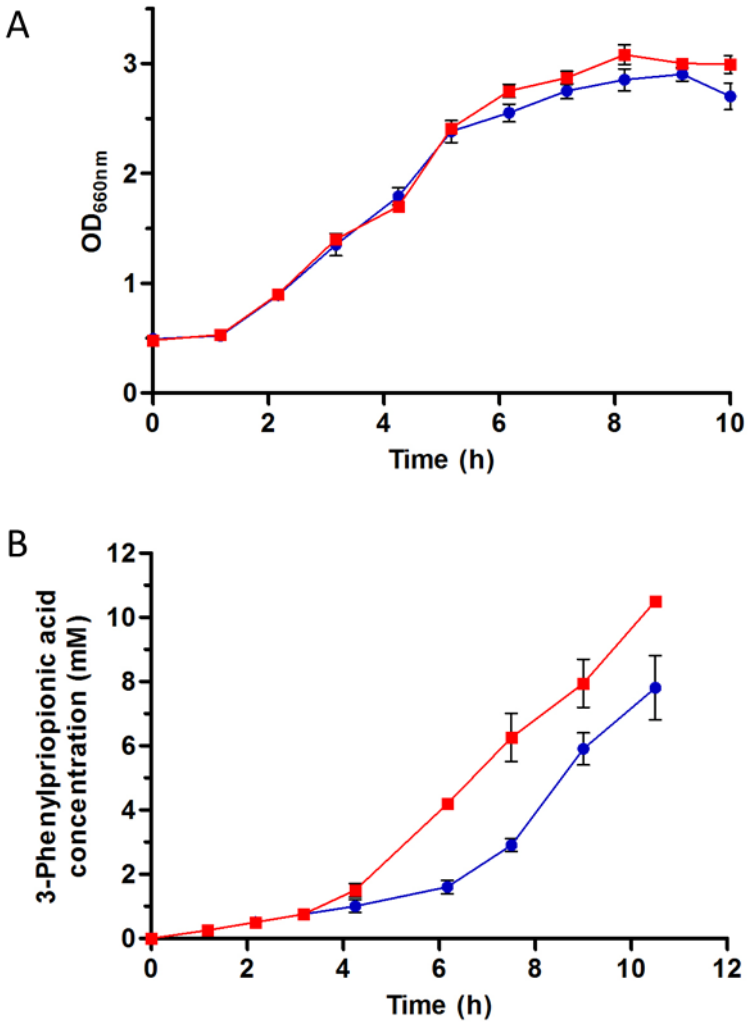


Fig. 5.4. **A.** growth of DSM795 (●) and *fldZ*::CT*erm*B (■) in modified Giesel medium supplemented with 12.1 mM L-phenylalanine. Average of three independent replicates. Error bars correspond to standard deviations. **B.** Concentration of 3-phenylpropionic acid in the growth medium of DSM795 (●) and *fldZ*::CT*erm*B (■) determined by GC-MS. Average of two independent replicates. Error bars correspond to standard error of mean.

It was found that growth rates of *C. sporogenes* DSM795 and *fldZ*::CT*erm*B in the modified medium were similar. In contrast to the previous experiments (Mordaka, 2010) where the original Giesel medium was used, no decrease of biomass production was observed for the mutant when compared to the wild type. Analysis of the medium footprints showed that despite inactivation of *fldZ* gene, the mutant fermented L-phenylalanine to 3-phenylpropionic acid and the formation of the product was even greater than in the wild type strain. No cinnamic acid was found in the residual medium. Thus, the experiment suggested that fldZ is not involved in the reductive branch of L-phenylalanine fermentation by the Stickland reaction.

5.1.2.2. Growth of DSM795 in a minimal defined medium

The growth of DSM795 and *fldZ*::CT*erm*B was tested in the minimal defined medium (the Lovitt medium; Lovitt *et al*., 1997a). The medium contained only compounds necessary for growth (such as buffering agents, salts, trace elements, vitamins and essential amino acids). To support the growth, the Lovitt medium was also supplemented with pairs of electron donors and acceptors tested for the fermentation.

Different amino acids and organic acid salts could serve as electron donors for reduction of methylene blue or brilliant cresyl blue (Stickland, 1934). It was shown that D-alanine was a good electron donor whereas L-alanine did not reduce the dye at all, suggesting that the enzyme involved in the oxidation of alanine was highly enantioselective. D-alanine was selected as the electron donor for the physiological experiments as it was oxidized by *C. sporogenes* to acetic acid in a simple two stage reaction and could not serve as electron acceptor in the Stickland fermentation (Stickland, 1935b).

To test if alanine could support the growth of *C. sporogenes* DSM795, the minimal Lovitt medium (10 mL) was supplemented with L- or D-alanine (final concentration 27 mM). The media were also supplemented with L-proline (final concentration 45 mM), which was previously shown to act as an excellent electron acceptor in *C. sporogenes* fermentation (Lovitt *et al.*, 1987b). The experimental cultures were set by inoculation with the overnight DSM795 pre-culture in the basal Lovitt medium (0.5 mL). After 1 h pre-incubation at 30°C with shaking, the aliquots (200 µL) were transferred into the wells of a 96-well plate and the growth of bacteria was observed for 44 h using automated NEPHELOstar plate reader (Fig. 5.5.).

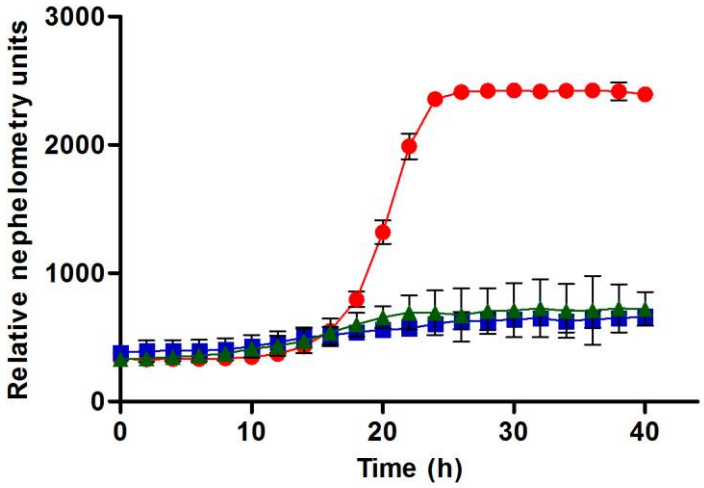


Fig. 5.5. Growth of *C. sporogenes* DSM795 in the Lovitt medium with 45 mM L-proline (▲) and Lovitt medium with 45 mM L-proline supplemented with 27 mM L-alanine (●) or 27 mM D-alanine (■). Average of five independent replicates. Error bars correspond to standard deviations.

The experiment showed that *C. sporogenes* can use L-alanine and L-proline as a pair of electron donor and acceptor to produce energy for growth. When the minimal medium was supplemented with D-alanine, no increase in the growth rate or biomass production was observed when compared to the basal medium. Therefore, in contrast to the findings by Stickland, 1934, D-alanine was not accepted as a substrate for fermentation.

5.1.2.3. Growth of DSM795 and *fldZ*::CT*erm*B with different fermentation substrates

To determine the role of the fldZ reductase in the metabolism of *C. sporogenes*, the growth of wild type DSM795 and *fldZ*::CT*erm*B mutant was tested in different variants of Lovitt medium. 2-Enoate reductase was proposed to be responsible for reduction of cinnamic acid in the L-phenylalanine pathway (Bühler *et al.*, 1980). Fermentation of L-tyrosine and L-tryptophan resulted in formation of 3-(*p*-hydroxyphenyl)propanoic acid and indole-3-propanoic acid, products corresponding to those produced in the reductive branch of Stickland reaction from L-phenylalanine (Jellet *et al.*, 1980). Some proteolytic *Clostridium spp.* also reduced L-leucine to give the final product 4-methylvaleric acid, whereas L-valine and L-isoleucine were not reduced at all and gave only products of their oxidation (Barker *et al.*, 1981). That suggested that L-tyrosine, L-tryptophan and L-leucine may be reduced through the same metabolic pathway as L-phenylalanine. L-Proline and glycine were also accepted as electron acceptors in the Stickland fermentation, but these two amino acids possessed their own reduction pathways (Schwartz and Müller, 1979; Venugopalan, 1980) and were selected for use as control substrates.

The Lovitt minimal medium was supplemented with L-alanine (as electron donor, final concentration 27 mM) and different amino acids served as electron acceptors: glycine, L-proline, L-phenylalanine, L-tryptophan, L-isoleucine, L-leucine or L-valine (final concentration 45 mM). L-Tyrosine was not used as the electron acceptor, because of its poor water solubility. Cultures were inoculated with an overnight pre-culture in the basal Lovitt medium, pre-incubated for 1 h at 30°C and samples (200 µL) were transferred into a 96-well plate where the growth of bacteria was observed for 44 h (Fig. 5.6.).

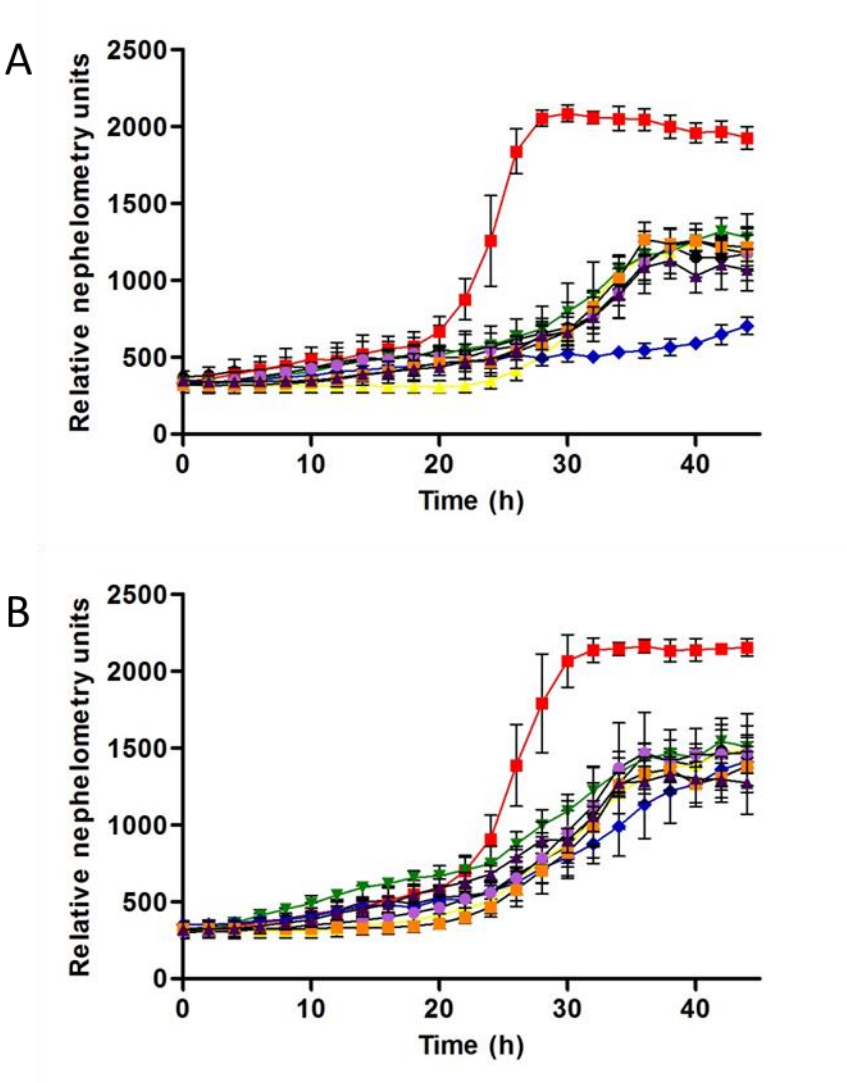


Fig. 5.6. Growth of *C. sporogenes* DSM795 (**A**) and *fldZ*::CT*erm*B (**B**) in Lovitt medium supplemented with 27 mM L-alanine as the electron donor. The electron acceptors (45 mM) were L-proline (■), glycine (▲), L-phenylalanine (▼), L-tryptophan (♦), L-isoleucine (●), L-leucine (■) and L-valine (▲). Control culture with no electron acceptor (●). Average of four independent replicates.

From all pairs of electron donors and acceptors only L-alanine and L-proline showed increased biomass production of the wild type and the mutant when compared to the control sample. The presence of L-tryptophan caused an inhibitory effect in the wild type but not in the mutant. When the medium was supplemented with glycine, phenylalanine, isoleucine, leucine and valine the mutant gave slightly (but statistically significant) higher biomass production.

A similar experiment was performed using a different electron donor, 27 mM glucose (Fig. 5.7.).

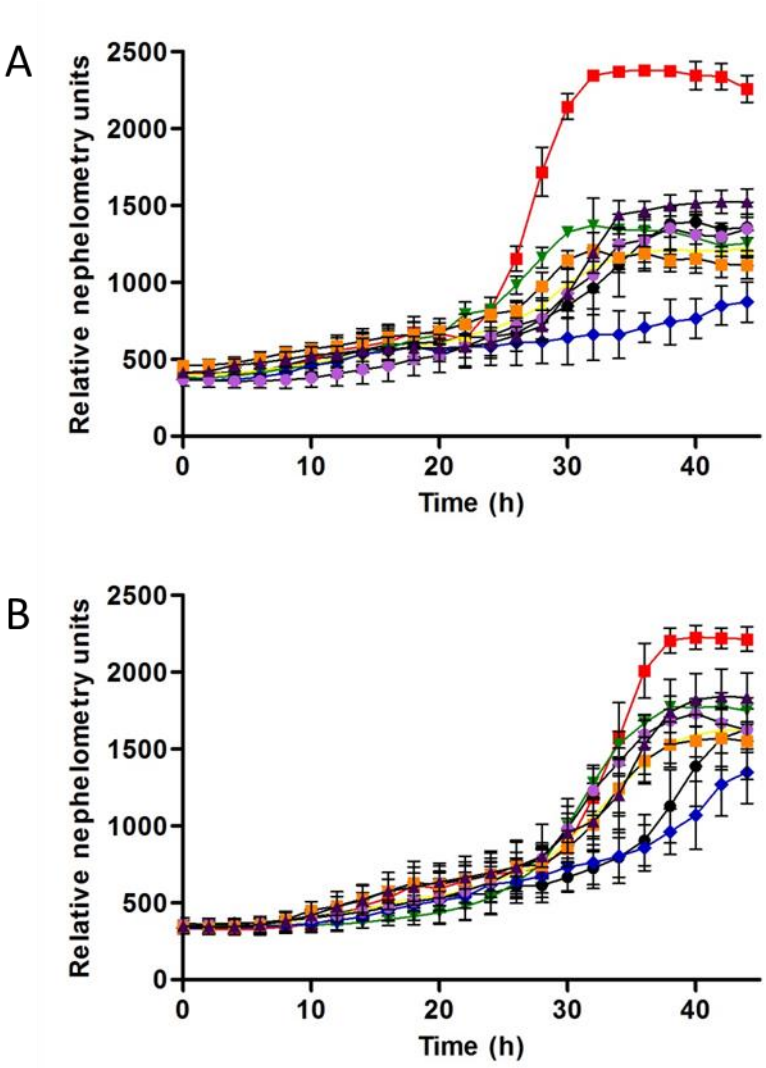


Fig. 5.7. Growth of *C. sporogenes* DSM795 (**A**) and *fldZ*::CT*erm*B (**B**) in Lovitt medium supplemented with 27 mM D-glucose as the electron donor. The electron acceptors (45 mM) were L-proline (■), glycine (▲), L-phenylalanine (▼), L-tryptophan (♦), L-isoleucine (●), L-leucine (■) and L-valine (▲). Control culture with no electron acceptor (●). Average of four independent replicates.

The pair of electron donor and acceptor that gave the best growth was D-glucose and L-proline confirming that the latter substrate is an excellent electron acceptor in the Stickland fermentation. L-Tryptophan inhibited the growth of the wild type strain causing an extended lag phase. Again, the *fldZ* mutant gave a slight (about 20%), but statistically significant (based on the t-tests) increase in the biomass production when compared to the wild type.

Results of the growth experiments showed unexpected physiological behaviour of the *fldZ*::CT*erm*B. The inactivation of the fldZ enoate reductase should limit the number of electron acceptors that can be used for fermentation. That should decrease the growth compared to the wild type strain. This suggested that fldZ reductase may not be the enzyme involved in the Strickland reaction.

5.1.2.4. Analysis of the final products of fermentation

To analyze the final products of amino acid fermentation in the wild type and the *fldZ* mutant, cultures grown in minimal medium supplemented with glucose (electron donor) and either L-phenylalanine or L-tryptophan (electron acceptors) were used for the footprinting analysis in the late stationary phase. Culture supernatants extracted into diethyl ether were derivatized with (trimethylsilyl)diazomethane (see method 4.8.2.8.) and analyzed using GC-MS.

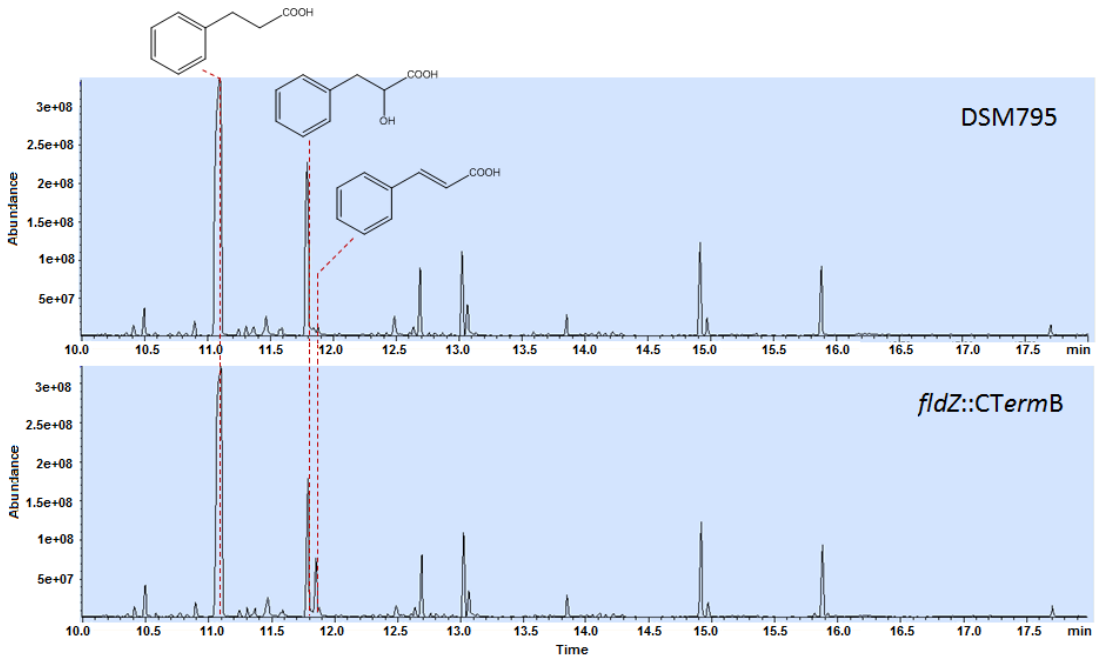


Fig. 5.8. Analysis of the final products of L-phenylalanine fermentation in the wild type DSM795 and the *fldZ*::CT*ermB* mutant grown in minimal medium. Unlabelled peaks were side products of the derivatization method.

Even though *fldZ*::CT*erm*B mutant had the *fldZ* gene knocked out, both strains gave similar products from the L-phenylalanine, namely 3-phenylpropionic acid and phenyllactic acid. The mutant accumulated also significant amounts of cinnamic acid, whereas the wild type did not (Fig. 5.8).

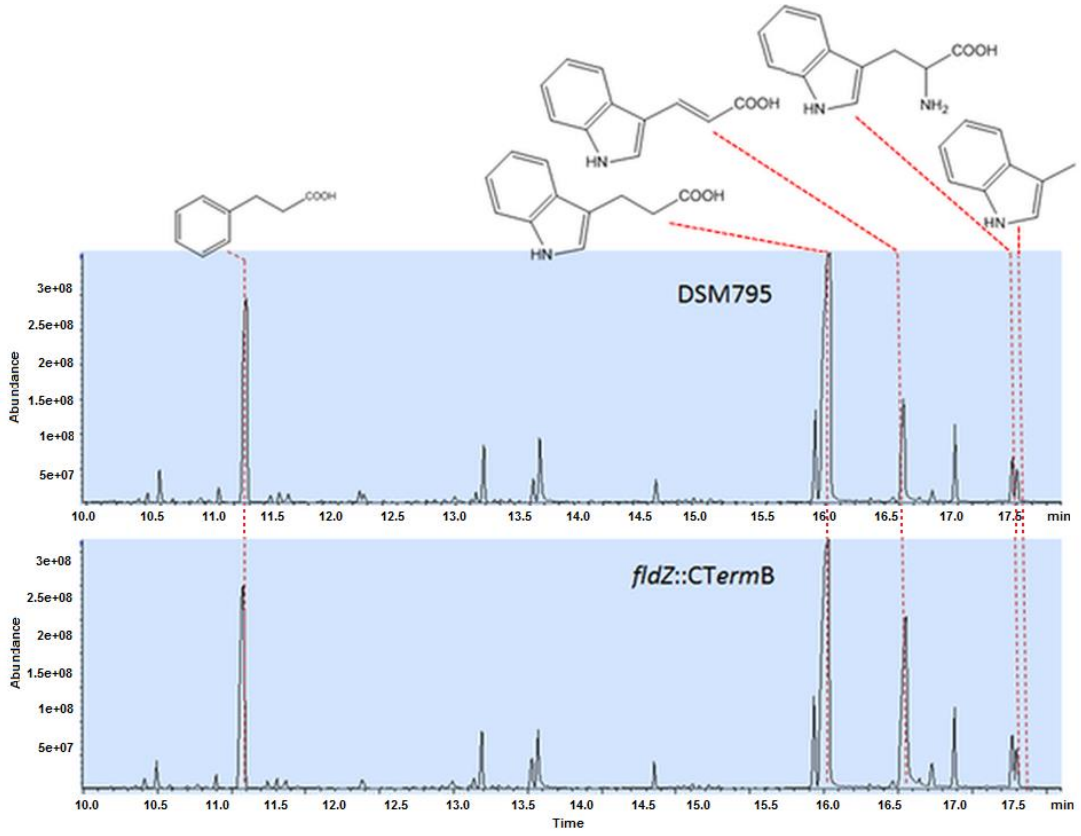


Fig. 5.9. Analysis of the final products of L-tryptophan fermentation in the wild type DSM795 and *fldZ*::CT*ermB* mutant grown in minimal medium. Unlabelled peaks were side products of the derivatization method.

When the medium was supplemented with L-tryptophan (Fig. 5.9.), *C. sporogenes* DSM795 and *fldZ*::CT*erm*B mutant produced 3-indolepropionic acid, 3-indoleacrylic acid and skatole. 3-Phenylpropionic acid, the product of phenylalanine fermentation, was also detected. This can be explained by the fact that the basal medium contained L-phenylalanine, as this amino acid is essential for growth of *C. sporogenes* and cannot be replaced with any other derivative. 3-Indolepropionic acid and 3-indoleacrylic acid were products of the reductive branch of tryptophan fermentation in the Stickland reaction. Skatole could be a product of the decarboxylation of indoleacetic acid that was formed in the oxidative branch of the Stickland fermentation.

The analysis of the final products of fermentation showed that surprisingly the mutant produced 3-phenylpropionic acid (when the medium was supplemented with L-phenylalanine and L-tryptophan) and 3-indolepropionic acid (when the medium was supplemented with L-tryptophan). These compounds were postulated to be products of the enoate reductase activity in the reductive branch of the Stickland reaction. Thus it was confirmed that fldZ enoate reductase is not essential for fermentation of L-phenylalanine and L-tryptophan by *C. sporogenes*.

5.1.2.5. Reduction of cinnamic acid by *C. sporogenes fldZ* mutant

Preliminary experiments with protein cell-free extracts of the *fldZ*::CT*erm*B mutant showed that cinnamic acid was not reduced in the spectrophotometric assay (Mordaka, 2010). However, further growth experiments in both complex and minimal defined media showed that *fldZ*::CT*erm*B produced 3-phenylpropionic and 3-indolepropionic acid, products of the cinnamate reductase activity in the reductive branch of the Stickland fermentation.

To investigate the scope for reduction of cinnamic acid by the mutant, DSM795 and *fldZ*::CT*erm*B strains were grown in the Giesel medium and harvested. Half of the culture was used for whole cell cinnamic acid biotransformation using hydrogen gas as the electron donor. Reactions were incubated for 24 h at 30°C, the products were derivatized using (trimethylsilyl)diazomethane and analyzed using gas chromatography (Fig. 5.10.).

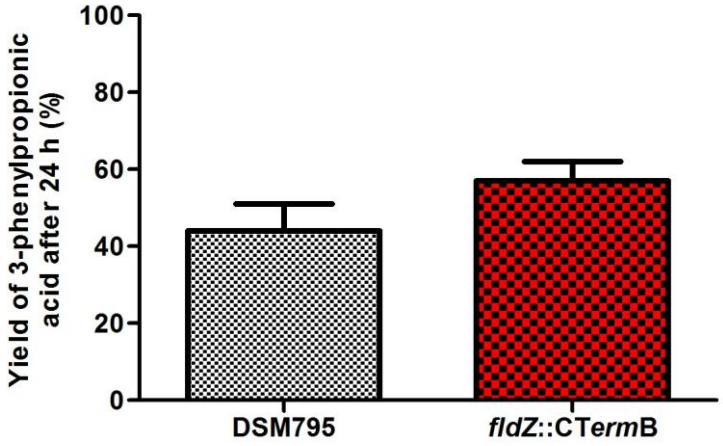


Fig. 5.10. Reduction of cinnamic acid using whole cells of *C. sporogenes* DSM795 and *fldZ*::CT*erm*B (grown in Giesel medium with 12.1 mM L-phenylalanine) and hydrogen gas as the electron donor. Error bars correspond to standard deviations.

Cinnamic acid was converted to 3-phenylpropionic acid by the whole cells of both strains. The yield of the reaction was greater for the mutant (57%) than for the wild type (44%).

The second part of the culture was used to prepare protein cell-free extracts. The cells were disrupted using the cell disruptor at pressure of 40,000 psi. The cell debris and insoluble proteins were separated from the soluble protein fraction by centrifugation at 75,000 x g for 30 min at 4°C. The supernatant was used in the spectrophotometric cinnamic acid reduction assay (Fig. 5.11.).

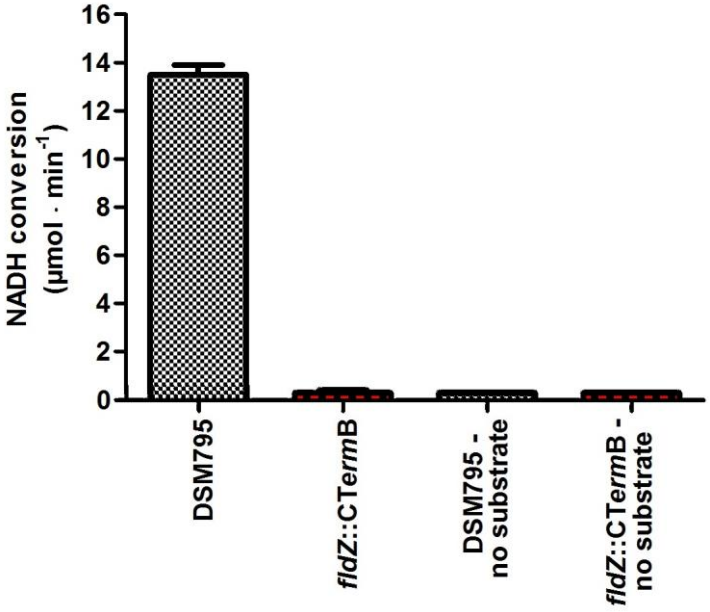


Fig. 5.11. Cinnamic acid reduction in the spectrophotometric assay using membrane-free protein extracts of *C. sporogenes* DSM795 and *fldZ*::CT*erm*B (grown in Giesel medium with 12.1 mM L-phenylalanine) and NADH as the electron donor.

Reduction of cinnamic acid coupled to consumption of NADH (resulting in a decrease of absorbance at 340 nm) was observed only for crude extracts of DSM795 (activity 13.5 µmol/min). No activity was detected when extracts of *fldZ*::CT*erm*B were used and in the control experiments without cinnamic acid.

Cinnamic acid biotransformations showed that the mutant reduced the substrate when whole cells were used but not with protein crude extracts. The wild type *C. sporogenes* reduced cinnamic acid in both types of reaction. Thus, *C. sporogenes* possesses at least two cinnamic acid reductases. One of them is the NADH-dependent soluble fldZ reductase. The second unknown enzyme may not accept NADH as the electron donor since no activity was observed in the spectrophotometric assay or may be a membrane attached/associated protein that was removed from the sample with the cell debris and insoluble proteins.

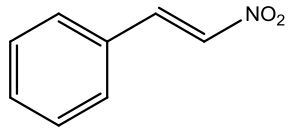
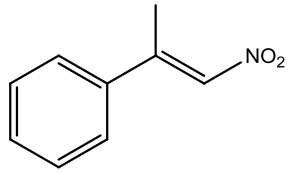
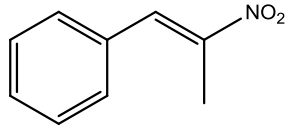
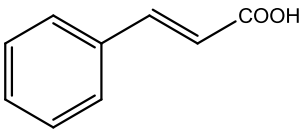
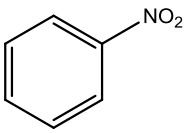
5.1.3. Biotransformations using DSM795 and *fldZ*::CT*erm*B

5.1.3.1. Reduction of nitroalkenes, cinnamic acid and nitrobenzene using harvested cells

To test if the protein encoded by *fldZ* gene is involved in reduction of C=C bonds, whole cells of *C. sporogenes* DSM795 and *fldZ*::CT*erm*B were used to reduce β-nitrostyrene, (*E*)-1-nitro-2-phenylpropene, (*E*)-1-phenyl-2-nitropropene and cinnamic acid. Since reduction of nitro groups was found to be catalyzed by flavin-containing enzymes, the

reduction of aromatic nitro compounds (represented by nitrobenzene) was tested as well. The effect of the growth substrate on the expression of reductases was also determined. Cells were cultivated in the basal Giesel medium or Giesel medium supplemented with 27 mM glucose or 12.1 mM L-phenylalanine. The cultures were harvested in the early stationary phase of growth and resuspended in 100 mM phosphate buffer. The reaction was started by injection of hydrogen gas as an electron donor. The products were analyzed after 72 h using chiral HPLC or GC-MS (Table 5.1.).

Table 5.1. Whole cell biocatalyst reduction of β-nitrostyrene, (*E*)-1-nitro-2-phenylpropene, (*E*)-1-phenyl-2-nitropropene, cinnamic acid and nitrobenzene using DSM795 and *fldZ*::CT*erm*B grown in Giesel medium. Data present means of three independent experiments with two replicates each. Standard errors of mean < 5%.

Substrate	Strain	Growth substrate	Yield (%)	Ee. (%)
 β-nitrostyrene	DSM795	-	11.6	-
		D-glucose	0.9	-
		L-phenylalanine	13.2	-
	fldZ::CT*erm*B	-	15.0	-
		D-glucose	6.4	-
		L-phenylalanine	13.4	-
 *(E)*-1-nitro-2-phenyl-propene	DSM795	-	33.0	≥ 99.9 *(R)*
		D-glucose	23.6	≥ 99.9 *(R)*
		L-phenylalanine	35.4	≥ 99.9 *(R)*
	fldZ::CT*erm*B	-	0.0	-
		D-glucose	0.0	-
		L-phenylalanine	0.0	-
 *(E)*-1-phenyl-2-nitro-propene	DSM795	-	6.3	23.0 *(S)*
		D-glucose	4.3	20.7 *(S)*
		L-phenylalanine	5.8	1.0 *(S)*
	fldZ::CT*erm*B	-	1.2	15.2 *(S)*
		D-glucose	1,4	16.0 *(S)*
		L-phenylalanine	5.0	0.2 *(S)*
 *(E)*-cinnamic acid	DSM795	-	16.5	-
		D-glucose	10.0	-
		L-phenylalanine	38.2	-
	fldZ::CT*erm*B	-	11.5	-
		D-glucose	11.7	-
		L-phenylalanine	39.6	-
 nitrobenzene	DSM795	-	0.0	-
		D-glucose	0.0	-
		L-phenylalanine	0.0	-
	fldZ::CT*erm*B	-	0.0	-
		D-glucose	0.0	-
		L-phenylalanine	0.0	-

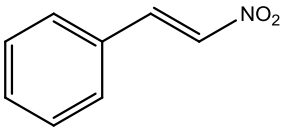
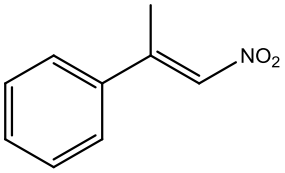
(E)-1-nitro-2-phenylpropene was reduced by the wild type with 35.5% yield and almost 100% enantiomeric excess, whereas no product was found in samples in the reaction containing *fldZ*::CT*erm*B cells. DSM795 reduced β-nitrostyrene with 13% yield and *(E)*-1-phenyl-2-nitropropene with 4.7%. When *fldZ*::CT*erm*B cells were used, yields were similar (13.4% and 5.0% respectively) but only for cells that were grown on L-phenylalanine. Mutants growing in the basal medium or on glucose showed reduced yields of (*E*)-1-phenyl-2-nitropropene hydrogenation product (1.2% and 1.4%). Cinnamic acid was reduced by both the wild type and the mutant and the reductase activity was induced by the presence of L-phenylalanine in the growth medium. No product of biotransformation was found when nitrobenzene was used as substrate.

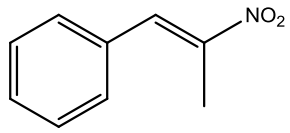
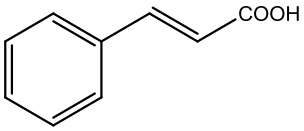
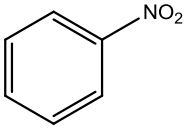
Results showed that fldZ enoate reductase is responsible for the reduction of C=C bond in *(E)*-1-nitro-2-phenylpropene, whereas the reduction of β-nitrostyrene, *(E)*-1-phenyl-2-nitropropene and cinnamic acid may be driven by some other enzyme(s). The other enzyme responsible for reduction of *(E)*-1-phenyl-2-nitropropene is more active when L-phenylalanine is present in the growth medium.

5.1.3.2. Reduction of nitroalkenes, cinnamic acid and nitrobenzene using protein extracts

Membrane-free protein extracts were used to reduce β-nitrostyrene, *(E)*-1-nitro-2-phenylpropene, (*E*)-1-phenyl-2-nitropropene, cinnamic acid and nitrobenzene. The cells used for preparation of protein extracts were grown in Giesel medium supplemented with 12.1 mM L-phenylalanine and were harvested in the early stationary phase of growth. In the reactions H_2, NADH and NADPH were used as the electron donors (Table 5.2.).

Table 5.2. Reduction of β-nitrostyrene, (*E)*-1-nitro-2-phenylpropene, (*E*)-1-phenyl-2-nitropropene, cinnamic acid and nitrobenzene using DSM795 and *fldZ*::CT*erm*B protein extracts. Data present means of three independent experiments with two replicates each. Standard errors of mean < 5%.

Substrate	Strain	Electron donor	Yield (%)	Ee. (%)
 β-nitrostyrene	DSM795	H_2	2.0	-
		NADH	71.4	-
		NADPH	72.2	-
	fldZ::CT*erm*B	H_2	3.5	-
		NADH	46.5	-
		NADPH	57.4	-
 *(E)*-1-nitro-2-phenyl-propene	DSM795	H_2	10.1	≥ 99.9 *(R)*
		NADH	≥ 99.9	≥ 99.9 *(R)*
		NADPH	57.1	≥ 99.9 *(R)*
	fldZ::CT*erm*B	H_2	0.0	-
		NADH	0.0	-
		NADPH	0.0	-

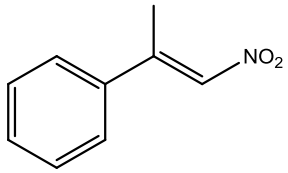
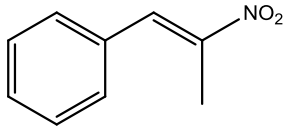
 *(E)*-1-phenyl-2-nitro-propene	DSM795	H_2	15.7	4.5 *(S)*
		NADH	58.1	11.5 *(S)*
		NADPH	50.5	4.0 *(S)*
	fldZ::CT*erm*B	H_2	4.3	3.2 *(S)*
		NADH	22.0	5.6 *(S)*
		NADPH	10.2	5.3 *(S)*
 *(E)*-cinnamic acid	DSM795	H_2	9.5	-
		NADH	≥ 99.9	-
		NADPH	68.9	-
	fldZ::CT*erm*B	H_2	0.0	-
		NADH	0.0	-
		NADPH	0.0	-
 nitrobenzene	DSM795	H_2	0.0	-
		NADH	46.4	-
		NADPH	24.6	-
	fldZ::CT*erm*B	H_2	0.0	-
		NADH	39.6	-
		NADPH	19.9	-

Using membrane-free protein extracts the yield of *(E)*-1-nitro-2-phenylpropene reduction by DSM795 was improved to almost 100% and almost 100% enantiomeric excess, when compared with whole cell biocatalysts assay. No product of *(E)*-1-nitro-2-phenylpropene reduction was found in samples of *fldZ*::CT*erm*B mutant. Reduction of β-nitrostyrene and *(E)*-1-phenyl-2-nitropropene was also more efficient than for whole cells and product formation was observed for both the wild type and the mutant. Cinnamic acid was reduced by protein extracts of DSM795 with a good yield (100% when NADH was used and 69% for NADPH), but no product was found when protein extract of the mutant was used. Nitrobenzene was reduced by both strains (no statistically significant differences of yields were observed between DSM795 and *fldZ*::CT*erm*B) but not when H_2 was used to start the biotransformation. NADH was better electron donor than hydrogen gas or NADPH in all of the reactions, with an exception for β-nitrostyrene.

5.1.3.3. Oxygen sensitivity of *C. sporogenes* phenylnitropropene reductases

Experiments on clostridial enoate reductases showed that the 2-enoate reductase in the reduced state was instantly inactivated by oxygen (Tischer *et al.*, 1979). The activity could be restored 3 h after removal of oxygen. To test the oxygen sensitivity of C=C double bond reductases, *C. sporogenes* membrane-free protein extracts were exposed to air for 3 h in an ice bath. Then the sample vials were closed with suba seals and the headspace of the vials was flushed with nitrogen for 1 h. After that the biotransformations were assembled under anoxic conditions in the anaerobic workstation (Table 5.3.).

Table 5.3. Reduction of (*E*)-1-nitro-2-phenylpropene and (*E*)-1-phenyl-2-nitropropene by protein extracts exposed to air. Data present means of three independent experiments with two replicates each. Standard errors of mean < 5%.

Substrate	Strain	Variant	Yield (%)	Ee. (%)
 *(E)*-1-nitro-2-phenyl-propene	DSM795	Anaerobic	≥ 99.9	≥ 99.9 *(R)*
		Aerobic	≥ 99.9	≥ 99.9 *(R)*
	fldZ::CT*erm*B	Anaerobic	0.0	-
		Aerobic	0.0	-
 *(E)*-1-phenyl-2-nitro-propene	DSM795	Anaerobic	57.1	11.1 *(S)*
		Aerobic	43.5	2.6 *(S)*
	fldZ::CT*erm*B	Anaerobic	21.0	5.6 *(S)*
		Aerobic	0.2	4.7 *(S)*

Reduction of *(E)*-1-nitro-2-phenylpropene by DSM795 extracts was not affected by the exposure to air suggesting that fldZ enoate reductase responsible for reduction of this substrate is not sensitive to oxygen or its activity was restored after removal of oxygen. On the other hand reduction of *(E)*-1-phenyl-2-nitropropene showed significantly lower yield for wild type and no reaction for the mutant, when their protein extracts were exposed to air. Thus, *(E)*-1-phenyl-2-nitropropene was reduced in the wild type by the fldZ reductase and the second enzyme that was irreversibly inhibited by the presence of oxygen.

5.1.4. Identification of *C. sporogenes* C=C double bond reductases

Reduction of the C=C double bond using DSM795 and the *fldZ*::CT*erm*B mutant showed that *C. sporogenes* possesses multiple reductase activities. One of them was fldZ 2-enoate reductase whereas other enzyme(s) remained unknown. Since attempts to purify the reductases have met with a limited success and no literature data suggested which other enzymes could be responsible for the reduction of nitroolefins by *C. sporogenes* DSM795, a different strategy was chosen to identify the reductases.

In 2010 a partial sequence of the *C. sporogenes* ATCC15579 genome was published as a part of the human gut microbiome sequencing project (Washington University Genome Sequencing Center; http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list_uids=5936). A genome of another *C. sporogenes* strain NCIMB10696 was sequenced by Prof. Nigel Minton's group (Centre for Biomolecular Sciences, University of Nottingham, personal communication). The genome sequences of these strains were analyzed for the presence of genes encoding hypothetical proteins, that showed homology

to C=C double bond reductases described in the literature. To confirm that these genes encode reductases, they were cloned, their protein products were expressed in *E. coli* and tested in the activity assays. This allowed for identification and characterization of new reductases that are present in *C. sporogenes* DSM795.

5.1.4.1. Identification of fldZ enoate reductase in *C. sporogenes*

Initially a 949 bp fragment of *C. sporogenes* encoding the fldZ enoate reductase was PCR amplified using primers designed for *fldZ* gene from *C. botulinum* strain Hall A 3502 (Dr. Benjamin Blount, personal communication; Appendix 8.1.). This DNA sequence was translated to an amino acid sequence (Appendix 8.8.2.) and used as a query sequence for browsing the *C. sporogenes* ATCC15579 genome using *blast p* tool (http://blast.ncbi.nlm.nih.gov). A hypothetical protein sequence CLOSPO_02780 (Csp15579.fldZ, Appendix 8.8.3.) was found to show 99% identity to the query. CLOSPO_02780 sequence was composed of 665 amino acids and showed 99% identity to the fldZ enoate reductase from *C. botulinum* str. Hall A 3502. The 1998 bp DNA fragment of genomic sequence encoding hypothetical protein CLOSPO_02780 was extracted from the Genbank (Csp15579.fldZ, Appendix 8.8.4.). PCR primers (CLOSPO_02780_f and CLOSPO_02780_r) were designed to allow the amplification of the full-length sequence gene. Initial PCR experiments using genomic DNA extracted from *C. sporogenes* DSM795 as a template were unsuccessful and no product was obtained. Detailed analysis of the *fldZ* gene from ATCC15579 and homologous sequences from *C. sporogenes* NCIMB10696 and *C. botulinum* ATCC3502 showed significant differences in the non-coding parts of these genes between different strains even within one genus (Appendix 8.8.5.). To identify the sequence of the *fldZ* gene in DSM795 it was necessary to design the forward primer to the gene located upstream of *fldZ* and the reverse primer to the gene located downstream of *fldZ*, because only these sequences showed significant homology between different strains of *Clostridia*. The gene was divided into three shorter parts to allow easier amplification with PCR and three sets of primers were designed: PM001 and PM002 (amplification of the coding sequence of the gene; product fldZ-ORF), PM003 and PM004 (5' flanking region containing the promoter and fragment encoding N terminus of the fldZ; product fldZ-5') and PM006 and PM008 (3' part of the gene and downstream region containing the gene terminator; product fldZ-3'). The primers were used in PCR on *C. sporogenes* DSM795 genomic DNA using the FailSafe Polymerase system containing different premixes for optimization of the reaction. After PCR, the reaction mixtures were separated by electrophoresis (Fig. 5.12-14.).

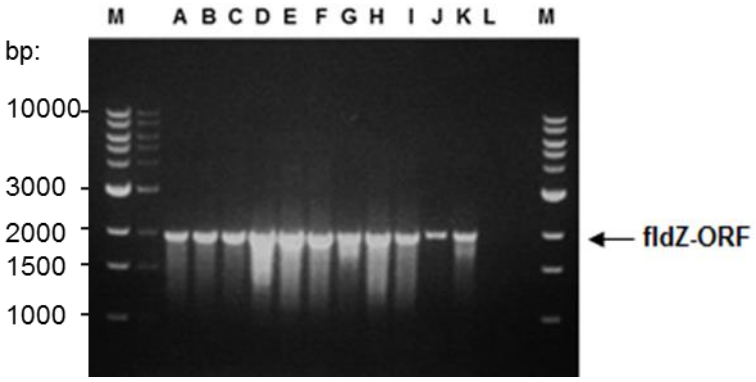


Fig. 5.12. Electrophoresis of fldZ-ORF PCR products. A-L: different premixes of the FailSafe Polymerase system. Products (10 µL of each) were run on 1% agarose gel with 1 kb DNA Ladder (NEB). The size of products was about 2000 bp.

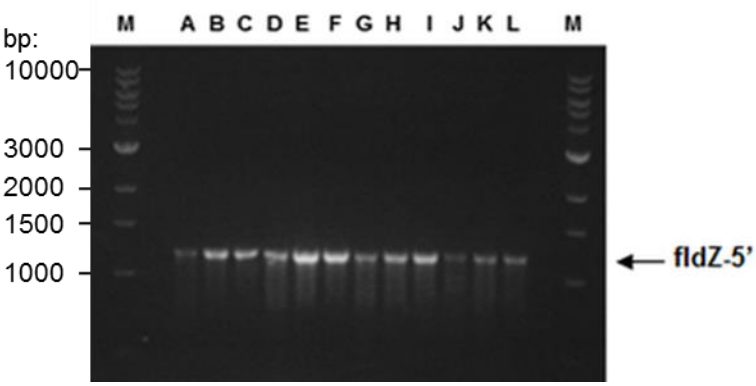


Fig. 5.13. Electrophoresis of fldZ-5' PCR products. A-L: different premixes of the FailSafe Polymerase system. Products (10 µL of each) were run on 1% agarose gel with 1 kb DNA Ladder (NEB). The size of products was about 1200 bp.

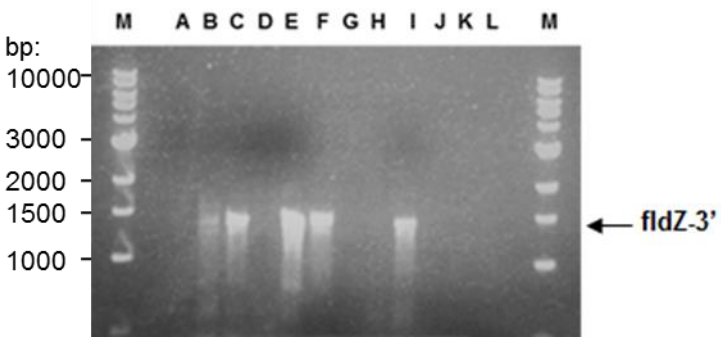


Fig. 5.14. Electrophoresis of fldZ-3' PCR products. A-L: different premixes of the FailSafe Polymerase system. Products (10 µL of each) were run on 1% agarose gel with 1 kb DNA Ladder (NEB). The size of products was about 1500 bp.

The electrophoresis showed the presence of PCR products of the expected length in almost all samples. To the further analysis three of the products obtained with each set of primers containing no side products were mixed together (samples B, C and K for fldZ-OFR, B, C, E, F and I for fldZ-5' and C, E, F and I for fldZ-3') and ligated into pJET1.2/blunt vector using CloneJET™ PCR Cloning Kit. Ligation mixtures were used to transform *E. coli* JM107

using TransformAid™ Bacterial Transformation Kit. Plasmids from positive transformants were purified and digested with the restriction enzyme *Bgl*II to detect the presence of PCR product. Plasmids containing inserts of the expected lengths were sent for sequencing using pJET1.2.for and pJET1.2.rev primers. All plasmids carried fragments of the *fldZ* gene with the consensus sequences of fldZ-ORF 2031 bp, fldZ-5' 1242 bp and fldZ-3' 1489 bp (Appendix 8.8.6.). These three fragments were aligned and the full *fldZ* gene sequence was obtained, containing 3274 bp (Appendix 8.8.7.). The analysis of the sequence showed the presence of the open reading frame encoding 665 amino acid sequence, the *C. sporogenes* DSM795 fldZ enoate reductase (Appendix 8.8.8.). The primary structure of the fldZ reductase was analyzed in the Protein Conserved Domain Tool (http:// www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Fig. 5.15.).

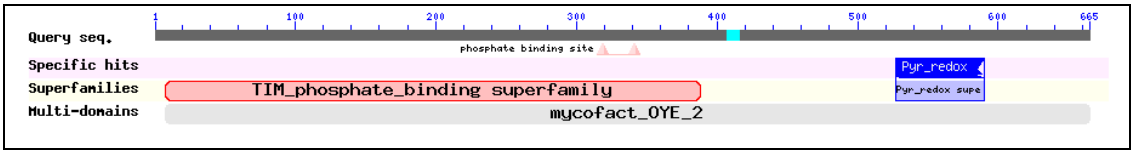


Fig. 5.15. Analysis of the fldZ reductase primary structure in the Protein Conserved Domain Tool.

The analysis showed that fldZ possessed conserved domains for binding of phosphate groups (TIM motif, characteristic for proteins interacting with cofactors, such as FMN) and NADH/FAD (pyridine nucleotide-disulphide oxidoreductases motif). The amino acid sequence of fldZ reductase showed 98% identity and 99% similarity to fldZ from *C. sporogenes* ATCC1559 and 99% identity and 99% similarity to fldZ from *C. botulinum* str. Hall A ATCC3502. Expression of fldZ in a heterologous host is described later (see 5.1.5.2.).

5.1.4.2. Identification of OYE-like reductase in *C. sporogenes*

Biotransformations using *fldZ*::CT*erm*B strain (see 5.1.3.1-2) showed that *C. sporogenes* contains more than one C=C double bond reductase. Genomes of some organisms encode more than one enoate reductase that can act as enantiocomplementary enzymes such as old yellow enzymes from *Saccharomyces cerevisiae* (Faber, 2004) or OPR1 and OPR3 from *Lycopersicon esculentum* (Hall *et al.*, 2007; Hall *et al.*, 2008). Thus, the amino acid sequence of fldZ enoate reductase was used as a query to find homologous proteins encoded by the genome of *C. sporogenes* ATCC15579. One sequence, CLOSPO_03444 was found showing 30% identity and 48% similarity to fldZ (Appendix 8.9.1.). CLOSPO_03444 contained 339 amino acid and showed significant homology to OYE-like reductases from *Bacillus subtilis*, *Lycopersicon esculentum* and *Saccharomyces*

cerevisiae. The gene sequence encoding CLOSPO_03444 was extracted from *C. sporogenes* ATCC15579 genome (Appendix 8.9.2.). The gene sequence was compared to corresponding genomic sequences from *C. botulinum* ATCC3502 and *C. sporogenes* NCIMB10696 and PCR primers were designed to the most conserved regions (primers PM011 and PM012). Primers were used in PCR on *C. sporogenes* DSM795 genomic DNA using the FailSafe Polymerase system containing different premixes for optimization of the reaction. Products of reactions were analyzed by gel electrophoresis (Fig. 5.16.).

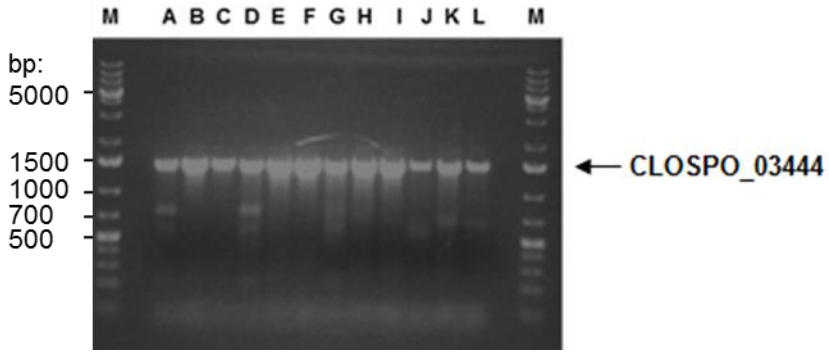


Fig. 5.16. Electrophoresis of CLOSPO_03444 PCR products. A-L: different premixes of the FailSafe Polymerase system. Products (10 µL of each) were run on 1% agarose gel with GeneRuler™ 1 kb Plus DNA Ladder (Fermentas). Bands are visible around 1500 bp.

Products of three reactions (B, C and F) were mixed together, purified by gel electrophoresis and recovered using QIAquick Gel Extraction Kit (QIAGEN). The purified DNA was ligated to pJET1.2/blunt vector using CloneJET™ PCR Cloning Kit. Ligation mixtures were used to transform *E. coli* JM107 using TransformAid™ Bacterial Transformation Kit. Plasmids from positive transformants were purified and digested with restriction enzyme *Bgl*II to detect the presence of PCR product. Plasmids containing inserts of expected length (about 1500 bp) were sent for sequencing using pJET1.2.for and pJET1.2.rev primers. The consensus sequence of the CLOSPO_03444 encoding gene was created by alignment of three independent PCR products sequences (Appendix 8.9.3.). The analysis of the gene sequence showed the presence of an open reading frame encoding 339 amino acids. The primary structure of the CLOSPO_03444 reductase was analyzed in the Protein Conserved Domain Tool (Fig. 5.17.).

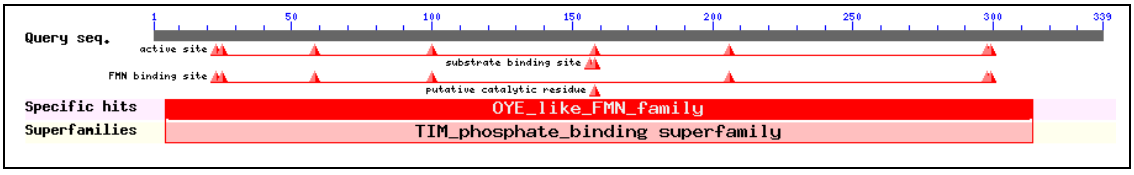


Fig. 5.17. Analysis of the CLOSPO_03444 reductase primary structure in the Protein Conserved Domain Tool.

The analysis showed that CLOSPO_03444 possessed conserved domains for binding of phosphate groups (TIM motif, characteristic for proteins interacting with cofactors, such as FMN) and showed homology to Old Yellow Enzyme (OYE)-like FMN binding domain. The OYE-like reductase CLOSPO_03444 from *C. sporogenes* showed 97% identity and 99% similarity to CLOSPO_03444 from *C. sporogenes* ATCC15579 and 97% identity and 99% similarity to the putative NADH/NADPH oxidoreductase from *C. botulinum* strain Hall A. Expression of fldZ in *E. coli* is described later (see 5.1.5.3.).

5.1.4.3. Identification of the hypothetical cinnamic acid reductase

In the reductive branch of L-phenylalanine fermentation by the Stickland reaction, phenylpyruvate is reduced to *(R)*-phenyllactate, which is then reversibly *syn*-dehydrated to cinnamic acid followed by reduction to 3-phenylpropionic acid. The analysis of the *C. sporogenes* ATCC15579 genome showed that the genes encoding enzymes involved in the pathway were localized in one gene cluster. It was found that the gene CLOSPO_00308 encoded fldA cinnamoyl-CoA:phenyllactate-CoA transferase responsible for formation of phenyllactate-CoA. The genes located downstream, CLOSPO_00309, CLOSPO_00310 and CLOSPO_00311, encoded subunits of the fldBC phenyllactate-CoA dehydratase and its oxygen-sensitive initiator protein fldI described by Dickert *et al.*, 2002. N-terminal sequences of fldH phenyllactate dehydrogenase (Dickert *et al.*, 2000) were found to be encoded by the genes CLOSPO_00316 and CLOSPO_00317 (Fig. 5.18.).

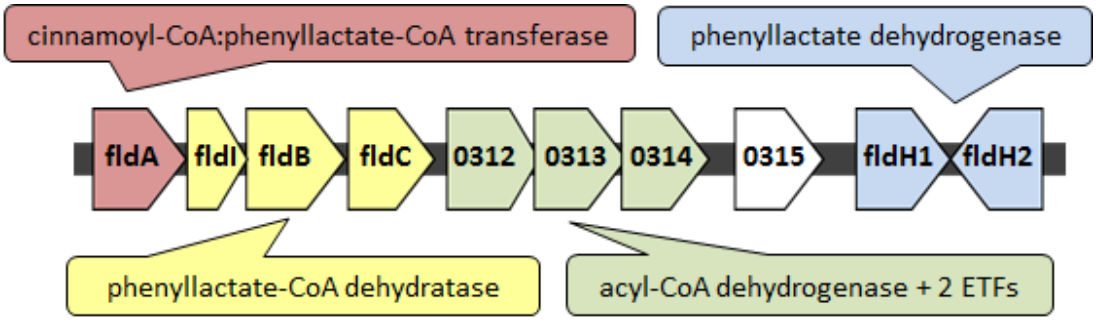


Fig. 5.18. Arrangement of genes encoding enzymes involved in the reductive branch of L-phenylalanine fermentation in the Stickland reaction in *C. sporogenes*.

The analysis of the gene cluster showed the presence of a previously undescribed operon encoding a hypothetical acyl-CoA dehydrogenase (CLOSPO_00312, sequence in Appendix 8.10.1.) with two electron transfer flavoproteins (ETF; genes CLOSPO_00313 and CLOSPO_00314). On the amino acid level, the acyl-CoA dehydrogenase showed significant homology (66% identity and 82% similarity) to CarC reductase responsible for reduction of caffeyl-CoA to dihydrocaffeyl-CoA in the acetogenic bacterium *Acetobacterium woodii*. CarC

was found to be transcribed together with two electron transfer flavoproteins that were found to be involved in the synthesis of ATP by a chemiosmotic mechanism using sodium ions as coupling ions during the caffeate respiration (Hess *et al.*, 2011).

The colocalization of CLOSPO_00312 with other enzymes involved in the L-phenylalanine reduction, a high homology to caffeyl-CoA reductase from *A. woodii* and the presence of two ETFs that could couple cinnamic acid reduction to the electron transfer phosphorylation may suggest that the acyl-CoA dehydrogenase is responsible for reduction of cinnamic acid in the Stickland fermentation. The substrate for this reaction would not be cinnamate, but rather cinnamoyl-CoA, which is the product of phenyllactate dehydration in the previous step of the pathway.

To identify the acyl-CoA dehydrogenase gene sequence in *C. sporogenes* DSM795, PCR primers (PM015 and PM016) were designed based on the CLOSPO_00312 gene sequence from *C. sporogenes* ATCC15579 (Appendix 8.10.2.). Primers were used in the PCR on *C. sporogenes* DSM795 genomic DNA using the KOD Polymerase system and different conditions (magnesium and template concentration, presence of 5% DMSO) were used for the optimization of the reaction (Fig. 5.19.).

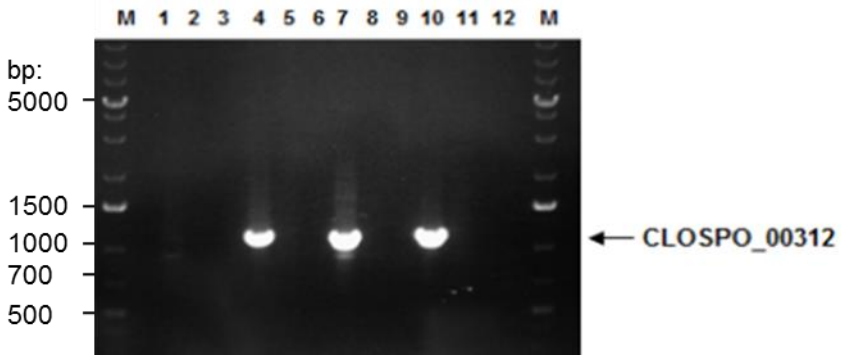


Fig. 5.19. Electrophoresis of CLOSPO_00312 PCR products. 1-12: different reaction conditions (magnesium and template concentration, presence of DMSO) in the KOD Polymerase system. Products (10 µL of each) were run on 1% agarose gel with GeneRuler™ 1 kb Plus DNA Ladder (Fermentas). Bands are visible around 1150 bp.

Products of three reactions (4, 7 and 10) were mixed together, purified by gel electrophoresis, excised from the gel and recovered using QIAquick Gel Extraction Kit. The purified DNA was ligated to pJET1.2/blunt vector using CloneJET™ PCR Cloning Kit. Ligation mixtures were used to transform *E. coli* JM107 using TransformAid™ Bacterial Transformation Kit. Plasmids from positive transformants were purified and digested with restriction enzyme *Bgl*II to detect the presence of the inserts. Plasmids containing inserts of expected length (about 1150 bp) were sent for sequencing using pJET1.2.for and pJET1.2.rev primers. The consensus sequence of the CLOSPO_00312 gene was created by

alignment of three independent insert sequences (Appendix 8.10.3.). The analysis of the gene sequence showed the presence of an open reading frame encoding 377 amino acids (Appendix 8.10.4.). The primary structure of the CLOSPO_00312 dehydrogenase was analyzed in the Protein Conserved Domain Tool (Fig. 5.20.).

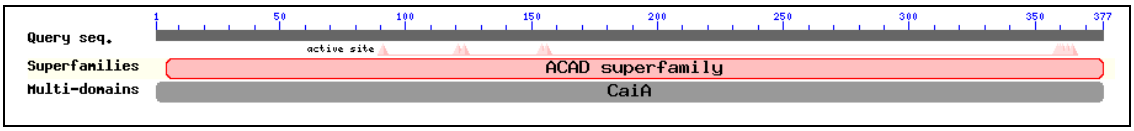


Fig. 5.20. Analysis of the CLOSPO_00312 acyl-CoA dehydrogenase primary structure in the Protein Conserved Domain Tool.

The analysis showed that CLOSPO_00312 had a structure characteristic to ACAD superfamily of acyl-CoA dehydrogenases accepting short chain acyl-CoA as substrates (Marchler-Bauer *et al.*, 2013). The reduced form of ACAD tends to be reoxidized in the oxidative half-reaction by electron-transferring flavoprotein, from which the electrons are transferred to the respiratory chain coupled with ATP synthesis.

The CLOSPO_00312 acyl-CoA dehydrogenase from *C. sporogenes* DSM795 showed 100% identity and 99% similarity to CLOSPO_00312 from *C. sporogenes* ATCC15579, 100% identity to the putative acyl-CoA dehydrogenase from *C. botulinum* strain Hall A and 67% identity and 82% similarity to CarC caffeyl-CoA reductase from *A. woodii* DSM1030.

Whilst it would be possible to express this gene, its enzymatic activity depends on co-expression with the electro transfer flavoproteins which was beyond the scope of this work.

5.1.5. Overexpression of *C. sporogenes* C=C double bond reductases

Biotransformations using whole cells and protein crude extracts of *C. sporogenes* showed that this bacterium contains unique enzyme activities. The analysis of the genomic sequences allowed for identification of genes encoding promising enzymes that may be useful for reduction of unsaturated compounds. Therefore, the fldZ enoate reductase and the OYE-like reductase were overexpressed in *E. coli* and their scope for reduction of enoates and nitroalkenes was tested.

5.1.5.1. Optimization of *E. coli* growth under anaerobic conditions

The presence of oxygen inactivated 2-enoate reductases from *C. kluyveri* and *C. tyrobutyricum*. The activity could be restored 3 h after the removal of oxygen (see 5.1.3.3; Tischer *et al.*, 1979). Attempts to overexpress clostridial 2-enoate reductases from *C. tyrobutyricum* and *C. thermoaceticum* (*Moorella thermoacetica*) using the *E. coli* expression

system in aerobic conditions resulted in a production of insoluble and inactive proteins (Rohdich *et al*., 2001) and it was possible that the *C. sporogenes* fldZ would behave similarly. Therefore, an attempt to overexpress *C. sporogenes* C=C double bond reductase in *E. coli* BL21(DE3)pLysS under anaerobic conditions was made. The conditions for anaerobic growth of *E. coli* K-12 (DSM5911, W3110, parental strain for BL21(DE3)pLysS) were optimized.

E. coli growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including electron acceptors such as oxygen, nitrate (Nouguchi *et al.*, 1997), fumarate (Spencer *et al.*, 1973) and dimethyl sulfoxide. The *E. coli* growth in complex and defined minimal media containing a variety of electron acceptors was tested in 96-well plate cultures and observed for 24 h using an automated NEPHELOstar plate reader (Fig. 5.21.).

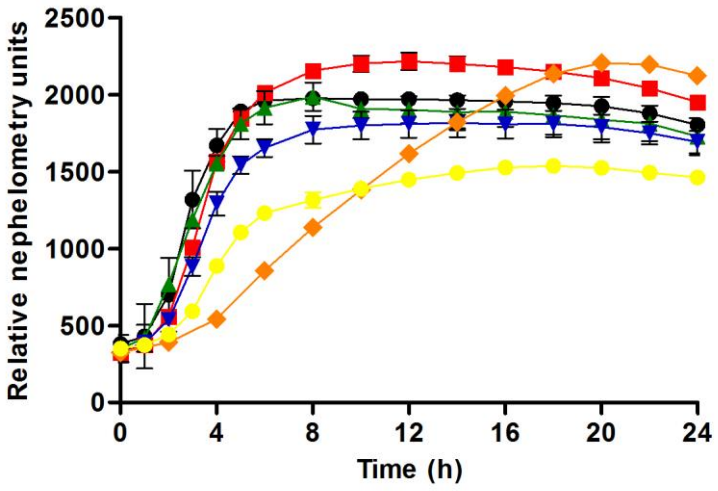


Fig. 5.21. Optimization of the *E. coli* K-12 growth under anaerobic conditions. The media were: Luria Bertani broth (●), Terrific Broth (■), Nouguchi medium (▲), sodium formate medium (▼), defined M9 medium (♦) and defined glycerol-fumarate GF medium (●). Average of five independent replicates.

The best growth (in case of the growth rate and biomass production) was observed for the Terrific Broth, which was the richest medium tested. The presence of nitrate (Nouguchi medium) or formate did not improve the growth when compared to a standard LB medium. Minimal defined media (M9 and glycerol-fumarate GF) were characterized by lower growth rates. However, the biomass production for M9 was the same as for the complex TB medium. Thus, TB medium was chosen as providing the best growth conditions and used in the subsequent experiments.

5.1.5.2. Overexpression and purification of the fldZ reductase

5.1.5.2.1. Expression of the fldZ reductase

The fldZ reductase was overexpressed in *E. coli* BL21(DE3)pLysS using the pET expression system. Considering previous limited attempts in expression of clostridial enoate reductases in *E. coli* (Rohdich *et al*., 2001) and significant differences in the codon usage between *E. coli* and *C. sporogenes*, the *fldZ* gene was optimized for expression using the GENEius sequence adaptation tool (http://www.eurofinsgenomics.eu/en/gene-synthesis-molecular-biology/geneius/sequence-optimisation.aspx) and the optimized *fldZ* gene was synthesized *de novo* (Eurofins MWG). The pUC57-Csp-fldZ-adapted plasmid carrying the optimized *fldZ* gene was used as the template in a PCR reaction. Two different sets of primers introducing different restriction sites in the products were used: PM017 and PM018 (introducing *Nde*I restriction site at the 5' end and *Not*I restriction site at the 3' end with a stop codon at the end of the open reading frame) and PM017 and PM019 (introducing *Nde*I restriction site at the 5' end and *Not*I restriction site at the 3' end without a stop codon at the end of the open reading frame). The PCR products were cloned into pJET1.2 vector and their sequences were verified by the DNA sequencing. Then, the inserts were excised using *Nde*I and *Not*I restriction enzymes and purified by electrophoresis. The pUC57-Csp-fldZ plasmid was also digested with *Nco*I and *Not*I enzymes and the insert was purified as well. These inserts were ligated into the pET20b(+) expression vector, previously digested with the enzymes corresponding to the inserts' sticky ends, dephosphorylated and purified. That allowed for creation of three expression constructs encoding the wild type fldZ, fldZ containing C-terminal HisTag (fldZ:CtH) and fldZ containing N-terminal pelB leader and C-terminal HisTag (fldZ:pelB). Moreover, the insert derived from the PCR with PM017 and PM019 primers was ligated into pET28b(+) vector. Then, the plasmid was digested with *Xba*I and *Not*I restrictases and transferred into pET20b(+) vector, previously linearized with the same enzymes. That created the fourth expression construct encoding fldZ reductase containing N-terminal HisTag (fldZ:NtH). The HisTag was introduced to make the purification easier using the HisTag bind resins, whereas the pelB leader was supposed to direct the protein product into the *E. coli* periplasm reducing its toxicity to the cells. The strains carrying the pET expression constructs were grown in a 100 mL scale and the plasmids were purified using the Plasmid Midi Kit. The plasmids were digested with *Xba*I and *Not*I restrictases and analyzed by gel electrophoresis to confirm the size of their inserts (Fig. 5.22, Table 5.4.). The expression constructs were also analyzed by DNA sequencing

using T7 promoter and T7 terminator primers (Appendix 8.11.) and used to transform BL21(DE3)pLysS expression strain.

Table 5.4. Comparison of the fldZ expression constructs. For the alignment of the protein sequences see Appendix 8.12.

Plasmid	Size of ORF (bp)	N-terminus	C-terminus	Predicted M_w of protein product (kDa)
fldZ	1998	-	-	73.02
fldZ:pelB	2097	pelB leader	HisTag	76.51
fldZ:NtH	2058	HisTag	-	75.19
fldZ:CtH	2031	-	HisTag	74.30

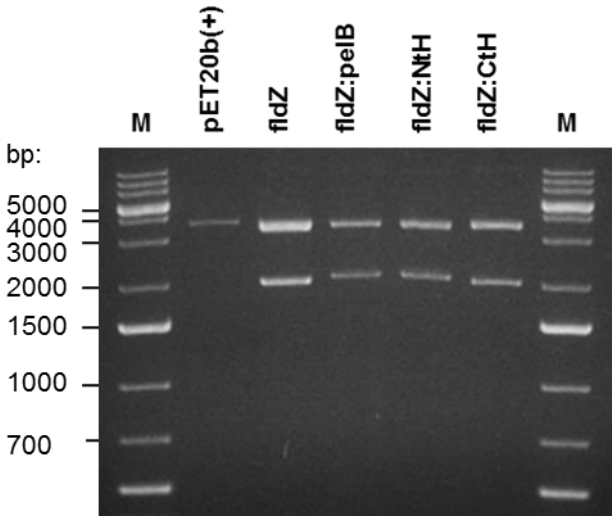


Fig. 5.22. Electrophoresis of pET20b(+) plasmids carrying fldZ inserts. The plasmids were digested with *Xba*I and *Not*I restriction enzymes. M - 1 kb Plus DNA Ladder (Fermentas).

Initially the wild type fldZ reductase was expressed at small scale (100 mL) in aerobic and anaerobic conditions at 30°C. The samples were taken before induction of the expression with IPTG and 1, 2, 3 and 5 h after induction. The protein extracts were prepared using the BugBuster reagent and the insoluble proteins were removed by centrifugation at 18000 x g for 20 min at 4°C. The protein extracts were analyzed using SDS-PAGE technique (Fig. 5.23.).

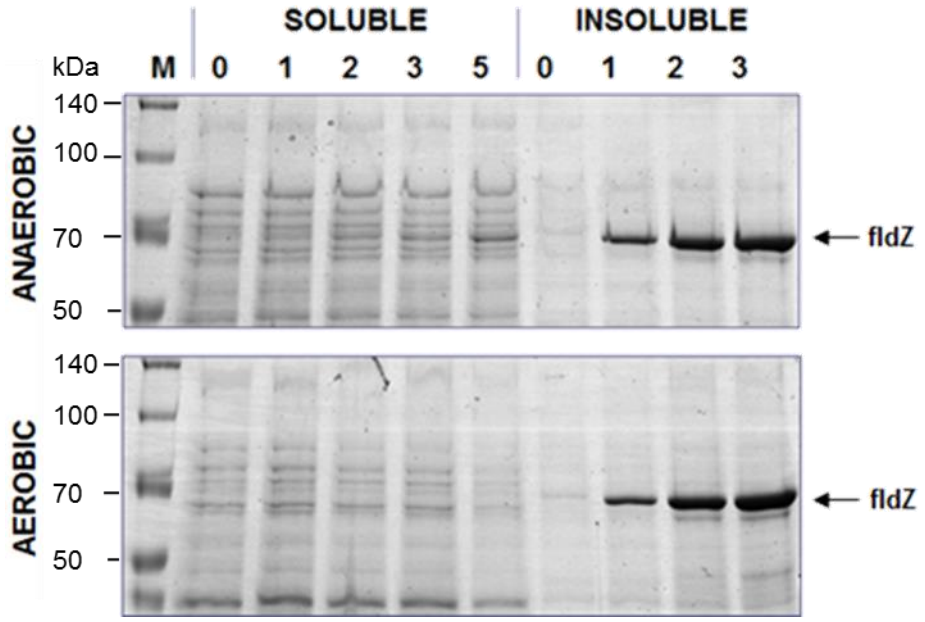


Fig. 5.23. Expression of the wild type fldZ reductase in aerobic and anaerobic conditions. M - PageRuler™ Prestained Protein Ladder (Fermentas).

Initial expression of reductase using untagged fldZ construct under anaerobic conditions resulted in formation of the soluble and insoluble products of an expected size about 73 kDa. In *E. coli* cells growing under aerobic conditions the *fldZ* gene was expressed as insoluble protein only.

Therefore fldZ, fldZ:pelB, fldZ:NtH and fldZ:CtH constructs were expressed in *E. coli* BL21(DE3)pLysS growing under anaerobic conditions. The cells were harvested 5 h after induction of the protein expression. The protein extracts were prepared with the BugBuster reagent and analysed by SDS-PAGE (5.24.).

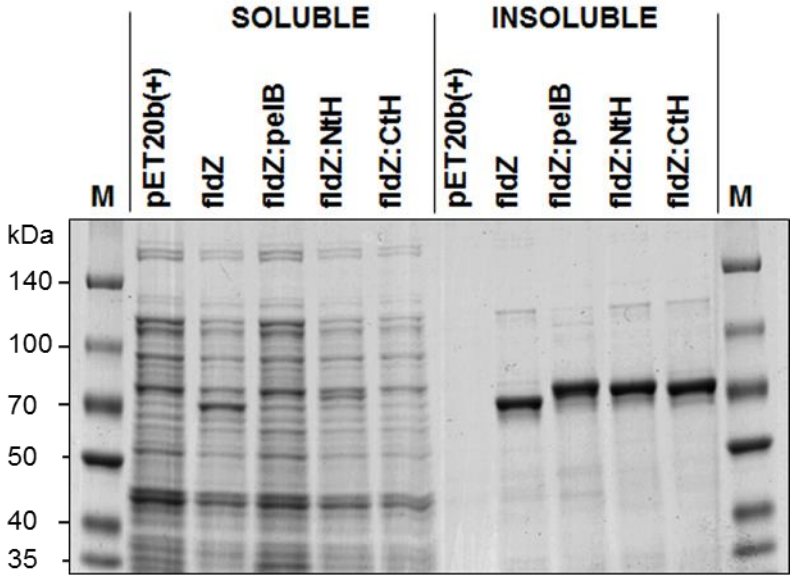


Fig. 5.24. Expression of fldZ, fldZ:pelB, fldZ:NtH and fldZ:CtH in anaerobic conditions. M - PageRuler™ Prestained Protein Ladder (Fermentas).

All constructs expressed the proteins of the expected molecular weight, but only the wild type fldZ and fldZ with the N-terminal HisTag were soluble. The activity of the protein extracts for reduction of cinnamic acid was tested in the spectrophotometric activity assay (Table 5.5.).

Table 5.5. Cinnamic acid reduction by protein extracts of fldZ, fldZ:pelB, fldZ:NtH and fldZ:CtH. For the example of spectral changes during the assay see Appendix 8.13.

Sample	Specific activity ($nmol \cdot s^{-1} \cdot mg^{1}$)	Relative activity
pET20b(+)	0.0	-
fldZ	6.3	100%
fldZ:pelB	0.0	-
fldZ:NtH	2.9	46%
fldZ:CtH	0.0	-

As expected the spectrophotometric assay showed the enoate reductase activity in protein extracts expressing fldZ and fldZ:NtH proteins. The specific activity for fldZ containing N-terminal HisTag was only a half of the wild type one. However, judging from the intensity of the fldZ:NtH band in the SDS-PAGE, the expression level of fldZ:NtH was also lower than the wild type and this probably caused decrease in specific activity. Alternatively, the presence of the N-terminal HisTag could cause structural changes in the enzyme and limit the activity of fldZ reductase. No reaction was observed in the control sample expressing an empty pET20b(+) and in fldZ:pelB and fldZ:CtH showing that only correctly folded, soluble fldZ reductase possessed the enzymatic activity.

5.1.5.2.2. Purification of fldZ-NtH using His-Bind Purification Kit

The fldZ reductase containing the N-terminal HisTag was successfully expressed as soluble and active protein. An attempt to purify fldZ to homogeneity using the Ni-NTA resin in column chromatography was made. An anaerobic culture of *E. coli* expressing fldZ:NtH (500 mL) was started from a pre-culture. Expression was induced by injection of IPTG when the culture reached an $OD_{660\ nm}$ of 0.3. The cells were harvested after 5 h at 30°C, centrifuged and washed with 50 mM phosphate buffer pH 7.0. After centrifugation the cells were resuspended in the protein extraction buffer (7 mL) containing tris(hydroxypropyl)phosphine as the reducing agent (final concentration 1 mM; compatible with the resin). The protein cell-free extract was produced using the cell disruptor at pressure 20,000 psi under nitrogen flow. The protein extract was centrifuged anaerobically at 75,000 x g for 20 min at 4°C and then transferred to the anaerobic cabinet. The soluble

protein fraction was loaded onto a polypropylene column containing the His·Bind resin and the column was let to drain under gravity flow in the cabinet. The resin was washed twice with the Binding buffer and one time with the Wash buffer. Then the protein was eluted with the elution buffer containing imidazole and the resin was stripped by applying the strip buffer. The protein fractions were analyzed by SDS-PAGE (Fig. 5.25.) and the enoate reductase activity was tested in the spectrophotometric assay (Table 5.6).

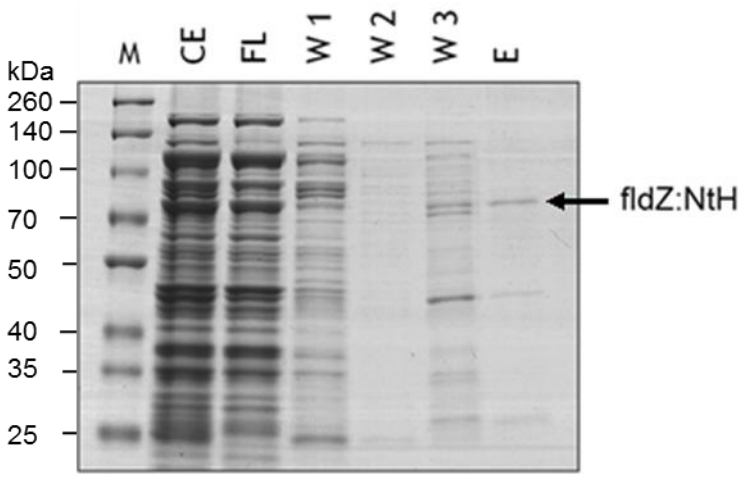


Fig. 5.25. Purification of fldZ:NtH using His-Bind Purification Kit. M - PageRuler™ Prestained Protein Ladder (Fermentas), CE - protein crude extract, FL - flow through, W1 - wash with Binding buffer, W2 - wash with Binding buffer, W3 - wash with Wash buffer, E - elution with the imidazole buffer.

Table 5.6. Results of the cinnamic acid reductase activity assay with the protein fractions from fldZ:NtH purification.

Protein fraction	Specific activity ($nmol \cdot s^{-1} \cdot mg^{-1}$)	Relative activity
Crude extract	5.1	100%
Flow through	0.0	-
Wash 1	0.0	-
Wash 2	0.0	-
Wash 3	0.0	-
Elution	0.0	-
Strip buffer	0.0	-

The SDS-PAGE analysis showed that fldZ:NtH was successfully purified, however the elution fraction was contaminated with two other proteins with lower molecular weights. No activity of the purified reductase was detected in the spectrophotometric assay.

The high concentration of imidazole in the elution buffer could have an inhibitory effect on the fldZ activity. To test that, the protein crude extract was mixed with the imidazole solution, but no decrease of the fldZ activity was observed in the assay (Table 5.7.).

Table 5.7. Attempts to restore the purified fldZ:NtH activity.

Protein fraction	Specific activity ($nmol \cdot s^{-1} \cdot mg^{-1}$)	Relative activity
Crude extract + Imidazole solution	5.1	100%
Elution + FAD/FMN	0.0	-
Elution + Flow through	0.0	-
Elution + 10% diluted Crude extract	0.4	8%

The purified reductase was also supplemented with the cofactors (FAD and FMN), but no reaction was detected. FldZ activity could also depend on other factors that were removed in the purification process. Thus, the purified fldZ:NtH was mixed with the flow through fraction and with diluted crude extract, but the activity of the reductase was not restored.

5.1.5.2.3. Purification of fldZ-NtH using Ni-NTA Spin Kit

The His-Bind Purification Kit used previously for purification of fldZ was not compatible with reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol. Thus, the loss of the enzymatic activity of the fldZ reductase could be a result of an insufficiently reducing environment during the purification process. Moreover, the His-Bind resin was sensitive to elevated temperatures, which did not allow pre-reducing it in the anaerobic cabinet before the chromatography and increased a risk to contaminate the reductase with oxygen. Thus, an attempt to purify the fldZ:NtH reductase with Ni-NTA Spin Kit (QIAGEN) was made. According to the manufacturer's note the Ni-NTA Spin Kit can be successfully used with up to 10 mM DTT. All buffers used in the purification process were autoclaved and cooled down under nitrogen flow in a sonicating water bath to ensure complete removal of oxygen. Then the buffers were transferred into the anaerobic cabinet, supplemented with DTT (2, 5 and 10 mM) and pre-reduced overnight.

E. coli cultures (100 mL) carrying pET20(+), fldZ and fldZ:NtH plasmids were grown under anaerobic conditions. The expression was induced with IPTG and the cells were harvested after 5 h at 30°C with shaking. The protein extracts were prepared in the extraction buffer containing the BugBuster reagent and different concentrations of DTT. The soluble protein fractions were purified using the Ni-NTA spin columns and analyzed by SDS-PAGE (Fig. 2.26.). The cinnamic acid reductase activity was tested in the spectrophotometric assay.

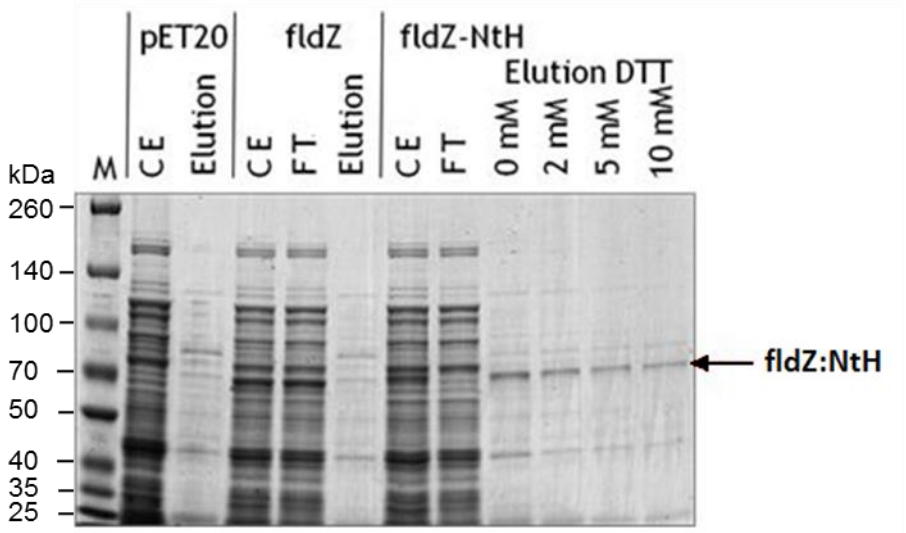


Fig. 5.26. Purification of fldZ:NtH using Ni-NTA Spin Kit. M - PageRuler™ Prestained Protein Ladder (Fermentas), CE - protein crude extract, FL - flow through, Elution - elution with the imidazole buffer containing DTT (from 0 to 10 mM).

The fldZ:NtH polypeptide was purified using the Ni-NTA spin columns. The highest yield of the purified protein was eluted when no DTT was present in the buffers. The higher reducing agent concentration the lower yields were observed, which might have been caused by DTT stripping the nickel ions from the resin and decreasing the binding capacity of the columns. The activity assay showed that none of the elution fractions was able to reduce cinnamic acid. Therefore, the loss of the fldZ activity was not a result of oxygen contamination or insufficient reducing environment during the purification process.

5.1.5.2.4. Purification of fldZ-NtH using Dynabeads His-Tag Isolation Kit

To elucidate the factor responsible for inactivation of the fldZ reductase during the HisTag purification process, the Dynabeads HisTag Isolation & Pulldown Kit was used. Dynabeads are magnetic beads covered with cobalt which guarantee highly specific interaction with histidine tag*s*. They allow elution of purified His-tagged proteins or the option to leave proteins on Dynabeads for use in the subsequent experiments. *E. coli* cultures (100 mL) were used for anaerobic overexpression of pET20b(+), fldZ and fldZ:NtH. The expression was induced with IPTG and the cells were harvested after 4 h at 30°C with shaking. The cells were harvested and re-suspended in the Dynabeads Binding Buffer supplemented with the BugBuster reagent and FAD/FMN. The soluble protein fractions (0.7 mL) were used for the purification process. The extracts were added to the polypropylene microcentrifuge vials containing the Dynabeads (50 µL), mixed and incubated for 5 min at 30°C. Then the tubes were placed on a magnet, the Dynabeads were collected on the wall and the supernatant was removed by pipetting. After four consecutive washing steps the protein was eluted with the Elution Buffer (100 µL). The protein fractions were analyzed by

SDS-PAGE (Fig. 5.27.). The activity of the crude extracts, crude extracts mixed with the beads and elution fractions were tested for the cinnamic acid reduction.

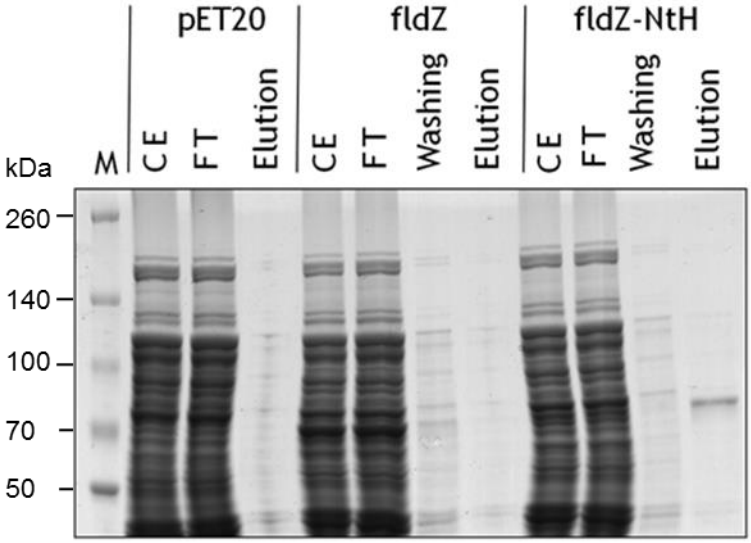


Fig. 5.27. Purification of fldZ:NtH using Dynabeads His-Tag Isolation Kit. M - PageRuler™ Prestained Protein Ladder (Fermentas), CE - protein crude extract, FL - flow through, Washing – washing with the Binding Buffer, Elution - elution with the buffer containing imidazole.

Using the Dynabeads the fldZ:NtH polypeptide was purified almost to homogeneity. However the purified protein did not show any enzymatic activity in the spectrophotometric assay. The wild type fldZ showed the reductase activity in the crude extracts. When the extract was mixed with the beads no change in activity was observed (fldZ + BEADS, Fig. 5.28.). Intriguing, when the beads were mixed with the fldZ:NtH crude extract, previously active fldZ:NtH crude extract immediately lost its activity (fldZ:NtH + BEADS).

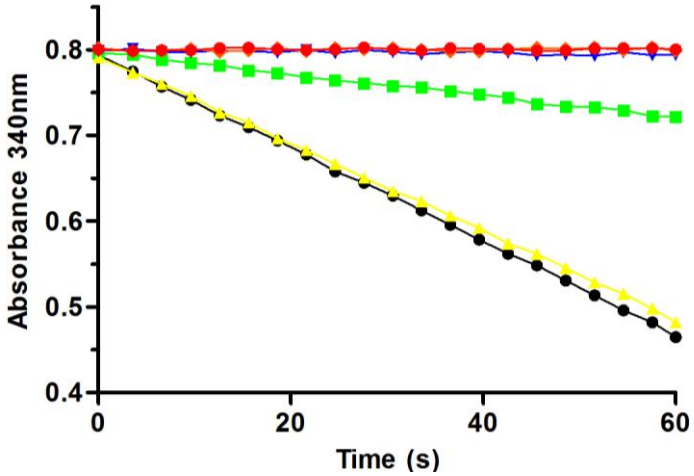


Fig. 5.28. Traces of the spectrophotometric cinnamic acid reduction assay using the protein fractions from purification with the Dynabeads His-Tag Isolation Kit. Protein fractions used in the assay: fldZ crude extract (●), fldZ:NtH crude extract (■), fldZ + BEADS (▲), fldZ:NtH + BEADS (▼), fldZ:NtH wash fraction (♦) and fldZ:NtH elution (●).

The presence of the Dynabeads inactivated the fldZ containing the N-terminal HisTag and did not affect the wild type fldZ reductase. This suggested that physical interaction between the fldZ:NtH and the histidine bind resin may cause the structural changes in the protein structure resulting in the loss of enzymatic activity.

5.1.5.2.5. Refolding of fldZ-NtH using Pierce Protein Refolding Kit

The enzymatic activity of inactive, misfolded or insoluble enzymes may be restored by a refolding process allowing the proteins to reach their native conformation. Thus, the fldZ:NtH reductase was purified for a large scale starting from a 500 mL *E. coli* culture using the His-Bind Purification Kit (as described in 5.1.5.2.2.). The N-terminal HisTag encoded by the expression construct was cleaved off by digestion with the thrombin endopeptidase for 3 h at 30°C. Then the fldZ was reconstituted in a matrix of refolding buffers (Pierce Protein Refolding Kit) containing FMN and FAD cofactors as well as different concentrations of denaturing agents such as guanidine and L-arginine and additives providing diverse redox environment (Table 5.8.). The refolding process was carried out for 5 and 24 h at room temperature with gentle shaking. The activity of the samples was tested in the cinnamic acid spectrophotometric assay.

Table 5.8. Results of the fldZ refolding using Pierce Protein Refolding Kit.

Buffer	Guanidine (M)	L-arginine (M)	Redox environment	Precipitation	Activity
1	0.0	0.0	5 mM DTT	+ (yellow)	0.0
2	0.0	0.4	2 mM GSH 0.2 mM GSSG	-	
3	0.0	0.8	2 mM GSH 0.4 mM GSSG	+ (white)	
4	0.5	0.0	2 mM GSH 0.2 mM GSSG	-	
5	0.5	0.4	2 mM GSH 0.4 mM GSSG	-	
6	0.5	0.8	5 mM DTT	+ (white)	
7	1.0	0.0	2 mM GSH 0.4 mM GSSG	-	
8	1.0	0.4	5 mM DTT	-	
9	1.0	0.8	2 mM GSH 0.2 mM GSSG	+ (white)	

The refolding process resulted in formation of precipitation in buffer 1 (yellow pellet) and 3, 6 and 9 (white pellets). No activity was observed in the cinnamic acid activity

assay for any of the samples. This may suggest that denaturing conditions used in the experiment were not sufficient to restore the active conformation of the enzyme. It is also possible that during the purification process the enzyme was deprived of the cofactors. The refolding buffers were supplemented with FMN and FAD. However, they did not contained agents which could allow reconstitution of the iron-sulphur cluster. Thus the fldZ reductase was not successfully restored.

5.1.5.2.6. Temperature and pH optimum of the fldZ reductase

The attempts to purify recombinant polyhistidine-tagged fldZ expressed in *E. coli* using the affinity purification procedures were unsuccessful. Therefore, the fldZ reductase was characterized using the protein extracts of *E. coli* expressing the wild type enzyme. A large scale culture of *E. coli* (1 L) expressing fldZ was grown under anaerobic conditions. The expression of the recombinant protein was induced with IPTG and the cells were harvested after 5 h at 30°C. The protein cell-free extract (7 mL) was prepared using the cell disrupter at the pressure of 20,000 psi. The sample was centrifuged at 75,000 x g for 30 min at 4°C and the soluble protein fraction was used in the activity assays and biotransformations. The extract was stored for 6 months in a cryogenic vessel containing liquid nitrogen without losing activity.

The activity of fldZ was tested in the cinnamic acid spectrophotometric assay at a range of temperatures (Fig. 5.29.; traces in Appendix 8.14.). The highest reaction rate was observed at 40-45°C. The higher temperature caused a gradual decrease in fldZ activity to completely inactivate the enzyme at 60°C.

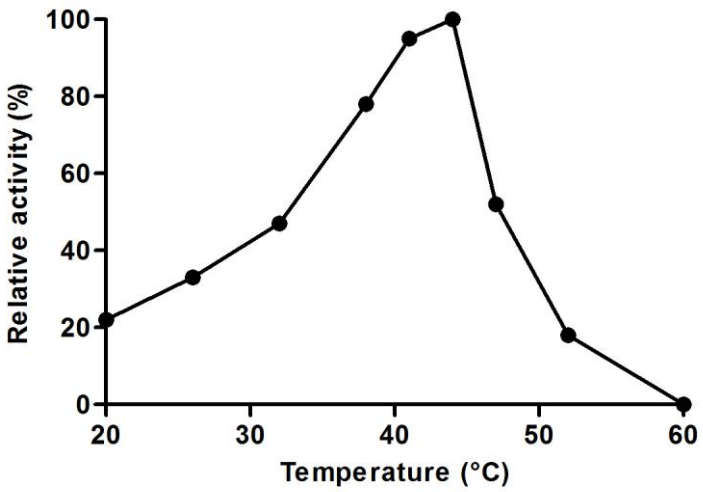


Fig. 5.29. The temperature profile of fldZ activity in the cinnamic acid spectrophotometric assay.

The activity tests were also performed at a range of pH varying from 4.5 to 8 in 50 mM potassium phosphate buffer (Fig. 5.30; traces in Appendix 8.14.).

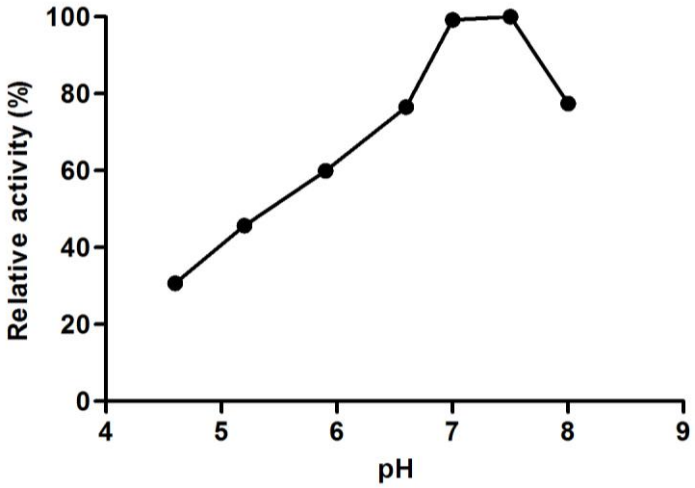


Fig. 5.30. The pH profile of fldZ activity in the cinnamic acid spectrophotometric assay.

It was found that the optimum pH for the fldZ activity was 7.0-7.5. These values differed from the ones published for the enoate reductase purified from *C. kluyveri* (optimum pH 6.0 in sodium citrate buffer and temperature 55°C; Tischer *et al.*, 1979).

5.1.5.2.7. Biotransformation of cinnamic acid by fldZ reductase

The *E. coli* protein extracts containing fldZ reductase were used to reduce cinnamic acid at the expense of NADH in anaerobic biotransformations. The reactions were performed in phosphate buffer pH 7.0 at 40°C. The samples of the supernatant were collected at intervals from 15 min to 7 h and after overnight incubation. The samples were derivatized with (trimethylsilyl)diazomethane and the substrate and product concentrations were determined by gas chromatography-mass spectroscopy (Fig. 5.31; for chromatograms and mass spectra of reaction products see Appendix 8.15.).

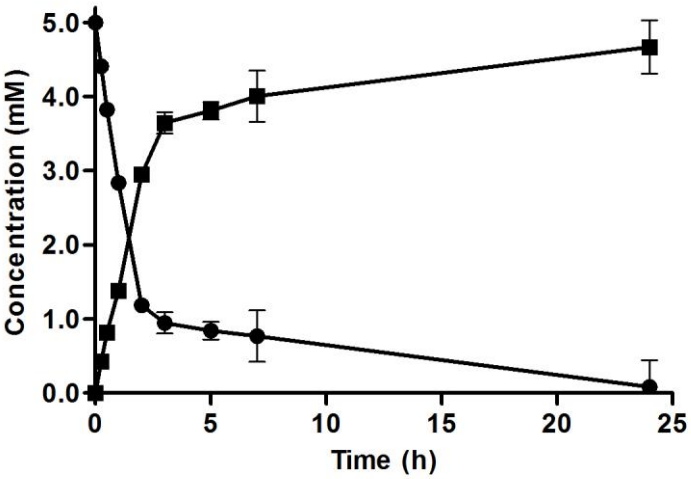


Fig. 5.31. Biotransformation of cinnamic acid in the expense of NADH using the crude extracts of *E. coli* expressing fldZ reductase (20 mg). Conversion of cinnamic acid (●) and formation of 3-phenylpropionic acid (■) were determined by gas chromatography-mass spectroscopy.

Cinnamic acid was reduced by protein extracts of *E. coli* expressing the wild type fldZ with almost 100% yield after overnight incubation. No 3-phenylpropionic acid formation was observed in the control experiments with *E. coli* expressing empty pET20b(+) (see Appendix 8.15). The biotransformations confirmed the results of the previous spectrophotometric activity assays and showed that fldZ was expressed as an active protein possessing C=C double bond reductase activity towards cinnamic acid.

5.1.5.2.8. Inhibition of the fldZ activity with oxygen

The test the inhibition of the fldZ reductase by the presence of oxygen, the protein extract of *E. coli* expressing the wild type enzyme was exposed to air for 15 min - 3 h. To avoid heat inactivation of the enzyme, the samples were kept in an ice bath throughout the exposure time. As a control fldZ incubated under anaerobic conditions at 4°C was used to test the possible loss of enzymatic activity in time. After the exposure to oxygen the samples were transferred into glass vials closed with suba seals and the headspace above the samples was flushed with nitrogen for 1 h. Then the protein extracts were used as biocatalysts in the cinnamic acid reduction biotransformation using NADH as the electron donor. The reactions were incubated for 2 h at 40°C. The biotransformation products were derivatized and analyzed using gas chromatography-mass spectroscopy (Fig. 5.32.).

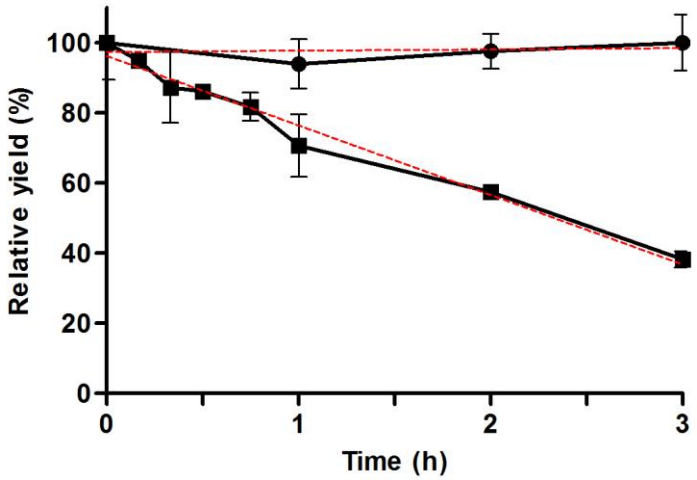


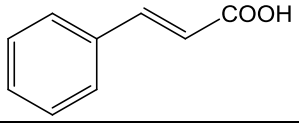
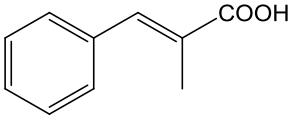
Fig. 5.32. Inactivation of the fldZ reductase by oxygen. The yields of the biotransformations were normalized to the yield of the control sample (fldZ not exposed to oxygen). Protein extracts exposed (■) and not exposed (●) to oxygen.

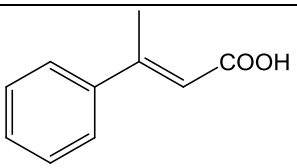
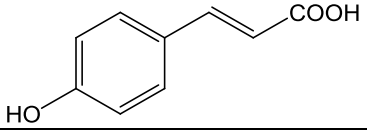
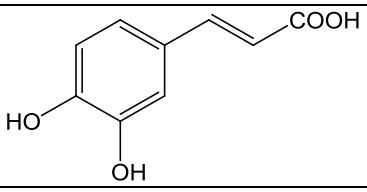
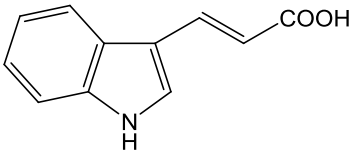
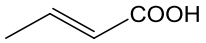
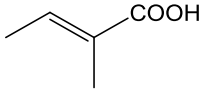
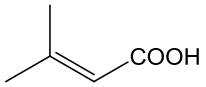
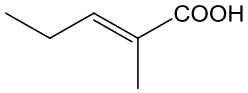
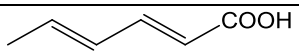
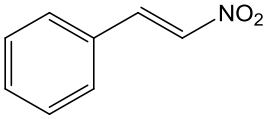
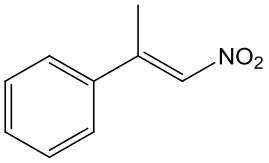
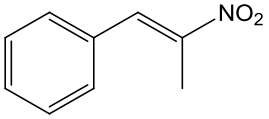
The biotransformations showed that the presence of oxygen had an inhibitory effect on the fldZ reductase activity. The reductase lost about a half of the activity after 2 h of exposure to oxygen. The activity could not be restored by 1 h incubation in anaerobic conditions.

5.1.5.2.9. Substrate range of fldZ

The fldZ reductase was tested for NADH oxidation in the presence of unsaturated aromatic and aliphatic carboxylic acids and phenylnitroalkenes. The activity was measured using the spectrophotometric assay (see 4.2.8.6.). Reactions containing the protein extracts of *E. coli* expressing the empty pET20b(+) were used as controls to subtract the background activity that was present in *E. coli*. Because of poor solubility in water of some chemicals tested, the stock solutions of all the substrates were prepared in tetrahydrofuran (THF) and diluted to give 1 mM concentration in the reaction mixtures (5% tetrahydrofuran final concentration v/v). THF was successfully used as a co-solvent in biotransformations using PETN reductase from *Enterobacter cloacae* (Dr. Fryszkowska, personal communication).

Table 5.9. Substrate range of the fldZ reductase

Substrate	Structure	Activity ($nmol \cdot s^{-1} \cdot mg^{-1}$)	Relative activity
cinnamic acid		8.3	100%
α-methyl-cinnamic acid		<0.05	<0.7%

β-methyl-cinnamic acid		0.13	1.6%
p-coumaric acid		3.64	43.3%
caffeic acid		0.07	0.8%
3-indoleacrylic acid		<0.04	< 0.5%
crotonic acid		0.0	-
2,3-dimethyl-acrylic acid		0.0	-
3,3-dimethyla-crylic acid		0.0	-
2-methyl-2-pentenoic acid		0.0	-
sorbic acid		0.0	-
β-nitrostyrene		0.0	-
(*E*)-1-nitro-2-phenylpropene		<0.07	0.8%
(E)-1-phenyl-2-nitropropene		<0.03	<0.4%

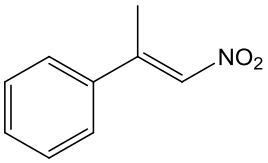
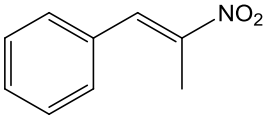
The activity tests showed that fldZ possessed a very narrow substrate range. The highest activity was observed for cinnamic acid. The presence of methyl groups in the alpha or beta positions in cinnamic acid drastically decreased the activity of the reductase to about 1%. *p*-Coumaric acid was a relatively good substrate (43% activity when compared to cinnamic acid). However, further alternation in the aromatic ring (caffeic acid and indoleacrylic acid) caused a significant decrease in the activity. FldZ did not accept aliphatic enoates at all, what may suggest that the aromatic ring plays a crucial role in the interaction

between the enzyme and the substrate. No activity was observed towards β-nitrostyrene, whereas phenylnitropropenes were poor substrates when compared to cinnamic acid (relative activity lower than 1%).

5.1.5.2.10. Reduction of phenylnitropropenes using fldZ

The protein extracts produced as described above were also used to measure reduction of two phenylnitropropene isomers *(E)*-1-nitro-2-phenylpropene and *(E)*-2-nitro-1-phenylpropene in the biphasic biotransformation. In the reaction NADH was used as the electron donor. The samples were incubated at 40°C and the yield of reactions was determined after 24, 48 and 72 h. The products were analyzed using chiral HPLC (Table 5.10).

Table 5.10. Reduction of phenylnitropropenes using the fldZ reductase.

Substrate	Sample	Yield (%)			Ee. (%)		
		24 h	48 h	72 h	24 h	48 h	72 h
 (*E*)-1-nitro-2-phenylpropene	fldZ	72.0 ±4.5	81.0 ±3.3	90.1 ±3.8	≥99 *(R)*	≥99 *(R)*	≥99 *(R)*
	pET20b(+)	6.3 ±0.1	7.5 ±0.2	7.7 ±0.4	54.8 ±4.5 *(R)*	52.6 ±0.6 *(R)*	51.4 ±9.1 *(R)*
 (*E)*-1-phenyl-2-nitropropene	fldZ	35.2 ±0.3	48.9 ±0.4	56.0 ±1.9	17.0 ±0.5 *(R)*	16.1 ±1.6 *(R)*	16.9 ±0.2 *(R)*
	pET20b(+)	10.2 ±4.2	17.2 ±2.8	17.0 ±0.6	16.8 ±5.5 *(R)*	17.2 ±1.6 *(R)*	20.3 ±2.3 *(R)*

The biotransformations showed that the wild type *E. coli* reduced both (*E*)-1-nitro-2-phenylpropene and (*E)*-1-phenyl-2-nitropropene with poor yields (7 and 17% after 72 h respectively) and moderate enantiomeric excess (51 and 20%). The expression of fldZ significantly improved the yields of biotransformations (90 and 56% respectively). Surprisingly, the enantiomeric excess for the product of (*E)*-1-phenyl-2-nitropropene reduction was ≥99% for *(R)*-enantiomer. No *(S)*-enantiomer was detected in the reaction mixture as a product of the background activity of *E. coli* enzymes shown in the control experiment. This may be caused by a limitation of the analytical method, where the *(S)*-product could not be detected at low concentrations in the presence of an excess of the

(R)-enantiomer. Therefore, it was shown that fldZ accepted both phenylnitropropenes as substrates in the reduction driven by NADH and the reductase demonstrated perfect enantioselectivity towards (*E)*-1-phenyl-2-nitropropene.

5.1.5.3. Overexpression and purification of OYE-like reductase

Old Yellow Enzyme-like reductase (OYE, CLOSPO_03444) was overexpressed in *E. coli* BL21(DE3)pLysS using the pET expression system. The pJET1.2 cloning plasmid carrying the nucleotide sequence encoding the open reading frame of OYE-like reductase (see 5.1.4.2.) was used as the template in a PCR with KOD polymerase. Two different sets of primers introducing different restriction sites in the products were used: PM020 and PM021 (introducing *Nde*I restriction site at the 5' end and *Not*I restriction site at the 3' end without the stop codon at the end of the open reading frame) and PM020 and PM021 (introducing *Nde*I restriction site at the 5' end and *Not*I restriction site at the 3' end without a stop codon at the end of the open reading frame). The PCR products were cloned into pJET1.2 vector and their sequences were verified by the DNA sequencing. Then, the inserts were excised using *Nde*I and *Not*I restrictases and purified by electrophoresis. These inserts were ligated into the pET20b(+) expression vector, previously digested with the *Nde*I and *Not*I, dephosphorylated and purified. That allowed creation of two constructs for the expression of the wild type OYE-like reductase and OYE containing a C-terminal HisTag (OYE:CtH).The C-terminal HisTag was used since other OYE-like reductases (such as PETNR from *Enterobacter cloacae*) were successfully overexpressed and purified using this strategy (Hulley *et al.*, 2010). The strains carrying the pET expression constructs were grown at a 100 mL scale and the plasmids were purified using the Plasmid Midi Kit. The plasmids were analyzed by DNA sequencing using T7 promoter and T7 terminator primers (Appendix 8.16.) and used to transform BL21(DE3)pLysS expression strain.

The wild type OYE and OYE:CtH reductases were expressed at a 100 mL scale in anaerobic conditions at 30°C. Samples were taken before induction of the expression with IPTG and 1, 3 and 5 h after induction. The cell-free protein extracts were prepared using the BugBuster reagent and the insoluble proteins were removed by centrifugation at 18000 x g for 20 min at 4°C. The protein extracts were analyzed using SDS-PAGE technique (Fig. 5.33.).

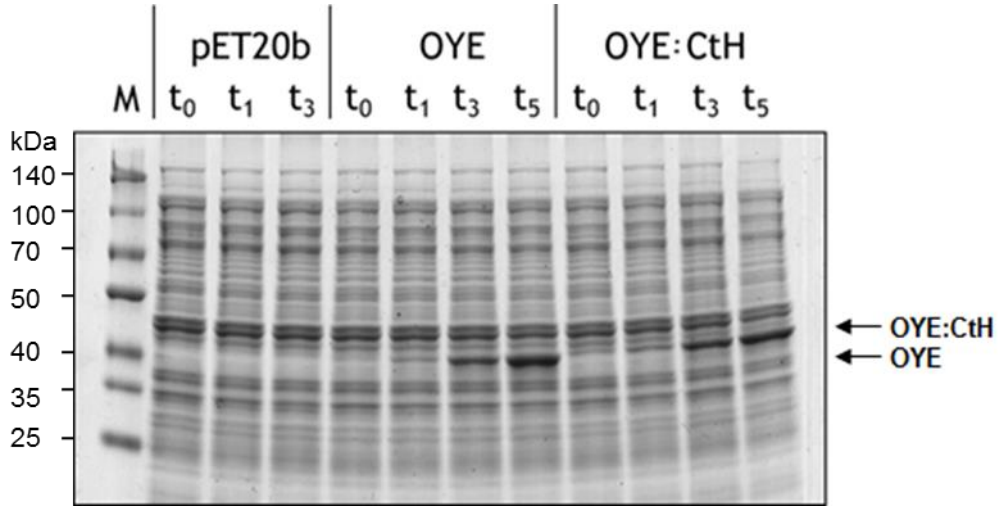


Fig. 5.33. Expression of the wild type OYE reductase and His-tagged OYE in anaerobic conditions. M - PageRuler™ Prestained Protein Ladder (Fermentas).

Expression of OYE and OYE:CtH in anaerobic conditions resulted in formation of soluble products of an expected size about 38 and 40 kDa respectively. Since the OYE reductase containing the C-terminal HisTag was successfully expressed as soluble protein, an attempt to purify the enzyme using the Dynabeads His-Tag Isolation Kit was made as described previously (5.1.5.2.4.). The protein fractions were analyzed by SDS-PAGE (Fig. 5.34.).

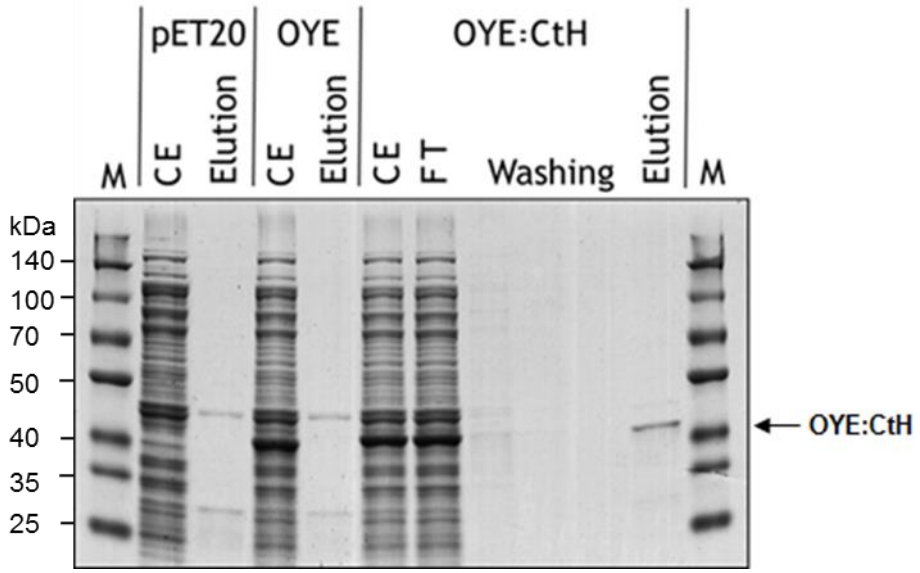


Fig. 5.34. Purification of OYE:CtH using Dynabeads His-Tag Isolation Kit. M - PageRuler™ Prestained Protein Ladder (Fermentas), CE - protein crude extract, FL - flow through, Washing - washing with the Binding Buffer, Elution - elution with the buffer containing imidazole.

The SDS-PAGE analysis showed that OYE:CtH polypeptide was successfully purified to homogeneity. The purified reductase was tested in the spectrophotometric activity

assays using a range of unsaturated compounds as substrates (cinnamic acid, β-nitrostyrene, *(E)*-1-nitro-2-phenylpropene, *(E)*-2-nitro-1-phenylpropene and cyclohexenone). The reactions were driven by NADH or NADPH. No conversion of the electron donors was observed in the reactions (data not shown). Therefore, the substrate range of the OYE-like reductase encoded by *C. sporogenes* remained unknown.

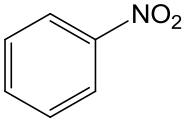
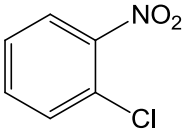
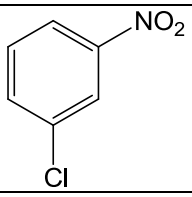
5.2. Reduction of aromatic nitro groups by *C. sporogenes*

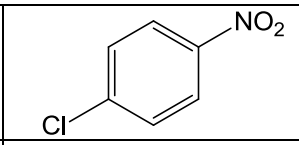
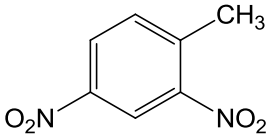
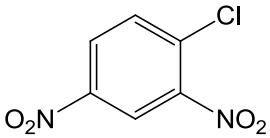
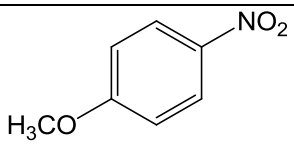
C. sporogenes was found to catalyse reduction of aliphatic (Angermaier and Simon, 1983a) and aromatic nitro groups (Dipeolu, 2008; Dipeolu *et al*., 2009). Attempts to purify the reductase responsible for NADH-dependent hydrogenation of the nitro group of *p*-nitrobenzoate showed the presence of two different enzymatic activities in the protein extracts of *C. sporogenes* (Angermaier and Simon, 1983b). However, the *C. sporogenes* nitroreductase has never been successfully purified and identified. Therefore, an attempt was made to identify and overexpress the nitroreductases present in *C. sporogenes* DSM795.

5.2.1. Reduction of aromatic nitro compounds using *C. sporogenes* crude extracts

Protein extracts of *C. sporogenes* DSM795 were used in biotransformations of aromatic nitro compounds to confirm the presence of nitroreductase activity. The culture used for preparation of protein extracts was grown in Giesel medium supplemented with 12.1 mM L-phenylalanine. The cells were harvested in the early stationary phase of growth and disrupted using the cell disruptor at pressure of 40,000 psi under nitrogen flow. The cell debris and insoluble proteins were separated from the soluble protein fraction by centrifugation at 75,000 x g for 30 min at 4°C. A small library of aromatic nitro compounds was tested as substrates for the reductase activity. In the reactions NADH and NADPH were used as the electron donors. The biphasic heptane/buffer biotransformations were prepared in anaerobic conditions and incubated for 24 h at 30°C. The reaction products were analyzed by gas chromatography-mass spectroscopy (Table 5.11.).

Table 5.11. Reduction of aromatic nitro compounds to amines using protein extracts of *C. sporogenes*. Results present means of three independent replicates with standard deviations.

Substrate	Structure	NADH		NADPH	
		Conv. (%)	Yield (%)	Conv. (%)	Yield (%)
nitrobenzene		86.0 ±1.8	6.5 ±0.8	80.4 ±5.8	4.1 ±0.5
2-chloro-nitrobenzene		60.5 ±3.8	1.8 ±0.1	55.8 ±1.6	1.2 ±0.1
3-chloro-nitrobenzene		65.7 ±3.8	5.9 ±0.2	60.8 ±5.7	4.5 ±0.2

4-chloro-nitrobenzene		50.4 ±2.5	17.2 ±0.9	43.3 ±1.7	8.5 ±0.3
2,4-dinitro-toluene		100.0	67.8[1)] ±7.3	100.0	99.3[1)] ±1.2
1-chloro-2,4-dinitrobenzene		100.0	55.8[2)] ±5.2	100.0	64.7[2)] ±5.1
p-nitroanisole		70.8 ±5.6	33.5 ±1.3	68.9 ±4.8	34.0 ±2.4

[1)]. yield for 2,4-diaminotoluene; [2)]. Yield for 4-chloro-1,3-diaminobenzene.

C. sporogenes DSM795 reduced aromatic nitro group with different yields depending on the substrate type and the electron donor used. The best substrates, 2,4-dinitro-toluene and 1-chloro-2,4-dinitrobenzene, contained two nitro groups attached to the aromatic ring and gave higher yields when NADPH was used. The products of their reduction were 2,4-diaminotoluene and 4-chloro-1,3-diaminobenzene respectively. *p*-Nitroanisole, the only substrate containing an electron donating group, was reduced with moderate yield about 34% for both NADH and NADPH. Nitrobenzene and chloro-nitrobenzenes were poor substrates giving higher yields when NADH was present in the reaction. The biotransformations confirmed that protein extracts of *C. sporogenes* DSM795 contain the nitroreductase activity. That activity may result from the presence of multiple enzymes preferring NADH or NADPH as the electron donor in the catalytic process.

5.2.2. Identification of nitroreductases in *C. sporogenes* genome

To identify nitroreductases, the genome of *C. sporogenes* ATCC15579 strain was screened for the presence of genes encoding proteins which show homology to nitroreductases known from the literature. Three protein sequences were used as a query: NRSal reductase from *Salmonella typhimurium*, NR reductase from *Enterobacter cloacae* and YqjM from *Bacillus subtilis*. Seven hypothetical genes were found which encoded unknown proteins annotated as hypothetical proteins: CLOSPO_01010, CLOSPO_ 01559, CLOSPO_01572, CLOSPO_01641, CLOSPO_01855, CLOSPO_02936 and CLOSPO_03559. The genes were named as *Cs*NTR from 1 to 7 respectively. The gene sequences were extracted from the database (Appendix 8.17.) and used to identify corresponding genes in the genomes of *C. sporogenes* NCIMB10696 and *C. botulinum* A str. Hall ATCC3502. The

sequence alignments were prepared for each gene and PCR primers were designed to the fragments of the genes conserved between different *Clostridium* strains (Table 5.12.).

Table 5.12. Hypothetical genes encoding nitroreductases identified in the genome of *C. sporogenes* ATCC15579. The primers used for identification of the genes in *C. sporogenes* DSM795 are listed.

Enzyme	Gene name	Size of ORF (bp)	Primers used for amplification	Expected PCR product (bp)
*Cs*NTR1	CLOSPO_01010	579	PM023, PM024	820
*Cs*NTR2	CLOSPO_ 01559	627	PM025, PM026	1500
*Cs*NTR3	CLOSPO_01572	516	PM027, PM028	605
*Cs*NTR4	CLOSPO_01641	564	PM029, PM030	849
*Cs*NTR5	CLOSPO_01855	546	PM031, PM032	850
*Cs*NTR6	CLOSPO_02936	498	PM033, PM034	624
*Cs*NTR7	CLOSPO_03559	549	PM035, PM036	601

Primers were used in PCR on *C. sporogenes* DSM795 genomic DNA using the FailSafe Polymerase system containing different premixes for optimization of the reaction. The reaction mixtures were pooled and the products of reactions were analyzed by gel electrophoresis (Fig. 5.35.).

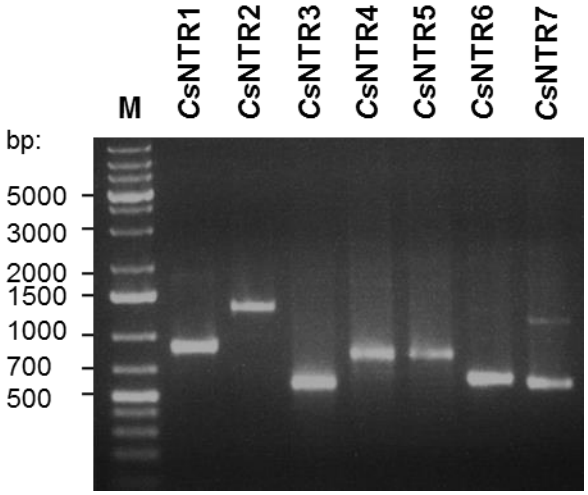


Fig. 5.35. Electrophoresis of *Cs*NTR1-7 PCR products. Products (10 µL of each) were run on 1% agarose gel with GeneRuler™ 1 kb Plus DNA Ladder (Fermentas).

The electrophoresis showed that PCR resulted in products of the expected length for each gene. The products were purified by gel electrophoresis, ligated into pJET1.2 vector and analyzed by DNA sequencing. The analysis showed that six of the PCR products contained genes with high homology to genes encoding nitroreductases. The PCR product for identification of *Cs*NTR2 did not show the presence of an open reading frame encoding nitroreductase-like protein. Thus it was assumed that this product is a result of an

unspecific PCR and that *C. sporogenes* DSM795 strain does not contain the gene encoding *Cs*NTR2 reductase. The analysis of the sequences showed the *Cs*NTR1 encoded an open reading frame of 179 aa., *Cs*NTR3 - 172 aa., *Cs*NTR4 - 173 aa., *Cs*NTR5 - 182 aa., *Cs*NTR6 - 166 aa. and *Cs*NTR7 - 183 aa. The primary structures of *C. sporogenes* nitroreductases were analyzed by the Protein Conserved Domain Tool (Fig. 5.36.).

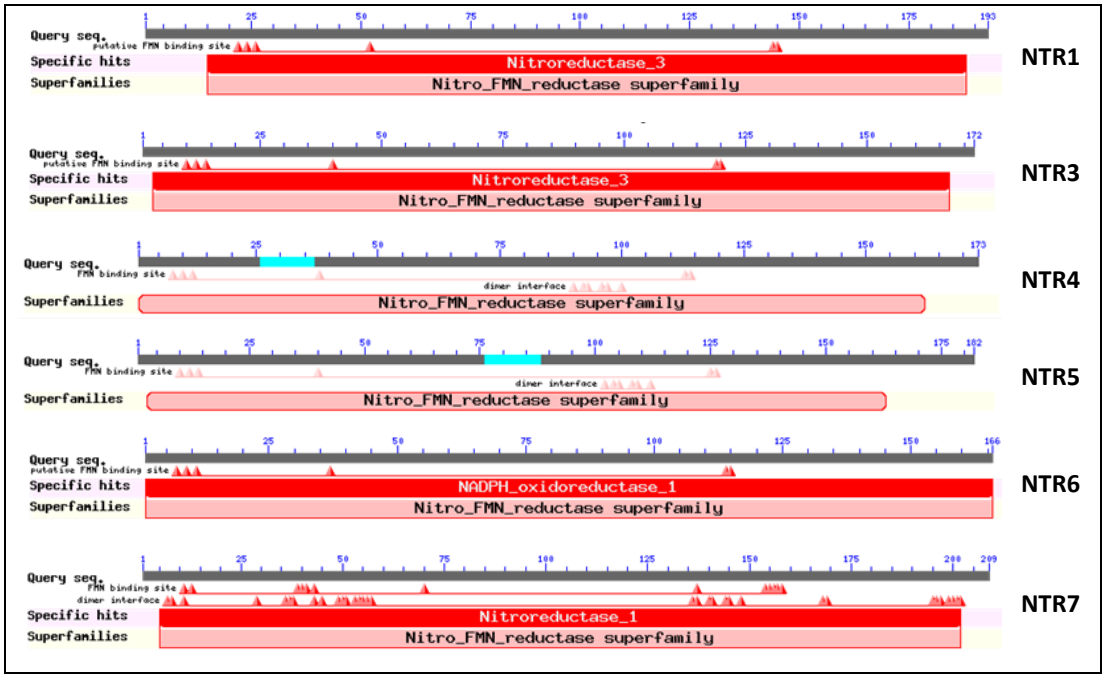


Fig. 5.36. Analysis of the *C. sporogenes* nitroreductases primary structures in the Protein Conserved Domain Tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?).

The analysis of the primary structure showed that all *Cs*NTR proteins possessed domains characteristic for enzymes reducing nitrocompounds (at the expense of NADH or NADPH) and containing FMN or FAD as cofactors.

5.2.3. Overexpression and activity of *C. sporogenes* nitroreductases

The gene sequences of *Cs*NTRs were optimized for expression in the *E. coli* host (Appendix 8.17.) and synthetic plasmids carrying DNA inserts encoding the optimized nitroreductase genes were created by Biomatik, USA, LLC. The plasmids were digested using *Nde*I and *Not*I restriction enzymes and the inserts were transferred into pET20b(+) expression vector. The strains carrying the pET expression constructs were grown at a 100 mL scale and the plasmids were purified using the Plasmid Midi Kit. The plasmids were sequenced using T7 promoter and T7 terminator primers and used to transform BL21(DE3)pLysS expression strain. The nitroreductases were expressed at 100 mL scale in anaerobic conditions at 30°C. Additionally, *C. sporogenes* OYE-like reductase was expressed to be tested for nitroreductase activity. The cells were harvested 5 h after induction of

expression. The protein extracts were prepared using the BugBuster reagent and the insoluble proteins were removed by centrifugation at 18000 x g for 20 min at 4°C. The protein extracts were analyzed using SDS-PAGE technique (Fig. 5.37.).

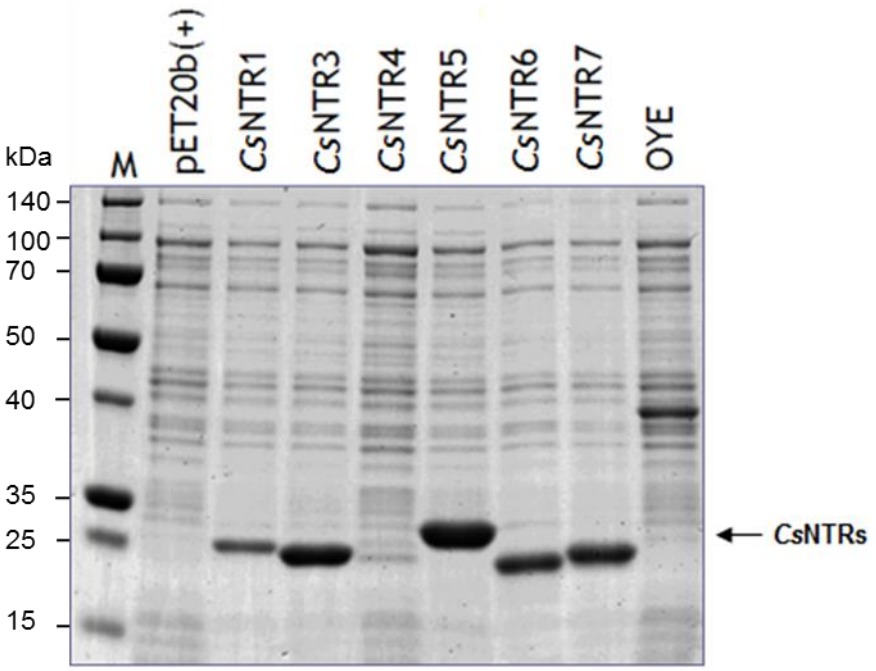
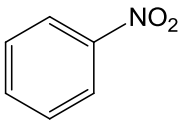
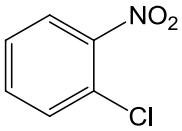
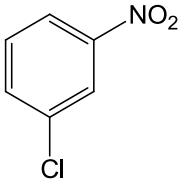
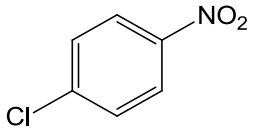
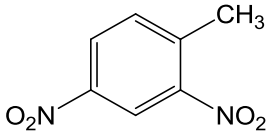
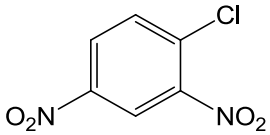


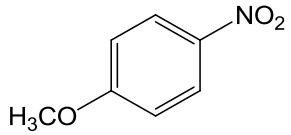
Fig. 5.37. Expression of the nitroreductases *Cs*NTR1-7 and the OYE-like reductase in *E. coli* in anaerobic conditions. M - PageRuler™ Prestained Protein Ladder (Fermentas).

The SDS-PAGE showed that all the *C. sporogenes* nitroreductases were successfully expressed as soluble proteins with very high yields, except for *Cs*NTR4, which was mainly insoluble (data not shown). An attempt to optimize *Cs*NTR4 expression by lowering the temperature of the culture and extending the incubation time before harvesting the cells did not improve the yield of protein produced (data not shown).

To test the activity of nitroreductases for reduction of aromatic nitro compounds, protein cell-free extracts of *E. coli* expressing empty pET20b(+) and *Cs*NTR constructs were used as biocatalysts in the biphasic aromatic nitro reduction biotransformation. In the reactions NADH and NADPH were used as the electron donors. The reactions were incubated for 24 h at 30°C. The conversion of the substrates and formation of the products were determined by gas chromatography-mass spectroscopy (Tab. 5.13.).

Table 5.13. Aromatic nitro reduction biotransformation using *Cs*NTR nitroreductases and OYE-like reductase from *C. sporogenes*. Results present means of three independent replicates.

Substrate	Sample	Conv. (%)		Yield (%)	
		NADH	NADPH	NADH	NADPH
 nitrobenzene	pET20b(+)	31.7	46.6	1.2	1.4
	*Cs*NTR1	29.4	55.7	0.1	0.3
	*Cs*NTR3	29.6	51.9	1.0	1.0
	*Cs*NTR4	31.4	49.2	2.0	1.5
	*Cs*NTR5	27.3	44.7	0.6	0.6
	*Cs*NTR6	25.2	50.2	0.2	0.2
	*Cs*NTR7	22.6	47.0	0.2	0.3
	OYE	32.1	46.9	1.6	1.5
 2-chloro-nitrobenzene	pET20b(+)	26.5	40.5	2.2	1.1
	*Cs*NTR1	25.9	40.3	0.5	0.2
	*Cs*NTR3	20.8	28.7	0.9	0.9
	*Cs*NTR4	19.9	29.6	1.7	1.2
	*Cs*NTR5	18.7	31.4	0.5	0.4
	*Cs*NTR6	18.4	31.6	0.3	0.2
	*Cs*NTR7	22.6	34.0	0.3	0.6
	OYE	25.2	33.9	1.5	1.2
 3-chloro-nitrobenzene	pET20b(+)	30.5	46.7	4.6	3.6
	*Cs*NTR1	27.0	41.7	1.7	1.9
	*Cs*NTR3	31.9	40.5	2.3	2.7
	*Cs*NTR4	28.8	45.0	3.9	4.0
	*Cs*NTR5	22.8	43.6	2.1	2.2
	*Cs*NTR6	25.1	35.2	2.1	2.2
	*Cs*NTR7	24.5	38.2	2.0	2.4
	OYE	31.7	43.5	3.5	3.9
 4-chloro-nitrobenzene	pET20b(+)	38.5	51.2	1.3	1.1
	*Cs*NTR1	35.9	50.1	0.1	0.1
	*Cs*NTR3	38.9	52.0	0.7	0.5
	*Cs*NTR4	39.4	45.7	1.8	1.0
	*Cs*NTR5	35.7	42.7	0.4	0.3
	*Cs*NTR6	37.8	46.9	0.1	0.2
	*Cs*NTR7	38.0	46.3	0.2	0.2
	OYE	38.3	49.3	1.0	0.8
 2,4-dinitrotoluene	pET20b(+)	53.0	18.2	n.d.	n.d.
	*Cs*NTR1	33.3	1.3	n.d.	n.d.
	*Cs*NTR3	36.0	20.2	n.d.	n.d.
	*Cs*NTR4	51.3	14.8	n.d.	n.d.
	*Cs*NTR5	39.3	8.5	n.d.	n.d.
	*Cs*NTR6	28.8	9.5	n.d.	n.d.
	*Cs*NTR7	34.0	7.1	n.d.	n.d.
	OYE	49.0	20.4	n.d.	n.d.
	pET20b(+)	28.0	40.2	4.6	4.5
	*Cs*NTR1	17.8	33.8	4.9	2.4
	*Cs*NTR3	10.6	35.3	3.9	2.8
	*Cs*NTR4	28.6	37.1	5.7	3.6
	*Cs*NTR5	15.5	44.9	4.4	2.3

1-chloro-2,4-dinitro-benzene	*Cs*NTR6	11.8	34.6	4.3	1.6
	*Cs*NTR7	5.0	21.5	4.3	1.8
	OYE	29.4	27.3	6.6	3.8
 *p*-nitroanisole	pET20b(+)	2.9	10.8	0.8	1.0
	*Cs*NTR1	3.8	11.8	0.4	0.6
	*Cs*NTR3	5.3	1.0	1.3	1.6
	*Cs*NTR4	7.6	18.8	1.9	1.4
	*Cs*NTR5	0.5	11.0	0.5	0.9
	*Cs*NTR6	1.7	11.0	0.5	0.4
	*Cs*NTR7	5.3	9.1	0.6	0.8
	OYE	13.3	11.6	0.7	1.1

n.d. - not determined.

The results of the biotransformations showed that none of the enzymes improved the reduction of aromatic nitro compounds when compared to background activity determined by the control reaction using *E. coli* extracts expressing empty pET20(+). The yield of 2,4-dinitrotoluene reduction could not be measured as the peak of 2,4-diaminotoluene was covered with a component coming from the protein extracts which had a similar mass spectroscopy fragmentation pattern. The substrate conversions in the reactions were much bigger than the yields showing that *E. coli* possessed enzymes converting the reaction substrates to unidentified alternative products. Therefore the substrate range of the *C. sporogenes* nitroreductases and the OYE-like reductase is unknown.

5.3. *C. sporogenes* proline reductase

Bifunctional compounds such as ω-aminocarboxylic acids are important intermediates in the chemical industry as they can be easily converted into polymers possessing unique physical and chemical properties. One especially demanded compound is 6-aminocaproic acid which is an intermediate in the polymerization of Nylon-6.

Proteolytic *Clostridia* can use L-proline as the electron acceptor in the Stickland type fermentation (Nisman, 1954). The L-proline reduction pathway contains two enzymes, a very active proline racemase and a D-proline reductase. The latter enzyme converts D-proline into a bifunctional compound 5-aminovaleric acid. The reaction may be driven by pyridine nucleotides (NADH/NADPH) or dithiols such as dithiothreitol (Barker, 1981).

Pipecolic acid is a homolog of proline and comprises a non-aromatic ring formed of five carbon atoms and one nitrogen atom. Analysis of the metabolic pathway databases (KEGG, BioCyc) showed that L-pipecolic acid may be easily biosynthesised in a one-step reaction from L-lysine using lysine cyclodeaminase from *Streptomyces hygroscopicus*. The reductive cleavage of the L-pipecolic acid ring in a process analogous to L-proline reduction would result in formation of valuable 6-aminocaproic acid. Therefore, a route for 6-aminocaproic acid biosynthesis starting from L-lysine was proposed and tested (Fig. 5.38.).

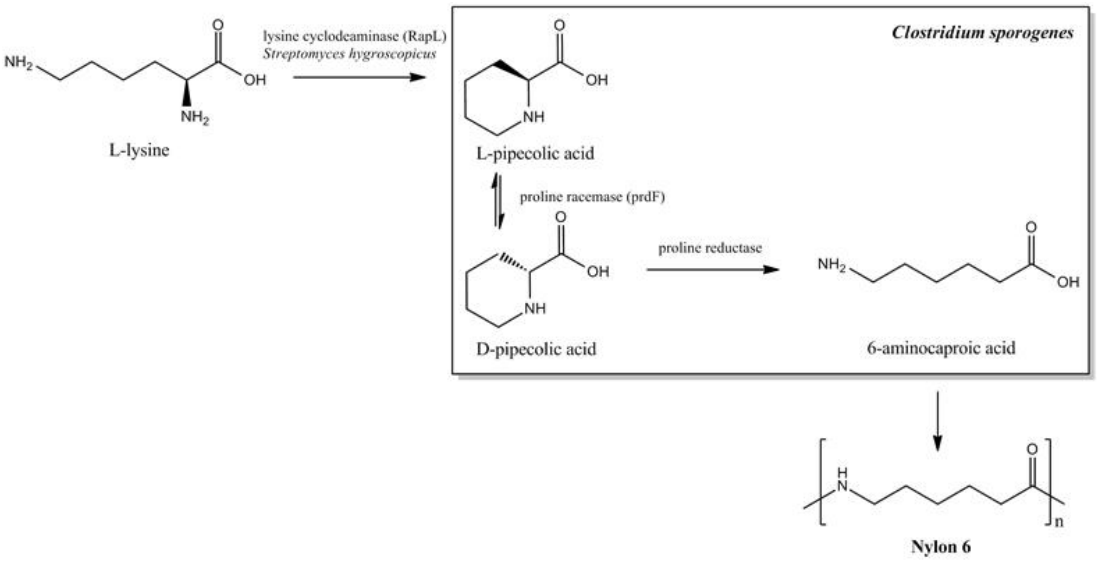


Fig. 5.38. Proposed route for biocatalytic synthesis of Nylon 6 using clostridial proline racemase and proline reductase.

The feasibility of the proposed pathway depends on the substrate range of clostridial enzymes, proline racemase (prdF) and proline reductase (prdAB). Therefore, an attempt to use *C. sporogenes* as a biocatalyst for reduction of pipecolic acid was made.

5.3.1. Growth of *C. sporogenes* on D,L-pipecolic acid

Growth experiments of *C. sporogenes* DSM795 in the minimal Lovitt medium showed that L-proline is an excellent fermentation substrate acting as the electron acceptor in the Stickland reaction and significantly increases the growth rate and biomass production of the culture (see 5.1.2.2.). Thus, to test if *C. sporogenes* can use D,L-pipecolic acid as an alternative electron acceptor in the fermentation, the growth of *C. sporogenes* was determined in minimal medium supplemented with L-alanine (as the electron donor) and L-proline and D,L-pipecolic acid (as the electron acceptors). The cultures (200 µL) were prepared in the wells of a 96-well plate and the growth of bacteria was observed for 50 h using automated NEPHELOstar plate reader in anaerobic conditions (Fig. 5.39.).

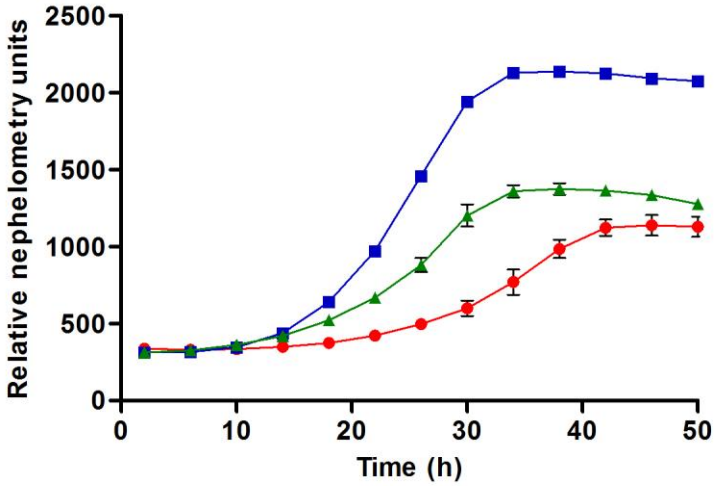


Fig. 5.39. Growth of *C. sporogenes* DSM795 in Lovitt medium supplemented with 27 mM L-alanine as the electron donor (●). 45 mM L-proline (■) or D,L-pipecolic acid (▲) were tested for acting as electron acceptors in the Stickland fermentation. Average of four independent replicates with error bars corresponding to standard deviations.

The growth experiments showed that D,L-pipecolic acid significantly increased the growth rate and the biomass production of the *C. sporogenes* cultures when compared to the control. However, it was not as good a fermentation substrate as L-proline.

5.3.2. Development of analytical method for detection of 6-aminocaproic acid

An analytical method for detection of 6-aminocaproic acid was developed based on the derivatization of amine groups with *o*-phthalaldehyde (Fig. 5.40.).

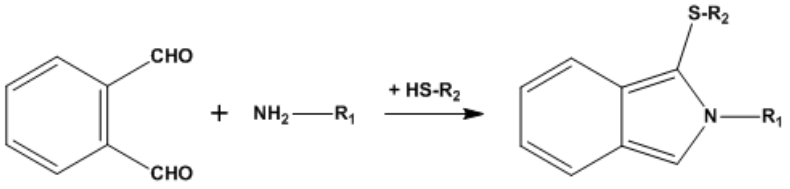


Fig. 5.40. The principle of primary amine derivatization using *o*-phthalaldehyde.

o-Phthalaldehyde (OPA) reacts exclusively with primary amine groups, but not with secondary amines. The derivatized compound can be detected by measuring the intensity of fluorescence at 455 nm after excitation at 340. L-proline, D,L-pipecolic acid, 5-aminovaleric acid and 6-aminocaproic acid were treated with OPA and the fluorescence of the reaction products was measured by FLUROstar plate reader. The fluorescence was detected at 470 nm due to lack of 455 nm filter in the device (Fig. 5.41.).

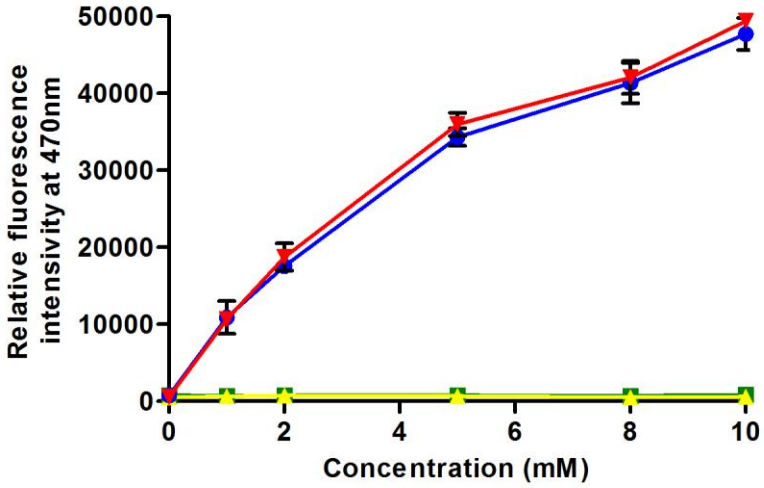


Fig. 5.41. Correlation between the fluorescence signal at 470 nm and the concentration of OPA-derivatized L-proline (■), D,L-pipecolic acid (▲), 5-aminovaleric acid (●) and 6-aminocaproic acid(▼). Error bars represents standard deviations.

The experiment showed almost linear correlation between the concentration up to 10 mM of OPA-derivatized 5-aminovaleric and 6-aminocaproic acids and the fluorescence signal at 470 nm. No fluorescence was observed for L-proline and D,L-pipecolic acid containing secondary amine functional groups.

5.3.3. Proline reductase expression and activity assays

C. sporogenes DSM795 was grown in the Giesel medium at 30°C. To induce the expression of proline racemase and proline reductase, the medium was supplemented with 27 mM D-glucose and 45 mM L-proline. The cells were harvested in the middle exponential and the early stationary phases of growth (4 h after inoculation, $OD_{660\ nm}$ 2.5 and 8 h after inoculation, $OD_{660\ nm}$ 5 respectively). The protein cell-free extracts were prepared using the cell disruptor at pressure of 40,000 psi. The membrane-free protein extracts were centrifuged at 75,000 x g for 20 min at 4°C and the soluble protein fractions were used in the proline reduction assay. DTT and NADH were used as the electron donors in the reactions. The heat inactivated protein extract was used as the negative control in the assay (Fig. 5.42.).

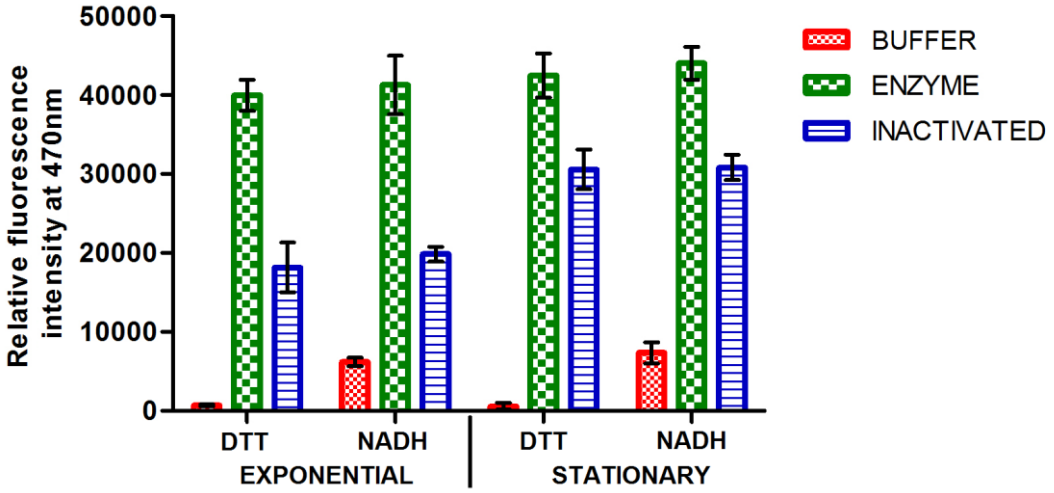


Fig. 5.42. L-proline reduction assay using protein extracts of *C. sporogenes*. Cells from exponential and stationary phases of growth were used to prepare the membrane-free protein extracts. Proteins were inactivated at 100°C for 20 min. DTT and NADH were used as electron donors. Data present means of four independent replicates with error bars representing standard deviations.

The proline reduction assay showed that proline racemase and reductase were successfully expressed during the *C. sporogenes* growth. Both DTT and NADH were good electron donors, however DTT showed a lower background in the control assay without the protein extracts. Although no difference in the L-proline reduction activity was observed between cells harvested at different phases of growth, the protein extract produced from the stationary phase cells showed a high fluorescence signal even when the reductase was inactivated by heat. That was probably caused by accumulation of 5-aminovaleric acid in the cells during *C. sporogenes* growth which was present later in the protein extracts.

Protein extracts of *C. sporogenes* grown on glucose/L-proline and harvested in the middle exponential phase of growth were used to reduce L-, D- and D,L-pipecolic acid in the expense of DTT (Fig. 5.43). L-proline was used as a control substrate, whereas the heat inactivated enzyme fraction was used as negative control.

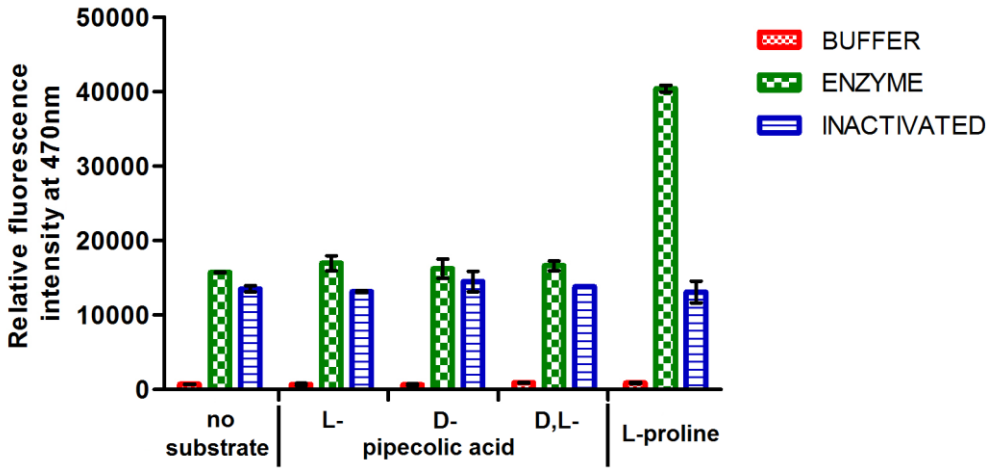


Fig. 5.43. Pipecolic acid reduction assay using protein extracts of *C. sporogenes* harvested in the exponential phase of growth. Proteins were inactivated at 100°C for 20 min. DTT was used as electron donor. L-proline was used as a positive control. Data present means of four independent replicates with error bars representing standard deviations.

The significant results showed that L-proline was successfully reduced to 5-aminovaleric acid but no reductase activity was observed in the presence of L-, D- or D,L-pipecolic acid. About a 20% increase in the fluorescence signal was observed for samples with the active proteins when compared to the ones with the heat inactivated extracts, even when no substrate was added to the reaction mixture. That could be explained by the presence of L-proline assimilated by the cells from the growth medium before production of the protein extracts. The residual L-proline was converted to the 5-aminovaleric acid in the assay and increased the fluorescence intensity. Therefore, the experiments showed that the proline reductase does not show activity towards L- and D-pipecolic acid.

6. Discussion

6.1. Biocatalytic reduction of C=C double bonds

Proteolytic Clostridia possess unique enzymatic activities as they can obtain energy and carbons from unusual reactions between two amino acids, where one of them acts as the electron donor and the second as the electron acceptor (discovered by Stickland, 1934; reviewed by Barker, 1980). A variety of electron acceptors, which can be used in this type of fermentation, make Clostridia an excellent source for screening of novel reductases. *C. sporogenes* was found to be an excellent biocatalyst for organic reductions in synthesis of high value chemicals. Its ability to hydrogenate activated alkenes, such as cinnamic acid and other α,β-unsaturated carboxylic acids (Bühler *el al.*, 1980; Giesel *et al.*, 1981; Bader *et al.*, 1982; Giesel and Simon, 1983; Bader and Simon, 1983; Dickert *et al.*, 2000) and nitroolefins (Fryszkowska *et al.*, 2008) was reported multiple times in the literature. The latter enzymatic activity was implemented into an industrially relevant chemoenzymatic route for synthesis of *(S)*-baclofen (Fryszkowska *et al*., 2010). However, proteins responsible for these enzymatic activities were never identified due to unsuccessful attempts of purification or lack of the *C. sporogenes* genome sequence (Fryszkowska *et al.*, 2008).

Proteolytic and saccharolytic Clostridia were found to reduce aromatic and aliphatic enoates (Tischer *et al.*, 1979; Bühler *el al.*, 1980) and genes encoding 2-enoate reductase were identified in *C. kluyveri*, *C. tyrobutyricum* and *M. thermoacetica* (Rohdich *et al.*, 2001). The gene encoding hypothetical 2-enoate reductase (*fldZ*) was also identified in *C. botulinum* by homology search (Sebaihia *et al.*, 2007). Since *C. sporogenes* is closely related to *C. botulinum* (separation of these taxa is now being questioned; Bradbury *et al*., 2012) it was likely that the fldZ homologue was responsible for the previously observed reductions of enoates and nitroolefins by *C. sporogenes*. A suitable way to test that would consist of identification, cloning and overexpression of *C. sporogenes* fldZ in a heterologous host. However, previous attempts to overexpress 2-enoate reductases in *E. coli* have met with limited success (Rohdich *et al.*, 2001). Therefore, an approach using production of genomic enoate reductase knock out mutants was chosen to confirm that fldZ is responsible for reduction of cinnamic acid and nitroolefins.

The *fldZ*::CT*erm*B mutant was created with the Clostron gene knock out system (Heap et al., 2007; Mordaka, 2010). In this thesis, successful insertional disruption of the *fldZ* gene in *C. sporogenes* was shown by the ability to grow on a selective medium, a series of polymerase chain reactions and Southern blot. The mutant was extremely stable for many generations, even without the selective pressure in the medium. Thus, the Clostron

was proved to be a very precise and efficient genetic tool for preparation of genomic knock out mutants in *C. sporogenes* and can give a significant impact on functional genomic studies in Clostridia.

To confirm that the fldZ enoate reductase is responsible for reduction of unsaturated compounds, wild type *C. sporogenes* and the *fldZ*::CT*erm*B mutant were used as biocatalysts. Cells grown on different energy substrates were used and biotransformations were driven using different electron donors. Inhibition of the reductase activities with air was also tested. The comparative analysis of reductase activities in the wild type and the mutant showed intriguing results (Tab. 6.1).

Table 6.1. Summary of C=C double bond reductase activities present in DSM795 and *fldZ*::CT*erm*B.

Biotransformation		**β-nitrostyrene**	***(E)*-1-nitro-2-phenylpropene**	***(E)*-2-nitro-1-phenylpropene**	***(E)*-cinnamic acid**
DSM795	Whole cells, H_2	+[1)]	+	+	+[1)]
	Cell-free extracts, NAD(P)H	+	+	+	+
fldZ::CT*erm*B	Whole cells, H_2	+[1)]	-	+[1,2)]	+[1)]
	Cell-free extracts, NAD(P)H	+[2)]	-	+[2,3)]	-

[1)] Yield improved by presence of L-phenylalanine in the growth medium; [2)] yield reduced when compared to the wild type; [3)] enzymatic activity was irreversibly inhibited by oxygen.

Biotransformations showed that *C. sporogenes* possesses at least three C=C double bond reductases characterized by different substrate specificities, expression patterns and sensitivities to oxygen.

FldZ was found to be capable of reduction of all unsaturated compounds tested. The expression of this enzyme was induced by amino acids present in the basal medium or L-phenylalanine. The enzyme could accept electrons delivered from all of electron donors that were used, however the greatest activity was observed for NADH. Biotransformations with protein extracts exposed to air showed that fldZ was not inactivated by oxygen or its activity was restored after removal of oxygen, which was previously reported for clostridial 2-enoate reductases (Tischer *et al.*, 1979).

C. sporogenes contains also another nitroalkene reductase, which reduced β-nitrostyrene and *(E)*-2-nitro-1-phenylpropene when whole cells and protein extracts of the mutant were used in the biotransformations. The expression of the unknown reductase was induced by growth on L-phenylalanine and its activity was irreversibly inactivated by oxygen. The presence of the second reductase was previously observed in the attempts to purify enzymes responsible for reduction of *(E)*-2-nitro-1-phenylpropene (Karl Fisher, personal communication; Appendix 8.7.).

The third C=C double bond reductase accepts cinnamic acid as a substrate. Confirming preliminary results published in Mordaka (2010), it was shown that in contrast to the wild type strain, the protein extracts of the mutant did not reduce cinnamic acid. However, biotransformations using whole cells of *fldZ*::CT*erm*B and experiments on L-phenylalanine fermentation in complex and minimal media showed that the mutant was able to produce 3-phenylpropionic acid. Thus, *C. sporogenes* contains two cinnamic acid reductases. FldZ is a soluble protein reducing cinnamic acid at the expense of NAD(P)H, whereas the second enzyme may not accept these electron donors. Cinnamic acid reduction was found to be coupled to vectorial transmembrane proton translocation allowing for ATP synthesis *via* electron transfer phosphorylation (Bader and Simon, 1983). It cannot be excluded that the second enzyme is a membrane associated/attached protein and could have been removed from the sample together with insoluble proteins and cell debris. It is also possible that the unknown enzyme does not accept free acid as the substrate, but acts on the coenzyme A derivative of cinnamic acid. Cinnamoyl-CoA can be enzymatically formed in two ways, as a result of activity of acyl-CoA ligase or CoA transferase (Dickert *et al.*, 2000). The reaction with acyl-CoA ligase requires the presence of CoA-SH and nucleoside triphosphate, whereas CoA transferase needs a CoA donor to activate cinnamic acid. Thus, these two reactions probably could not occur when protein extracts were used, but are more likely to be driven by metabolically active whole cells.

Evidence for the presence of fldZ and two other enzymes led to the analysis of *C. sporogenes* newly published genome, which allowed identification of two hypothetical proteins showing homology to flavin containing C=C double bond reductases, fldZ 2-enoate reductase (CLOSPO_02780) and OYE-like reductase (CLOSPO_03444), which were subsequently cloned, overexpressed and characterized.

FldZ belongs to a family of reductases consisting of about 660 amino acids and containing FAD and iron-sulphur cluster as cofactors. It shows the highest homology to 2-enoate reductase from proteolytic *C. botulinum* str. A (yellow highlight, Fig. 6.1.) and 2-

enoate reductases from saccharolytic *C. tyrobutyricum, C. kluyveri* and *M. thermoacetica* strains (green). On the other hand the OYE-like CLOSPO_03444 reductase is similar to classical C=C double bond reductases consisting of proteins composed of about 330-400 amino acids and possessing FMN as the cofactor (red).

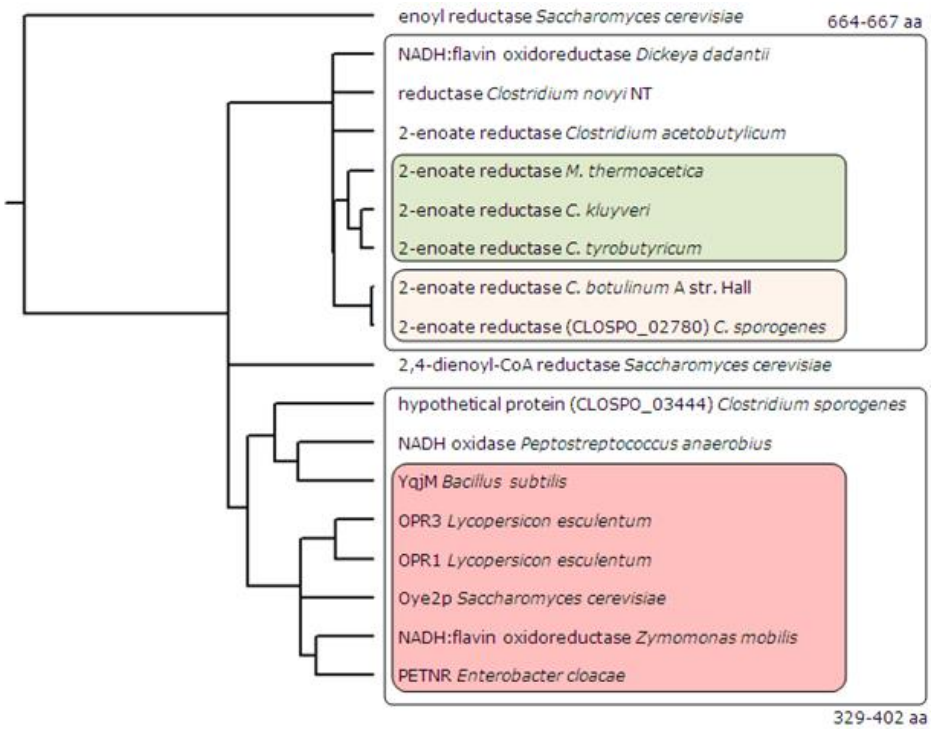


Fig. 6.1. Phylogenetic tree showing the evolutionary relationships among various C=C double bond reductases based upon similarities and differences in their primary amino acid sequences. The tree was created using the parsimony method with PHYLIP software. Yellow - 2-enoate reductases from proteolytic Clostridia, green - 2-enoate reductases from saccharolytic Clostridia, red - classical OYE-like reductases. For the list of sequences used to create the tree see Appendix 8.18.

The N-terminal domain of clostridial enoate reductases shares the structure with classical OYE-like C=C double bond reductases (FMN binding motif and substrate/catalytic site; for alignment and details see Appendix 8.19.). Clostridial enoate reductases contain also four conserved cysteine residues responsible for interaction with the iron-sulphur (Fe-S) cluster. The C-terminal part of fldZ is occupied by a small NADH binding domain within a larger FAD binding domain.

To characterize the catalytic activity, fldZ was cloned and overexpressed in *E. coli*. It was the first attempt to express a recombinant enoate reductase encoded by proteolytic *Clostridium*. Previous attempts to produce 2-enoate reductase from saccharolytic strains have met with limited success (Rohdich *et al.*, 2001). The expression was possible thanks to optimization of the *E. coli* growth under anaerobic conditions and optimization of the

codon usage of the *fldZ* gene. Although the growth rate and biomass production of *E. coli* were much smaller when compared to aerobic conditions, the optimized medium and growth conditions allowed for production of significant amounts of soluble recombinant proteins.

For easier purification using affinity resins, fldZ was His-tagged. Attempts to purify the enzyme using three different purification methods, gravity-flow columns, spin columns and magnetic beads, were unsuccessful. This might be caused by structural changes in the secondary and tertiary structure of the reductase which were forced by binding process of the histidine tag to the resin. Thus, the reductase was treated with a range of strong and weak denaturants under different redox conditions, but the activity was not restored. A note must be made that this protein refolding technique would be suitable only if the enzyme was inactive because of the misfolded structure.

However, the loss of activity could also be caused by stripping off or disruption of the iron-sulphur cluster by the interaction with the resin containing immobilized cobalt or nickel. Fe-S clusters are crucial components of oxidoreductases, as they mediate transfer of electrons within the enzyme, and are assembled biosynthetically by special enzymatic systems (Johnson *et al.*, 2005) which were not present in the purified enzyme sample. Incubation of the iron-sulphur-depleted enzymes (such as aconitase-type hydrolase from *Methanocaldococcus jannaschii* or NADH-cytochrome *c* reductase from *Pseudomonas arvilla*) with ferrous ions and sulfide in the presence of 2-mercaptoethanol was found to led to reconstitution of iron-sulphur clusters and restoration of the enzymatic activity (Yamaguchi *et al.*, 1981; Drevland *et al.*, 2007). Therefore, this method should be tested for reconstitution of the activity of the purified His-tagged fldZ in the future.

Alternatively, fldZ can be expressed as a fusion protein containing other tagging sequences than polyhistidine-tag. The pET vector system offers a wide range of N-terminal, C-terminal and internal fusion tags that can facilitate the purification of the target protein. T7-tag (11 amino acids) and S-tag (15 amino acids) allow rapid purification based on their interactions with monoclonal antibodies and S-protein respectively. The fusion proteins can be eluted under denaturing conditions disrupting interactions between the tag and the resin. To maintain mild conditions, the fusion proteins can also be released by digestion with protease recognising the cleavage site located in the linker encoded by the vector (Kim and Raines, 1993). GST-tag encoding 220 amino acids of the schistosomal glutathione S-transferase domain has been reported to enhance solubility of its fusion proteins. Tagged proteins can be eluted from the chromatography medium under mild, non-denaturing

conditions using reduced glutathione that often additionally preserves both protein structure and function (Harper and Speicher, 2011). Strep-tag is a short tag of 8 undisturbing amino acids encoding improved streptavidin polypeptide, which usually does not require removal to restore full protein activity. It specifically binds to StrepTactin ligand immobilized on the base matrix and allows purification under physiological conditions with elution performed with desthiobiotin, which displaces the fusion protein at the biotin-binding site. Strep-tag is recommended for expression of metalloproteins, membrane proteins and sensitive protein complexes with multiple subunits (Schmidt and Skerra, 2007). Thus, fldZ containing different tags should be overexpressed in *E. coli* and active fldZ fusion proteins should be purified by the respective affinity purification methods.

Since attempts to purify the fldZ reductase have met with the limited success, the enzyme was characterized using protein extracts of *E. coli* expressing the non-His-tagged wild type reductase. Cinnamic acid was chosen as the substrate in activity assays and biotransformations, as protein extracts of the wild type *E. coli* strain does not possess cinnamic acid reductase activity. The pH and temperature optima were determined as 7.0-7.5 and 40-45°C respectively, which tallied with previous results reported by Bühler *et al.*, 1980 for partially purified *C. sporogenes* 2-enoate reductase, but significantly different than optimal reaction conditions of 2-enoate reductases from saccharolytic Clostridia (Tischer *et al.*, 1979). Biotransformation of cinnamic acid with fldZ at the expense of NADH showed that the substrate is reduced to 3-phenylpropionic acid in stoichiometric amounts and the reaction is complete after 24 h. The enzyme was stable under anaerobic conditions at 4°C for at least few hours without losing activity. FldZ was found to be deactivated by the presence of oxygen and lost a half of the activity after 2 h exposure to air. Surprisingly the activity was not successfully restored after 1 h incubation under nitrogen flow. That differs from the results obtained for the *(E)*-1-nitro-2-phenylpropene reduction using *C. sporogenes* protein extracts exposed to air, which were discussed earlier. However, different amount and type of biocatalysts, different substrates and their concentrations, and different exposure and reaction times make these two experiments unable to be compared.

The activity tests showed that fldZ possesses a very narrow substrate range. The highest reductase activity was observed towards cinnamic acid and *p*-coumaric acid. Cinnamic acid substituted with methyl groups at α- and β-carbons, caffeic acid, 3-indoleacrylic acid and phenylnitropropenes were accepted as substrates, but the activity was very poor. Aliphatic 2-enoates and β-nitrostyrene were not reduced at all. This

confirmed previously published findings that 2-enoate reductase of proteolytic Clostridia are characterized by a narrow substrate range, in contrast to reductases from saccharolytic Clostridia, which can accept both aromatic and aliphatic unsaturated carboxylic acids (Tischer *et al.*, 1979; Bühler *et al.*, 1980).

FldZ was also tested for reduction of *(E)*-1-nitro-2-phenylpropene and *(E)*-2-nitro-1-phenylpropene in biphasic biotransformation at the expense of NADH. Protein extracts of the wild type *E. coli* reduced both substrates with poor yields, possibly due to the presence of endogenous NEM reductase, which was previously shown to reduce unsaturated compounds such as 1-nitrocyclohexene (Williams *et al.*, 2004). However, expression of the fldZ reductase significantly improved the yields of reduction for both substrates. It should be noted that despite background *E. coli* activity showing poor enantiomeric excess, hydrogenation of *(E)*-1-nitro-2-phenylpropene by protein extracts of *E. coli* expressing fldZ was characterized by a perfect enantioselectivity.

The analysis of the *fldZ*::CT*erm*B mutants showed that *C. sporogenes* possesses another C=C double bond reductase that can reduce β-nitrostyrene and *(E)*-2-nitro-1-phenylpropene. The OYE-like reductase (CLOSPO_03444) would be a perfect candidate for this enzymatic activity as homologous flavin containing reductases such as PETNR, KYE1, YersER and NRSal were found to reduce both these substrates (Toogood *et al.*, 2008; Yanto *et al.*, 2010; Yanto *et al.*, 2011). The OYE-like reductase was successfully expressed as a soluble protein containing C-terminal His-tag and purified to homogeneity using affinity purification method. However, the reductase did not show perceptible activity towards unsaturated substrates in the spectrophotometric activity assays. Thus, the substrate range of the OYE-like reductase is unknown. It cannot be excluded that the enzyme was inactive because of the presence of His-tag inhibiting the catalytic activity, which was shown previously for NRSal reductase from *Salmonella typhimurium* (Yanto *et al.*, 2010). On the other hand, the presence of the C-terminal His-tag in PETN reductase from *E. cloacae* did not affect the activity of the enzyme (Hulley *et al.*, 2010). In the future to test the OYE-like reductase activity, crude protein extracts of *E. coli* expressing the wild type reductase should be used. As shown previously for the fldZ reductase, a barely perceptible activity towards nitroolefins in the spectrophotometric activity assays does not confirm that fldZ cannot efficiently reduce these substrates in biphasic biotransformations. Thus, biotransformations of nitroolefins with OYE-like reductase at the expense of NAD(P)H should be performed in the future as well.

Biotransformations using whole cells and physiological studies with the fldZ mutant showed that fldZ acts as a cinnamic acid reductase accepting NADH as the electron donor. However, it was also shown that fldZ is not involved the L-phenylalanine fermentation *via* the Stickland reaction and that *C. sporogenes* possesses another cinnamic acid reductase with different properties to fldZ. The analysis of the *fld* gene cluster encoding enzymes involved in the reduction of L-phenylalanine showed the presence of a hypothetical protein annotated as acyl-CoA dehydrogenase in an operon together with two electron transfer flavoproteins, which may be responsible for reduction of cinnamoyl-CoA to 3-phenylpropionoyl-CoA (Fig. 6.2. A). A similar reductase carC was found to catalyze reduction of caffeyl-CoA to hydrocaffeyl-CoA by anaerobic bacterium *A. woodii* (Hess *et al.*, 2011). CarC is also co-transcribed with two electron transfer flavoproteins, which couple reduction of caffeyl-CoA with a chemiosmotic mechanism using sodium ions to produce ATP *via* electron transfer phosphorylation (Biegel *et al.*, 2011). A similar process was described in *C. sporogenes*, in which reduction of cinnamic acid was coupled to generation of a proton motive force leading to ATP synthesis (Bader and *et al.*, 1982). Moreover, fldA CoA transferase responsible for activation of phenyllactic acid in the previous step of the L-phenylalanine reduction pathway was shown to be more active when 3-phenylpropionyl-CoA was used as the CoA donor rather than cinnamoyl-CoA (Dickert *et al.*, 2000). This may suggest that during fermentation cinnamic acid is reduced as a CoA derivative rather than a free acid. Therefore a new model of L-phenylalanine reduction in the Stickland reaction can be proposed with reduction of cinnamoyl-CoA by acyl-CoA reductase annotated now as fldD (Fig. 6.2. B).

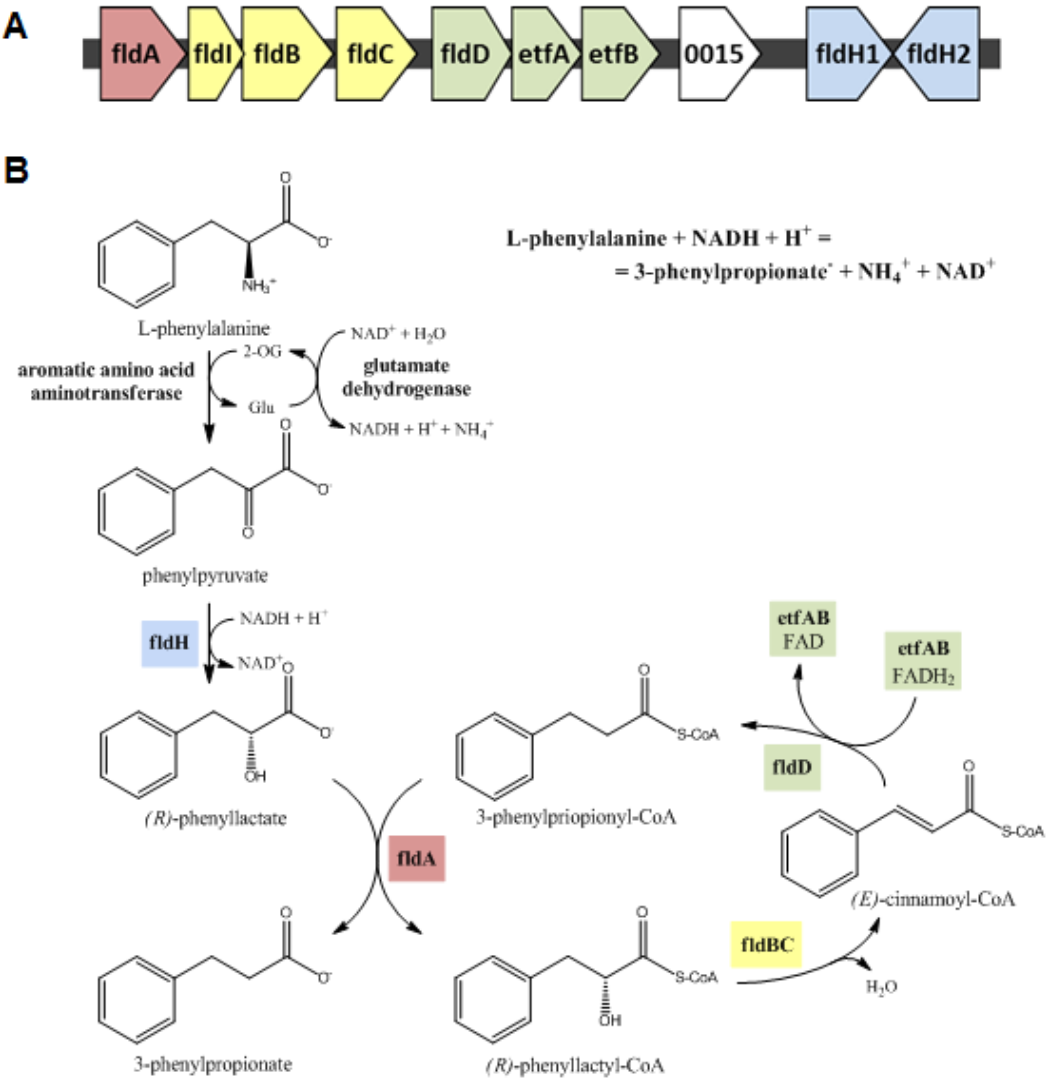


Fig. 6.2. Arrangement of the *fld* gene cluster in *C. sporogenes* ATCC15579 (**A**). Proposed model of L-phenylalanine reduction *via* the Stickland reaction in *C. sporogenes* (**B**). FldH – phenyllactate dehydrogenase; FldA – 3-phenylpropionyl-CoA:phenyllacte CoA transferase; fldBC – phenyllactate-CoA dehydratase, fldD – cinnamoyl-CoA reductase, etfAB – electron transfer flavoprotein.

According to the new pathway, the amino group is transferred from L-phenylalanine to 2-oxoglutarate resulting in 3-phenylpyruvic acid formation, which is subsequently reduced to *(R)*-phenyllactic acid by phenyllactate dehydrogenase (fldH). Phenyllactate is activated by transfer of CoA group from 3-phenylpropionyl-CoA mediated by 3-phenylpropionyl-CoA:phenyllactate CoA transferase (fldA) and dehydrated by phenyllactate-CoA dehydratase (fldBC) to cinnamoyl-CoA. Then, cinnamoyl-CoA is reduced to 3-phenylpropionyl-CoA by cinnamoyl-CoA reductase (fldD) using electrons delivered by electron transfer proteins (etfAB), which couple the reaction to creation of the proton motive force and ATP synthesis. In the last step of the pathway, 3-phenylpropionic acid is released after transfer of the CoA group to *(R)*-phenyllactate by fldA.

To test this model, *C. sporogenes* physiology needs to be analysed by constructing the fldD deficient strain as well as the double mutants genes for both cinnamic acid reductases knocked out. Recently a new method for creation of multiple knock out mutants in Clostridia was developed based on the modified Clostron gene knock out system (Kuehne *et al.*, 2010). Thus, this technique can now be used to produce single and double mutants for fldZ, fldD, etf1 and etf2 proteins. Overexpression of the fldD reductase in *E. coli* should also be performed. However the activity of fldD will depend on finding suitable electron donors and optimization of the cofactor regeneration system for this reaction.

Since fldZ was shown not to be essential for L-phenylalanine fermentation *via* the Stickland reaction, its physiological function remains unknown. It is unlikely that fldZ is involved in dissimilation of amino acids, since homologous 2-enoate reductases are also present in saccharolytic strains such as *C. kluyveri* and *C. tyrobutyricum*. Preliminary experiments on growth of the *fldZ*::CT*erm*B mutant in complex media showed decreased biomass production when glucose was used as the growth substrate (Mordaka, 2010), whereas no difference was observed between the wild type and the mutant growing on L-phenylalanine. It is possible that fldZ may be responsible for regeneration of the intracellular NAD^+ pool by reducing unsaturated carboxylic acids when an excess of the electron donor such as glucose is present in cells. If fldZ is knocked out, the pool of NAD^+ becomes limited, *C. sporogenes* has no alternative but to switch to production of butyrate, butanol and ethanol. This would result in decreased ATP production and in decreased growth as observed experimentally. When the medium is balanced and stoichiometric amounts of electron donors and acceptors are present, the growth substrates are fully metabolized *via* the Stickland reaction and no differences in the growth between DSM795 and *fldZ*::CT*erm*B are observed. This hypothesis will be tested in the future by series of growth experiments in media containing different concentrations of electron donors and acceptors for fermentation. A note must be made that although fldZ and fldD catalyse similar reactions, the presence of fldZ would not disrupt the L-phenylalanine reduction pathway as starting from the phenyllactate dehydration stage all enzymes in the Stickland reaction act on CoA derivatives, not on free acids.

The physiological experiments in minimal defined media showed that *C. sporogenes* grown on L-tryptophan accumulated skatole as a fermentation product. At present, skatole is recovered from coal tar and used as a fragrance and fixative in many perfumes and as an aroma compound. A few *Clostridium* species were found to produce skatole as a result of decomposition of protein materials (Rosenberger, 1958), but this activity was never

reported in *C. sporogenes*. Skatole could be the product of decarboxylation of 2-indoleacetic acid produced in the oxidative branch of the Stickland reaction. Thus, *C. sporogenes* can be used for biosynthetic production of skatole starting from renewable feedstock thanks to 2-indoleacetic acid decarboxylase activity. Since this enzymatic activity is present, it is possible that *C. sporogenes* can also be used for production of more industrially relevant chemicals such as toluene and *p*-cresol, which would be final products of L-phenylalanine and L-tyrosine oxidative fermentation respectively. Footprint analysis of the L-phenylalanine fermentation in minimal medium did not show the presence of toluene. However, the analytical method used for detection of fermentation products was not compatible with chemicals characterized with such a low boiling point (Pons *et al.*, 1984). On the other hand formation of *p*-cresol could not be determined as L-tyrosine was not used in the growth experiments due to its poor water solubility. Similar reactions have been reported in other Clostridia grown on L-phenylalanine (Pons *et al.*, 1984) and L-tyrosine respectively (Selmer and Andrei, 2001).

6.2. Biocatalytic reduction of aromatic nitro groups

Reduction of nitrobenzene to aniline by whole cells of *C. sporogenes* at the expense of hydrogen gas was reported by Dipeolu (Dipeolu, 2008; Dipeolu *et al.*, 2009). However, this enzymatic activity was not detected in biotransformations using whole cells of *C. sporogenes* presented herein. On the other hand, when protein extracts were used in the monophasic biotransformation with an ionic liquid as a co-solvent, *C. sporogenes* produced aniline at the expense of NADH and NADPH, but not with hydrogen gas. Therefore, the nitrobenzene reductase was present in *C. sporogenes*, but was not detected when whole cells were used probably due to insufficient amount of biocatalysts used in the reaction or inability to use reducing equivalents delivered by hydrogen gas.

In further experiments a small library of aromatic nitro compounds was used to test the reductase activity of *C. sporogenes* in biphasic biotransformations. The substrates were reduced with different yields depending on the substrate structure and electron donor used in the reaction.

A strategy was chosen for identification for *C. sporogenes* nitroreductases. Since no data about potential nitroreductases was present in the literature, the computational analysis of *C. sporogenes* genome was undertaken to identify enzymes showing homology to proteins reducing nitrogen-containing compounds from other organisms. Then, the six

promising enzymes were overexpressed and their nitroreductase activities were tested in biotransformations.

Biocatalytic reduction of aromatic nitro compounds with *E. coli* protein extracts showed poor amine product formation, however the conversion of substrates was significant. A high background activity was observed even for the strain expressing empty vector, which might be a result of activity of endogenous *E. coli* nitroreductases. Two types of nitroreductases have been identified in this organism, oxygen-insensitive and oxygen-sensitive (Linwu *et al.*, 2009). However none of them was ever examined for reduction of aromatic nitro compounds tested in this study.

The most probable route for aromatic nitro group reduction is by addition of pair of electrons forming nitroso and hydroxylamino intermediates before forming the amine group (Roldán *et al.*, 2008; Fig. 6.3.). Moreover, nitroso and hydroxylamino groups can form an azoxy intermediate as a result of spontaneous dehydration (Yanto *et al*., 2010). Some of the substrates can also undergo side reactions such as dechlorination or demethylation (Dipeolu, 2008).

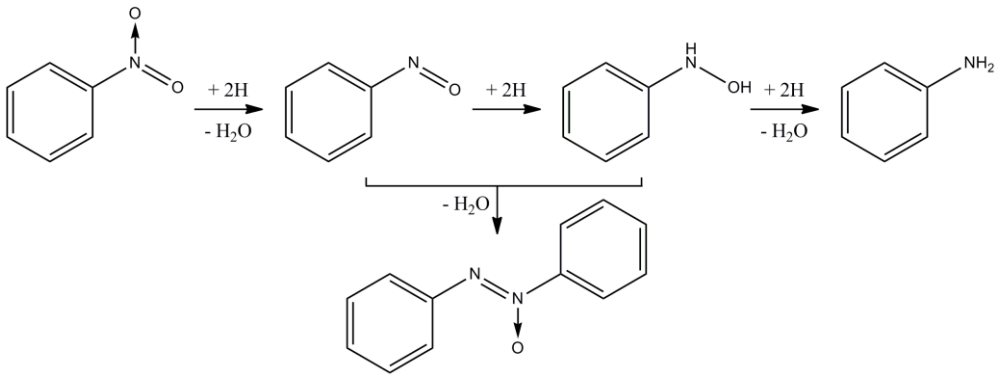


Fig. 6.3. Proposed reaction scheme for nitrobenzene reduction (adapted from Yanto *et al*., 2010).

The presence of the recombinant *C. sporogenes* nitroreductase (*Cs*NTR1 and 3-7) did not significantly change substrate conversions or amine product yields when compared to the control reactions. However, *Cs*NTR nitroreductases were expressed and tested separately. It cannot be excluded that in *C. sporogenes*, *Cs*NTR reductases act synergistically and different enzymes are responsible for different stages of reduction of nitro groups to amines. Using the current analytical method, intermediates of the nitro reduction could not be identified as protein cell-free extracts were used as biocatalysts, which gave high background in the gas chromatography analysis, and no analytical standards were available to determine their retention times. Therefore, *C. sporogenes* nitroreductases should be purified and their activity towards reduction of aromatic nitro compounds and

intermediates will be tested separately and as a blend. Alternatively, the nitroreductases could be co-expressed and tested in whole cell biotransformations.

6.3. Outlook for bio-nylon derived from renewable feedstock

Nylon 6 fibres are used in a broad range of products requiring strength and elasticity (Mark, 2009). Currently, Nylon 6 is synthesised by ring- opening polymerization of ε-caprolactam at 260°C under atmosphere of nitrogen. There are a few disadvantages of using caprolactam as the substrate for the synthesis of Nylon. For the industrial scale caprolactam can be synthesized from cyclohexenone (by condensation with hydroxylamine followed by acid-catalyzed rearrangement of an oxime to an amide; Gawley, 1988) or cyclohexane (by synthesis of cyclohexenone oxime by photoreaction of nitrosyl chloride; Naylor and Anderson, 1953). Thus, the substrates for caprolactam synthesis are derived mainly from non-renewable resources such as petroleum and other fossil fuels. Moreover, in contrast to 6-aminocaproic acid (a monomer of Nylon), caprolactam has been annotated as a carcinogenic substance. However, its harmfulness is not clear and now being questioned by International Agency for Research on Cancer (http://monographs.iarc.fr/ ENG/Classification/ClassificationsAlphaOrder.pdf; 2012).

The method of 6-aminocaproic acid biosynthesis presented in this thesis has a major advantage over the currently used Nylon synthesis route. It starts from L-lysine which, in contrast to caprolactam, is a renewable resource and has been manufactured using genetically engineered *Corynebacterium glutamicum* with production exceeding 750,000 tons per year in 2006 (Pfefferle *et al.*, 2003; Wittmann and Becker, 2007). Conversion of L-lysine to pipecolic acid by lysine cyclodeaminase followed by reduction to 6-aminocaproic acid by D-proline reductase would provide a simple and carbon efficient route, which fulfils the main principles of the green chemistry (Anastas and Warner, 1998).

The expression of D-proline reductase was successfully induced in *C. sporogenes* and the enzyme was tested for reduction of D- and L-pipecolic acid. Although the crude extracts of DSM795 reduced proline to 5-aminovaleric acid, the enzyme did not show any activity towards pipecolic acid.

Initially, growth experiments in minimal medium showed that the presence of pipecolic acid significantly increased the growth rate and biomass production of *C. sporogenes*. However, it was not accepted by D-proline reductase as a substrate and could not serve as the electron acceptor for energy conservation in the Stickland fermentation. D,L-Pipecolic acid was never reported to be an intermediate in any of clostridial metabolic pathways. Pipecolic acid is an important precursor of microbial secondary metabolites such

as immunosuppressant rapamycin, the antitumor agent swainsonine, the peptide antibiotic virginiamycin and the anthelmintic agent marcfortine (He, 2006). In *Pseudomonas putida* and human brain pipecolic acid is involved in L-lysine degradation pathway to α-aminoadipate, which is subsequently converted *via* glutaryl-CoA to acetyl-CoA (IJlst *et al.*, 2000; Revelles *et al.*, 2005). Since Clostridia metabolize L-lysine to acetate, butyrate and ammonia *via* a cleavage of the six-carbon chain (Barker, 1981), the presence of the former metabolic pathway is unlikely. Thus, the stimulatory effect of pipecolic acid on the *C. sporogenes* growth remains unclear and requires further analysis such as footprints of cultures grown on pipecolic acid.

To make the 6-aminocaproic acid biosynthesis route feasible two conditions need to be met. At first, a route for D-pipecolic acid synthesis needs to be established as all existing routes for converting lysine into pipecolic acid result in formation of the L-enantiomer (Fig. 6.4.).

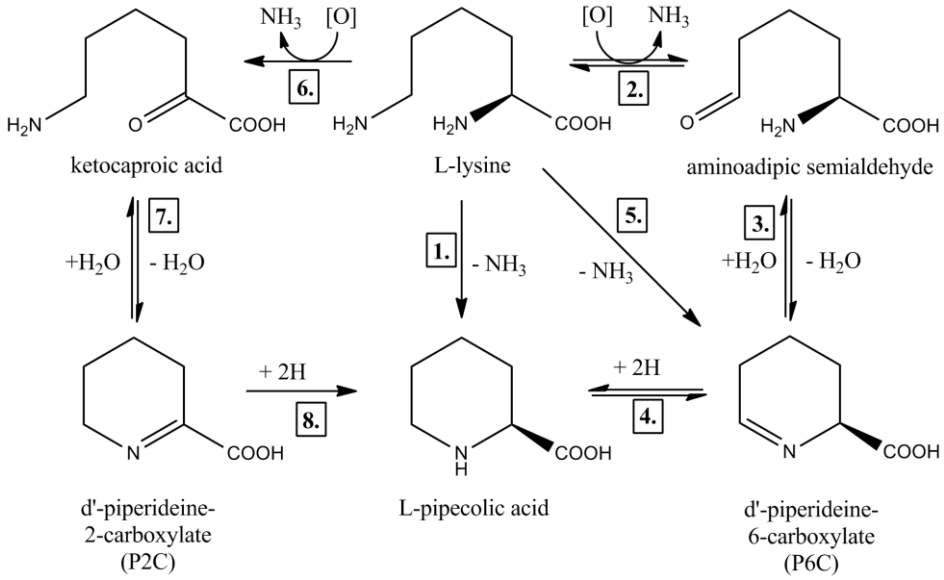


Fig. 6.4. Possible routes for biocatalytic synthesis of pipecolic acid (Miller and Rodwell, 1971; He, 2006). Enzymes: **1**. L-lysine cyclodeaminase; **2.** saccharopine synthase and saccharopine oxidase; **3.** spontaneous reaction; **4.** Δ'-pyrroline-5-carboxylate reductase; **5.** L-lysine ε-aminotransferase; **6.** L-lysine α-oxidase; **7.** spontaneous rection; **8.** Δ'-piperideine-2-carboxylate reductase.

The proline racemase, which is responsible for interconversion of L- and D-proline (Stadtman and Elliott, 1957), needs to be tested for activity towards L- and D-pipecolic acid. *C. sporogenes* protein extracts will be incubated with a solution of L-pipecolic acid and the rotation of the polarized light will be determined. A note must be made that pipecolic acid was found to have an inhibitory effect on the activity of homologous proline racemase from *C. sticklandii* (Cardinale and Abeles, 1968). Therefore, another route for D-pipecolic acid synthesis must be considered. D-Pipecolic acid can possibly be obtained by oxidation of L-

lysine α-amino group (Fig. 6.4., reaction 6.) followed by enantioselective hydrogenation of imine, Δ'-piperideine-2-carboxylate, using a chiral transition metal catalyst (Willoughby and Buchwald, 1992). However, this method would employ a chemical reaction step and make the synthesis route more complex and less environmentally friendly.

The second challenge that needs to be accomplished is evolution of the D-proline reductase to accept pipecolic acid as a substrate for the reductive cleavage of the ring. Naturally occurring enzymes are shown to be excellent biocatalysts, but many of their properties need to be tailored to fulfil requirements of specific applications. Thus, protein engineering methods are used to alter enzymes properties such as activity, selectivity, specificity, stability and solubility (Rubin-Pitel and Zhao, 2006). Using rational design approach is technically easy and inexpensive. However, the limited knowledge about the structure of D-proline reductase and the mechanism of enzyme catalysis may make this method unsuitable. On the other hand, random mutagenesis using directed evolution does not require structural knowledge of a protein, but analysis of large numbers of mutants is essential (Reetz, 2011). The fluorescence method for detection of 6-aminocaproic acid described in this thesis can be easily adapted to a high-throughput format and used for screening of reductases accepting D-pipecolic acid as a substrate. This approach can also be used to change the enantioselectivity of the D-proline reductase to make it accept L-enantiomers of proline and pipecolic acid, which would limit the whole biosynthetic pathway just to two reactions (Reetz, 2011).

6.4. Conclusions

As a whole, the presented work revealed interesting aspects of *C. sporogenes* physiology and enzymatic activities that were not previously reported. It has given a new insight into the metabolic pathway by which *C. sporogenes* obtains carbon and energy for growth on certain amino acids. This can be used for better understanding of metabolism of Clostridia and potentially result in generation of the new medicines against clostridial pathogens. The fldZ reductase was succesfully overexpressed and was shown to be an efficient biocatalyst in the enantioselective reduction of nitroolefins and hydrogenation of aromatic enoates. That allows clostridial enoate reductases to be fully exploited in industial applications. A new idea about using clostridial enzymes for biosynthesis of Nylon 6 from renewable feedstocks has also been presented. Even though attempts to oxerexpress and characterize novel C=C double bond and aromatic nitro group reductases have met with the limited success, *C. sporogens* turns out to be a very promising subject for futher research as a source of industrially desirable enzymes.

7. Literature

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8. Appendices

8.1. Generation of *C. sporogenes* fldZ knockout mutant

C. sporogenes DSM795 fldZ knockout mutant was designed and generated by Dr. Benjamin Blount and Prof. Nigel Minton (Clostridia Research Group, Centre for Biomolecular Sciences, University of Nottingham). To create the strain with the inactivated gene encoding the clostridial enoate reductase, the first generation ClosTron mutagenesis method was chosen as the most efficient and convenient one (Heap *et al*., 2007). The data in section 8.1. were provided by Dr. Benjamin Blount.

8.1.1. Sequencing of *fldZ* gene in *C. sporogenes* DSM795

A 969 bp region of *fldZ* was PCR amplified on genomic DNA from *C. sporogenes* DSM795 using the FailSafe PCR system with primers designed using the *C. botulinum* Hall A ATCC3502 sequence (primers ENR-F1 and ENR-R1 897 - 1865 bp). PCR product was agarose gel purified and excised. Gel band was purified using a QIAGEN gel extraction kit. The PCR product was then sequenced using the ENR-F1 and ENR-R2 primers. The 949 bp region was successfully sequenced as follows:

>*fldZ C. sporogenes* DSM 795 (partial sequence)
TCCATGCATATGGATCAAAAATATTTTTACAATTATCAGCAGGCTTTGGTAGAGTAAGTATACCATCT
ATTGTAGGAAAAGTGGCAGTAGCACCTTCTAAAATTCCACATAGATTTTTACCAGGAGTAACTTGTCG
TGAGTTAACTACAGAAGAAGTAAAAGAATATGTAAAAGCCTTTGGTGAATCAGCAGAAATTGCAAAAA
AAGCCGGATTTGATGGTGTAGAAATTCATGCAGTACATGAAGGATACTTATTAGATCAATTTGCTATT
TCTTTCTTTAACCATCGTACTGATGAATATGGTGGATCATTAGAAAATCGTTTAAGATTTGCCTGTGA
GGTTGTACAAGAAATTAAGAAACGTTGTGGACAAGACTTCCCAGTTTCACTTAGATACAGTATAAAAA
GCTTCATTAAGGATTGGTGTAAGGGTGGCTTACCAGATGAAGAATTTGAAGAAAAAGGAAGAGATATT
CCAGAAGGAATTGAAGCTGCTAAAATACTTGTTGCAGCAGGATATGATGCTTTAAATGGAGATGTTGG
GTCTTATGATTCATGGTATTGGAGTCATCCACCAATGTATCAAAAGAAGGGATTATACCTTCCATACA
ATGAAATACTTAAAAAGGTAGTAGATGTACCTATTATTACAGCAGGAAGAATGGAAGACCCTGAACTA
TCAAGTGATGCAATTTTGTCAGGAAAAACAGATATGATTGCTTTAGGAAGGCCACTTCTTGCAGATGC
AGAAATTCCAAATAAGATTTTTGAAGATAAATATGATAAAGTTAGACCTTGTTTATCTTGTCAAGAAG
GGTGTATGGGAAGATTACAGAATTTTGCAACAGTATCCTGTGCAGTAAATCCTGCCTGTGGACGTGAA
AAAGAGTATGGACTAAAAAAAGCAGAACAGATTAAGAAAGTTCTTGTTGTAGGTGGCGGTGTTGC

8.1.2. Retargeting pMTL007C-E2

The *C. sporogenes fldZ* fragment sequence was entered into the intron design tool, using the Perutka algorithm (access at http://clostron.com). The output for the best hit was as follows:

```
Sequence                                        Pos         Score
-30                          +1          +15
AATGAAATACTTAAAAAGGTAGTAGATGTACCTATTATTACAGCA  641|642s    6.158

fldZ-IBS: AAAAAAGCTTATAATTATCCTTAGTAGTCGATGTAGTGCGCCCAGATAGGGTG
fldZ-EBS1d: CAGATTGTACAAATGTGGTGATAACAGATAAGTCGATGTACCTAACTTACCTTTC-
TTTGT
fldZ-EBS2: TGAACGCAAGTTTCTAATTTCGATTACTACTCGATAGAGGAAAGTGTCT
```

Expected fldZ targeting region (PCR product):

AAAAAAGCTTATAATTATCCTTAGTAGTCGATGTAGTGCGCCCAGATAGGGTGTTAAGTCAAGTAGTT
TAAGGTACTACTCTGTAAGATAACACAGAAAACAGCCAACCTAACCGAAAAGCGAAAGCTGATACGGG
AACAGAGCACGGTTGGAAAGCGATGAGTTACCTAAAGACAATCGGGTACGACTGAGTCGCAATGTTAA
TCAGATATAAGGTATAAGTTGTGTTTACTGAACGCAAGTTTCTAATTTCGATTACTACTCGATAGAGG
AAAGTGTCTGAAACCTCTAGTACAAAGAAAGGTAAGTTAGGTACATCGACTTATCTGTTATCACCACA
TTTGTACAATCTG

The insertion was targeted between bases 641 and 642 of the fragment in a sense direction. This corresponded to bases 936 and 937 of the full gene. Alternative unused sites were as follows:

GCCGGATTTGATGGTGTAGAAATTCATGCAGTACATGAAGGATAC	236|237s	5.699
AATATGGTGGATCATTAGAAAATCGTTTAAGATTTGCCTGTGAGG	327|328s	5.676
CTGCTGATTCACCAAAGGCTTTTACATATTCTTTTACTTCTTCTG	162|163a	5.622
GGAAAAACAGATATGATTGCTTTAGGAAGGCCACTTCTTGCAGAT	731|732s	5.359
ATTGAAGCTGCTAAAATACTTGTTGCAGCAGGATATGATGCTTTA	515|516s	5.308
CTTCTTGCAGATGCAGAAATTCCAAATAAGATTTTTGAAGATAAA	764|765s	5.291
TAGCAAATTGATCTAATAAGTATCCTTCATGTACTGCATGAATTT	240|241a	5.081
CCACTTCTTGCAGATGCAGAAATTCCAAATAAGATTTTTGAAGAT	761|762s	4.979
TTTCACGTCCACAGGCAGGATTTACTGCACAGGATACTGTTGCAA	855|856a	4.707
GCAGAAATTGCAAAAAAAGCCGGATTTGATGGTGTAGAAATTCAT	218|219s	4.220
AGAATGGAAGACCCTGAACTATCAAGTGATGCAATTTTGTCAGGA	689|690s	3.680
AATACCATGAATCATAAGACCCAACATCTCCATTTAAAGCATCAT	534|535a	3.415
TATCAGCAGGCTTTGGTAGAGTAAGTATACCATCTATTGTAGGAA	63|64s	3.391
TTTGAAGAAAAAGGAAGAGATATTCCAGAAGGAATTGAAGCTGCT	482|483s	3.318
CATGCAGTACATGAAGGATACTTATTAGATCAATTTGCTATTTCT	260|261s	3.316
GATATTCCAGAAGGAATTGAAGCTGCTAAAATACTTGTTGCAGCA	500|501s	3.192
TTCCTTCTGGAATATCTCTTCCTTTTTCTTCAAATTCTTCATCTG	456|457a	3.131
CTCCATTTAAAGCATCATATCCTGCTGCAACAAGTATTTTAGCAG	507|508a	3.101
GAAATACTTAAAAAGGTAGTAGATGTACCTATTATTACAGCAGGA	644|645s	2.984

The *fldZ* targeting vector pMTL007C-E2::*Csp-fldZ*-936s was synthesised by Eurofins Genetic Service.

8.1.3. Insertional inactivation of *fldZ*

Initially pMTL007C-E2::*Csp-fldZ*-936s was transformed into *E. coli* TOP10 and then subsequently into the *E. coli* CA434 conjugal donor strain by electroporation. A stationary overnight culture of *E. coli* CA434 pMTL007C-E2::*Csp-fldZ*-936s was centrifuged, washed with PBS buffer and re-suspended with stationary overnight *C. sporogenes* DSM795. The mixture was spotted onto anaerobic 1% agar TYG media and incubated anaerobically for 8 h. Plates were washed with phosphate saline buffer (PBS) and the resultant slurry was plated onto TYG agar with cycloserine (250 μg/mL) to select against the *E. coli* donor and thiamphenicol (15 μg/mL) to select for recipients of the plasmid. Plates were incubated anaerobically for 48 h and thiamphenicol resistant colonies were re-streaked onto TYG agar with cycloserine (250 μg/mL) and erythromycin (10 μg/mL) to select for insertion of the intron.

Six erythromycin resistant colonies were selected and genomic preparations were performed on overnight cultures. The cultures were spun down, resuspended in 5% Chelex 100, boiled at 100°C for 10 min and then spun down again. The supernatants were transferred to fresh microtubes. PCR screening was performed on the genomic preparations to determine correct insertion with primer pairs as follows:

F-junction screening:
ENR-F1: 5′ – GACAGAAAGAGTCCATGCATATGG – 3′
EBSUni: 5′ – CGAAATTAGAAACTTGCGTTCAGTAAAC – 3′

R-junction screening:
ENR-R1: 5′ – TCCATTCCTGCAACACCGCCACC – 3′
ErmRAM-R: 5′ – ACGCGTGCGACTCATAGAATTATTTCCTCCCG – 3′

Flanking screening:
ENR-F1: 5′ – GACAGAAAGAGTCCATGCATATGG – 3′
ENR-R1: 5′ – TCCATTCCTGCAACACCGCCACC – 3′

ErmRAM Screening:
ErmRAM-F: 5′ – ACGCGTTATATTGATAAAAATAATAATAGTGGG – 3′
ErmRAM-R: 5′ – ACGCGTGCGACTCATAGAATTATTTCCTCCCG – 3′

Two of the colonies (*fldZ*::CT*erm* B and E) showed a band of a size approximate to the 871 bp product expected for the F-junction screening (Fig. 8.1). There were no bands present in the R-junction screening. The two colonies did not show a wild type band in the flanking screen, indicating that the gene has been interrupted.

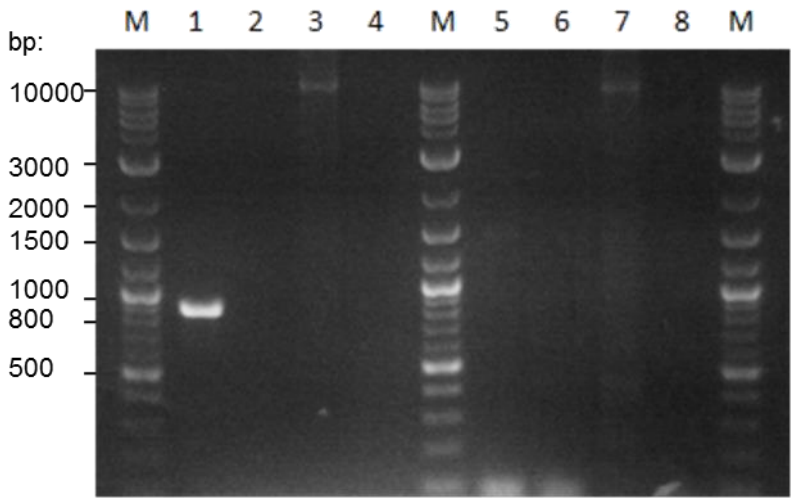

Fig. 8.1. Electrophoresis of the PCR products. Reaction mixtures (10 µL) were run on 1% agarose gel with NEB 2-log ladder (**M**). F-junction screen PCR templates: **1** – *fldZ*::CT*erm*B, **2** – *C. sporogenes* DSM795, **3** - pMTL007C-E2::*Csp-fldZ-936s*, **4** – dH_2O. R-junction screen PCR templates: **5** – *fldZ*::CT*erm*B, **6** – *C. sporogenes* DSM795, **7** - pMTL007C-E2::*Csp-fldZ-936s*, **8** – dH_2O.

The ErmRAM screen yielded a product of 900 bp and no 1300 bp product indicating that the only ErmRAM present was that in the insertion and that the strains had been cured of the plasmid (Fig. 8.2).

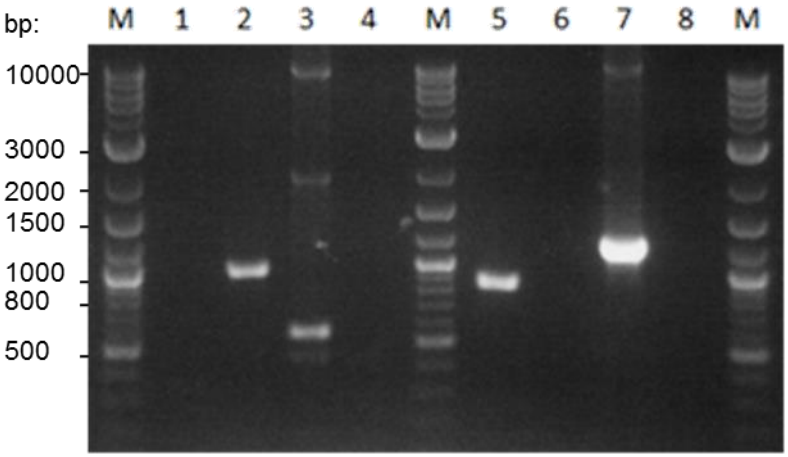

Fig. 8.2. Electrophoresis of PCR products. Reaction mixtures (10 µL) were run on 1% agarose gel with NEB 2-log ladder (**M**). Flanking screen PCR templates: **1** – *fldZ*::CT*erm*B, **2** – *C. sporogenes* DSM795, **3** - pMTL007C-E2::*Csp-fldZ-936s*, **4** – dH_2O. ErmRAM screen PCR templates: **5** – *fldZ*::CT*erm*B, **6** – *C. sporogenes* DSM795, **7** - pMTL007C-E2::*Csp-fldZ-936s*, **8** – dH_2O.

To confirm this, the strains were restreaked onto thiamphenicol selective media and showed no growth. The PCR product from the F-junction screening was sequenced by using the ENR-F1 and EBSUni primers (Fig. 8.3).

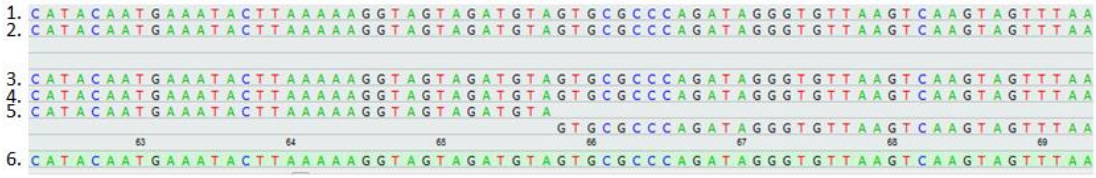

Fig. 8.3. Sequencing of F-junction screening PCR product using ENR-F1 and EBSUni primers: **1** and **2** – *fldZ::*CT*erm*B colony B EBSUni and ENR-F1 sequencing reads, **3** – *fldZ::*CT*erm*B colony E ENR-F1 sequencing read, **4** – *C. sporogenes fldZ* sequence 5' of insertion, **5** – ClosTron insert sequence, **6** – consensus sequence.

The sequencing confirmed that integration of the intron into *fldZ* gene was successful. Colony B was stored at -80°C as *C. sporogenes* DSM 795 *fldZ*::CT*erm*B.

8.2. Optimization of the *C. sporogenes* high-throughput growth experiments

Nephelometry is a method of measuring light scattered by particles suspended in solutions at right angles to the light beam (Fig. 8.4.). It is widely used in different analytical approaches in clinical immunology, drug discovery and clinical chemistry. It was also found to be useful in the monitoring of microbial growth in high-throughput systems (Angersbach *et al*., 2005).

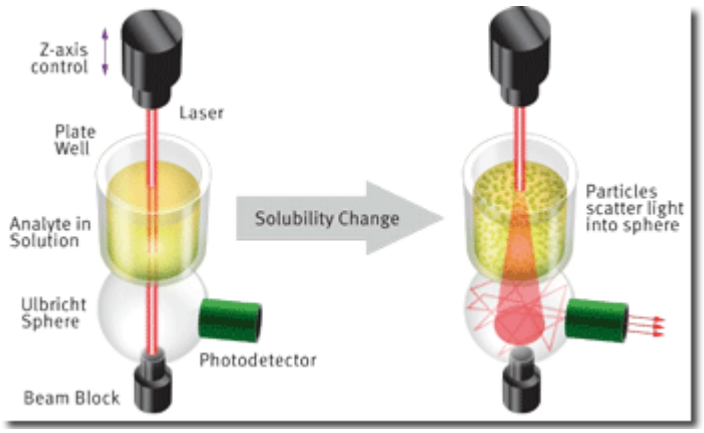

Fig. 8.4. Diagram presenting the principle of NEPHELOstar measurement (Angersbach *et al.*, 2005). A clear solution (on the left hand side) causes minimal light scattering resulting in low nephelometry signal. High density solution with particles scatters the light and results in high signal (right).

The NEPHELOstar (BMG LABTECH) is a microplate nephelometer using a red laser diode (633 nm) as the light source. It was shown that data obtained by measuring scattering of light using a nephelometer were comparable to optical density readings using a transmission reader. A good correlation between the two methods was shown for *Corynebacterium glutamicum*, a linear correlation up to OD_{600nm} 4 (Angersbach *et al*., 2005), and *Clostridium tyrobutiricum* (Rebros *et al.*, 2009).

To test the correlation between optical density readings at 660 nm using a spectrophotometer (polystyrene cuvettes, 10 mm pathlength, Agilent 8453 spectrophotometer) and nephelometry measurements in 96-well plates using a NEPHELOstar, *C. sporogenes* DSM795 was grown in TYG medium. Overnight cultures were centrifuged, the residual medium was decanted and the cells were washed with 50 mM potassium phosphate buffer pH 7.0. The cells were centrifuged again and resuspended in the same buffer to give an $OD_{660\ nm}$ 2.5. A series of dilutions in the buffer was prepared to give samples in a range of $OD_{660\ nm}$ from 0.1 to 2.1. The samples (200 µL) were transferred into the wells of a 96-well plate (Corning) and the nephelometry signal was measured using

a NEPHELOstar plate reader. The nephelometry signal was also determined when the plate was covered with a Breathe-Easy film (Diversified Biotech) and a lid (Fig. 8.5.).

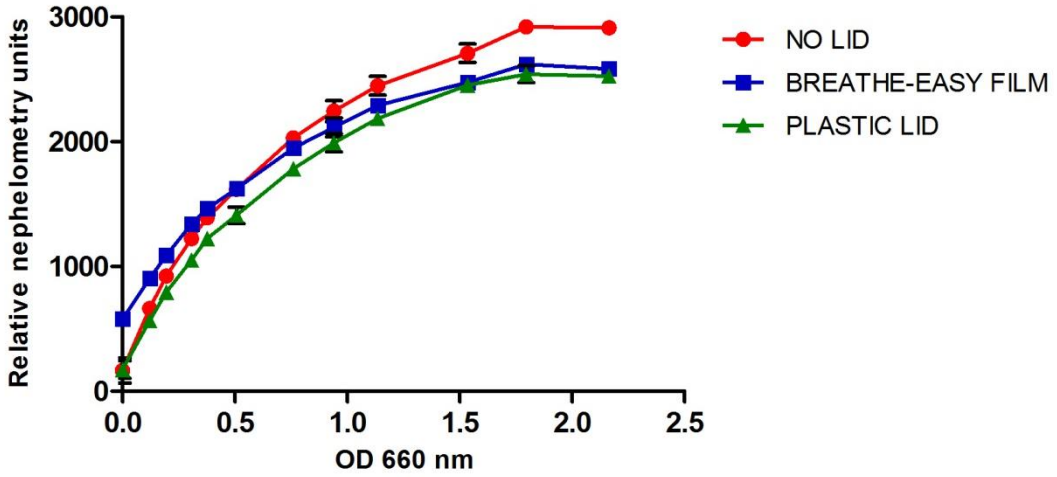

Fig. 8.5. Correlation between optical density of *C. sporogenes* DSM795 and nephelometry signal measured by a NEPHELOstar plate reader.

It was shown that there is a good correlation between these two analytical methods up to OD_{660} 1. For optical densities higher than 1.5, the device reached the highest level of sensitivity resulting in almost constant nephelometry signal. Presence of the Breathe-Easy film significantly increased the background of the measurement when compared with the plate only and the plate covered with a lid. Although it made the measurement less sensitive for low concentrated samples, a Breathe-Easy film was used in the experiments to provide necessary sterility of the wells, prevent cross-contamination between different wells of one plate and prevent loss of samples by evaporation.

A NEPHELOstar allows the operator to set the laser diode parameters such as intensity of the beam (gain, from 0 to 240) and its diameter (beam focus, from 0.5 to 4.5 mm). Both factors were optimized to give the best correlation between the nephelometry signal and the optical density of *C. sporogenes* cells in the range of $OD_{660\ nm}$ from 0 to 1 (Fig. 8.6 and 8.7). For the determination of laser intensity, the nephelometry signal was normalized to the signal of a sample with $OD_{660\ nm}$ 0.85. The low laser gain (10-30) showed decreased slope of the calibration curve what lowered the sensitivity of the measurement. When the gain was increased to 40 and more no further improvement of the sensitivity was observed. A similar experiment with changing the laser beam width showed that the best correlation occurs for the beam set to 2.0 mm. Both parameters (gain 40 and beam focus 2.0 mm) were used in the subsequent *C. sporogenes* growth experiments.

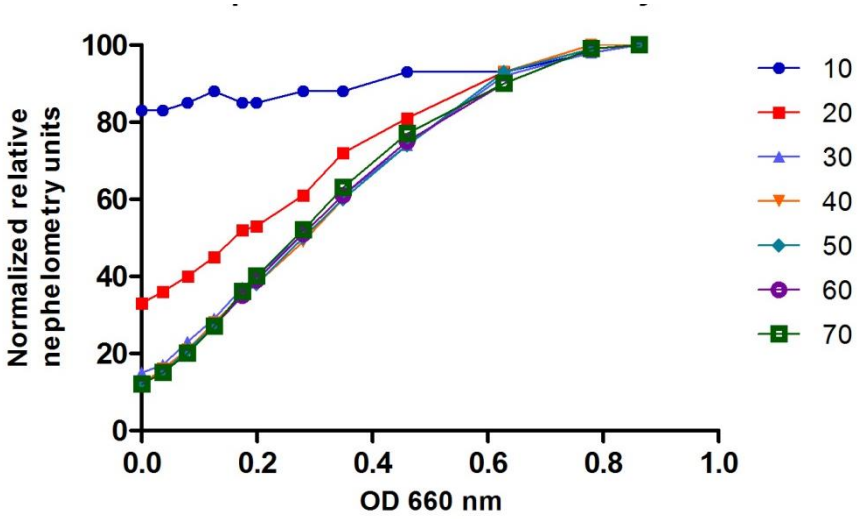

Fig. 8.6. Correlation between the nephelometry signal and optical density (660 nm) at different laser intensities of the NEPHELOstar diode. Values were normalized (as 100%) to the nephelometry signal of the sample with $OD_{660\ nm}$ 0.85.

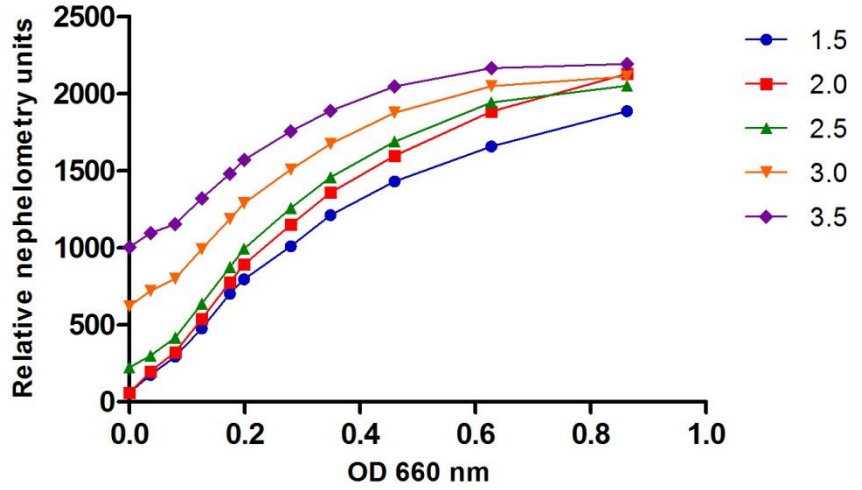

Fig. 8.7. Correlation between the nephelometry signal and optical density (660 nm) at different laser bean width (from 1.5 to 3.5 mm).

To prove that NEPHELOstar can be used as a reliable device for monitoring of *C. sporogenes* growth under anaerobic conditions, the plate reader was placed in an AtmosBag (Sigma, size L) and flushed with nitrogen for 2 h before use. In the anaerobic cabinet *C. sporogenes* DSM795 pre-culture in TYG medium (10 mL) was started from a single colony taken from a TYG agar plate and cultivated overnight at 30°C with shaking. The pre-culture (from 0 to 800 µL) was used to inoculate experimental cultures in TYG medium (final volume 10 mL). After short adaptation phase (1 h at 30°C with shaking, 200 rpm) the cultures (200 µL) were transferred into wells of a sterile 96-well plate and the wells were sealed using a Breathe-Easy film. The plate was taken out of the anaerobic cabinet and transferred into a NEPHELOstar plate reader. The cultures were incubated at

30°C for 24 h with orbital shaking (shaking width 4 mm). The light scattering in the wells was measured every 20 min (2 s per well, period delay 0.5 s). The gain was set at 40, with the laser beam focus at 2.5 mm. The average of growth curves of the triplicate cultures are presented in Fig. 8.8.

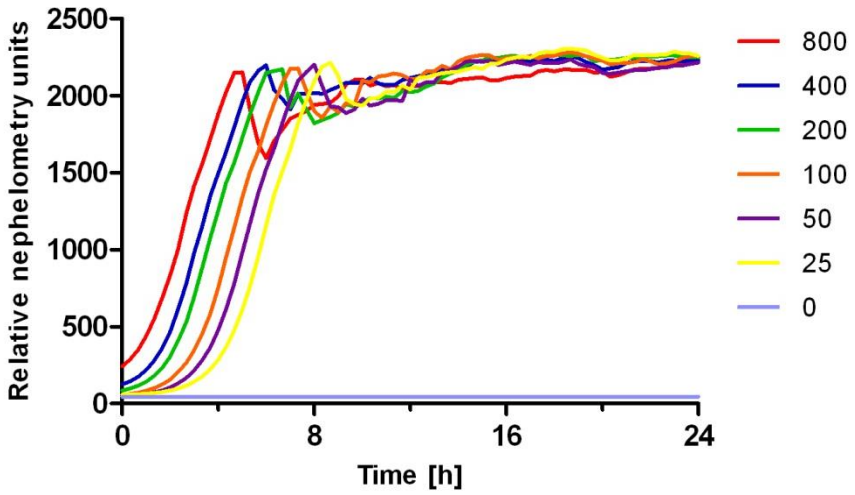

Fig. 8.8. Growth curves of *C. sporogenes* DSM795 obtained from monitoring cell growth by laser nephelometry. The series present the volume of the pre-culture used for inoculation of the experimental culture. The data present an average of at least three independent samples.

Reproducible growth curves were obtained up to the value of 2000 relative nephelometry units. Later, some fluctuation of the growth curves occurred probably due to flocculation of high density microbial cultures (characteristic for *C. sporogenes* DSM795) and condensation of water beneath the Breathe-Easy film. The described application showed that NEPHELOstar can be used to successfully and rapidly monitor *C. sporogenes* cell growth in 96-well plates.

8.3. Dry-weight biomass calibration curve for *C. sporogenes*

To determine the standard biomass calibration curve, a 500 mL culture of *C. sporogenes* DSM795 was maintained until the early stationary phase of growth. The culture was centrifuged at 10000 rpm for 10 min, the residual medium was decanted and the cells were resuspended in deionised water to the theoretical OD_{660nm} value 10 based on the OD readings of the growing culture (UVmini 1240, Shimadzu). A series of dilutions was prepared in a range of optical density values from 0.5 to 8. OD_{660nm} of samples was measured again using the UV spectrophotometer to avoid pipetting errors leading to volume discrepancies. The samples were transferred to pre-dried and pre-weighed 27 mL glass vials. The vials were dried to constant weight (for about 48 h) and the OD values were plotted against the dry-weight values for each sample (Fig. 8.9.).

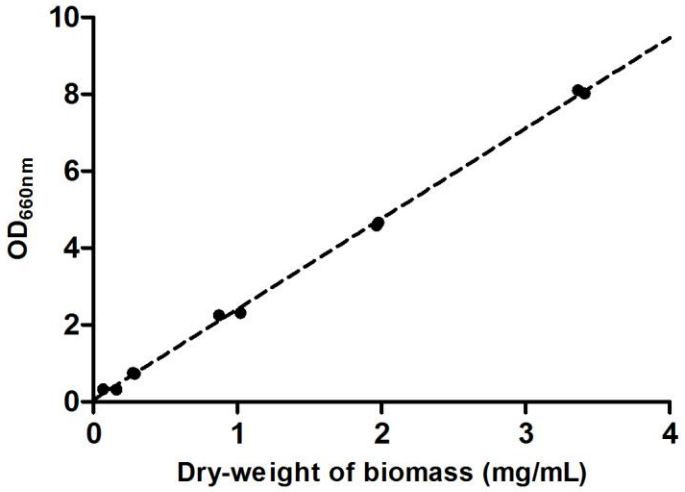

Fig. 8.9. Standard curve of optical density versus cell dry weight of *C. sporogenes* DSM795.

By linear regression it was shown that 1 mg/mL of the *C. sporogenes* dry biomass equals to 2.3 units of OD_{660nm}. This value is similar to the data obtained by Dipeolu, 2005 and Mordaka, 2010.

8.4. Protein assay calibration curve (an example)

Total protein concentration in the samples was determined by using DC Protein Assay (Bio-Rad). Bovine serum albumin protein standard (BSA; Bio-Rad or Sigma) was dissolved in the same buffer as used for the preparation of experimental samples. Some of the protein extraction buffers contained different concentrations of reducing agents (such as DTT, THP), cofactors (FMN, FAD) or detergents (BugBuster), which changed the sensitivity and background of the assay. To avoid errors, the calibration curve was freshly prepared for each experiment together with the unknown protein samples. BSA (2 mg) was dissolved in the buffer (1 mL) and a series of dilutions was prepared (from 0.2 to 1.5 mg/mL). Each sample (0.1 mL) was transferred into a clean and dry 15 mL test tube and mixed with Reagent A' (0.5 mL) and Reagent B (4 mL). After 15 min incubation, absorbance at 750 nm was determined using the spectrophotometer (Agilent 8453). An example of the protein assay calibration curve is presented in Fig. 8.10.

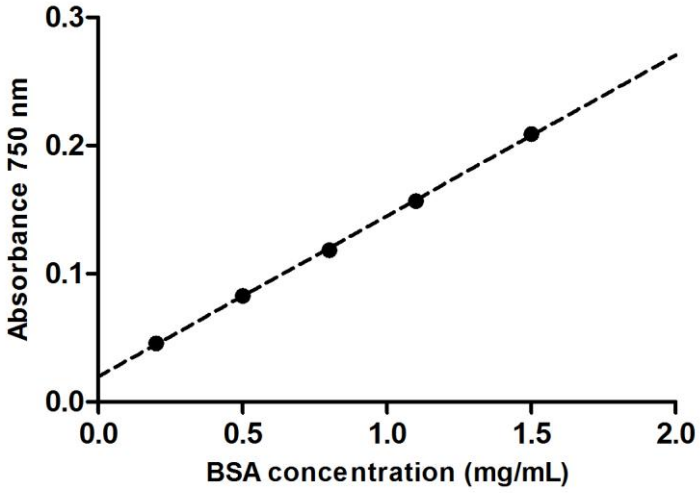

Fig. 8.10. An example of the protein assay calibration curve. The trend line equation was determined to be y = 0.125x + 0.019.

The trend line was determined using a linear regression approach. The concentrations of the unknown protein samples in the range between 0.2 and 1.5 mg/mL were calculated based on the equation of the trend line.

8.5. Calibration curves for cinnamic acid and 3-phenylpropionic acid

Calibration curves of cinnamic and 3-phenylpropionic acid were prepared according to the method presented in section 4.2.8.2.

1. Calibration curve for cinnamic acid (derivatized with (trimethylsilyl)diazomethane, peak area was normalized to the peak area of the internal standard, *tert*-butylbenzene):

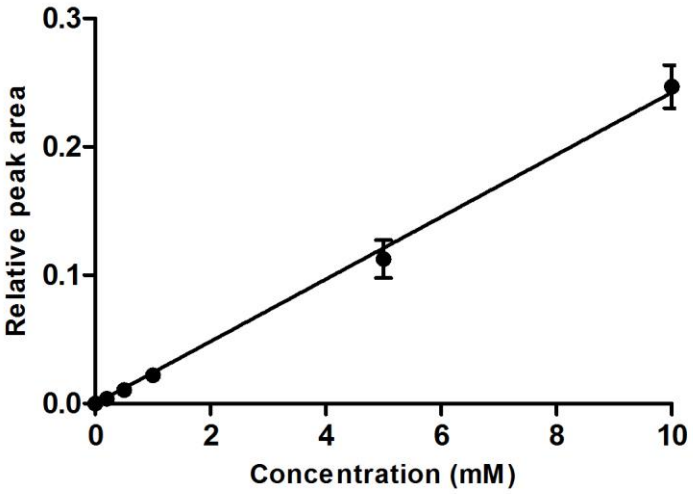

Fig. 8.11. Calibration curve for cinnamic acid.

2. Calibration curve for 3-phenylpropionic acid (derivatized with (trimethylsilyl) diazomethane, peak area was normalized to the peak area of the internal standard, *tert*-butylbenzene):

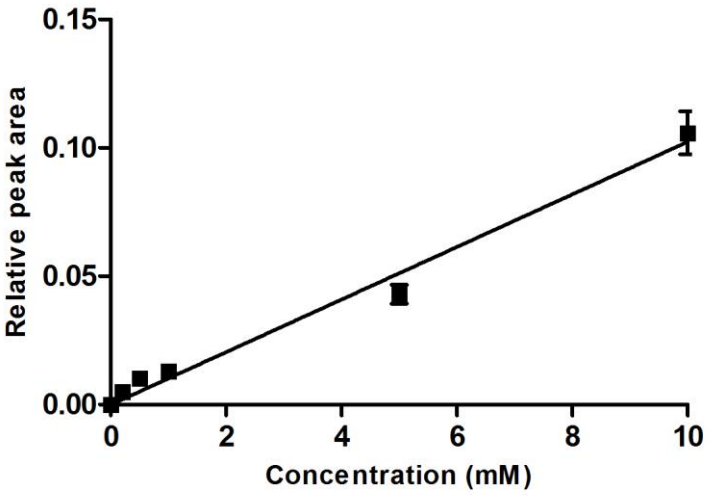

Fig. 8.12. Calibration curve for 3-phenylpropionic acid.

8.6. Calibration curves for aromatic nitro reduction

Calibration curves for substrates and products of the aromatic nitro reduction biotransformation were prepared as described in section 4.2.8.4. The peak areas were normalized to the peak area of the internal standards, limonene (for heptane fraction) or 3,4-diaminotoluene (for aqueous fraction).

1. Calibration curves for nitrobenzene and aniline:

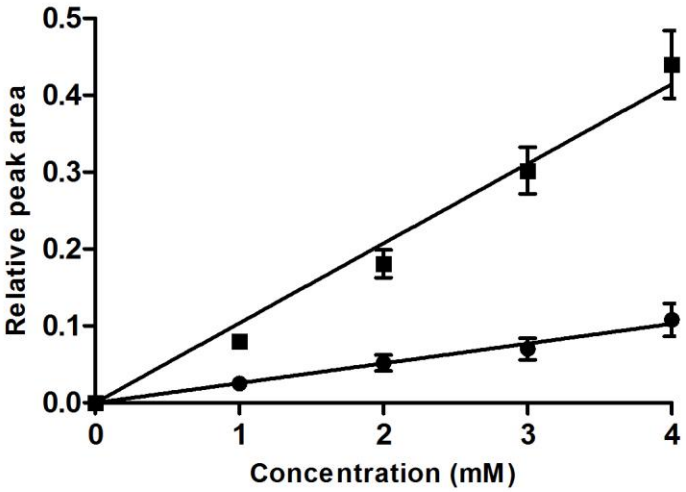

Fig. 8.13. Calibration curve for aniline (●) and nitrobenzene (■) in the heptane phase.

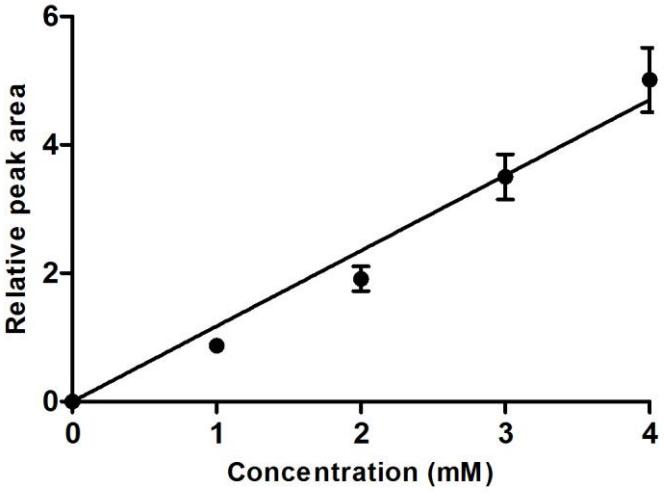

Fig. 8.14. Calibration curve for aniline (●) in the aqueous phase.

2. Calibration curve for 2-chloronitrobenzene and 2-chloroaniline:

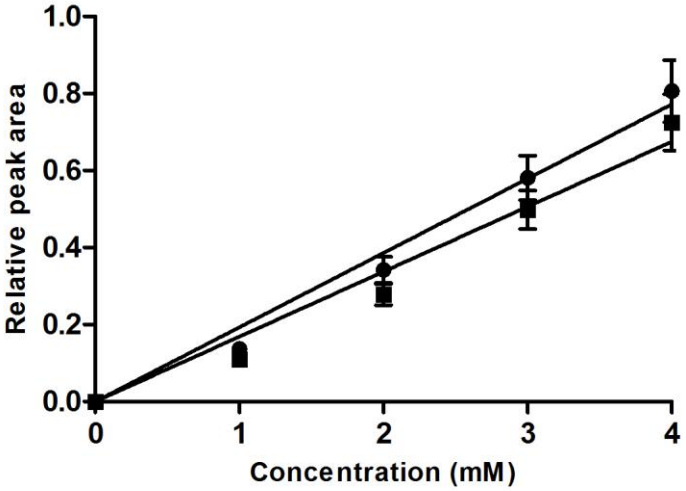

Fig. 8.15. Calibration curve for 2-chloroaniline (●) and 2-chloronitrobenzene (■) in the heptane phase.

3. Calibration curve for 3-chloronitrobenzene and 3-chloroaniline:

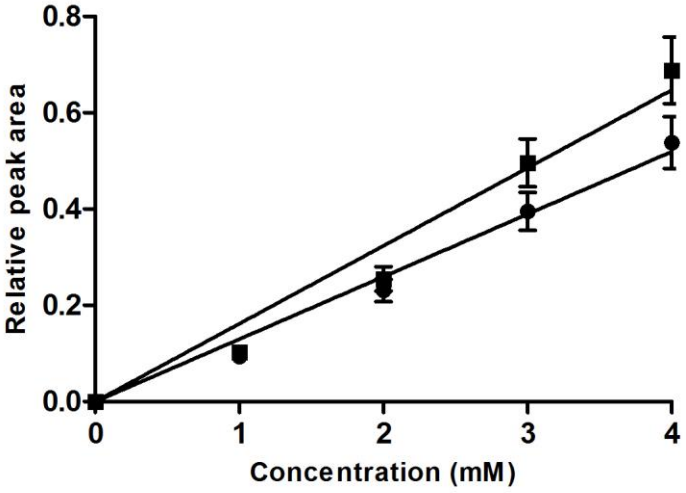

Fig. 8.16. Calibration curve for 3-chloroaniline (●) and 3-chloronitrobenzene (■) in the heptane phase.

4. Calibration curve for 4-chloronitrobenzene and 4-chloroaniline:

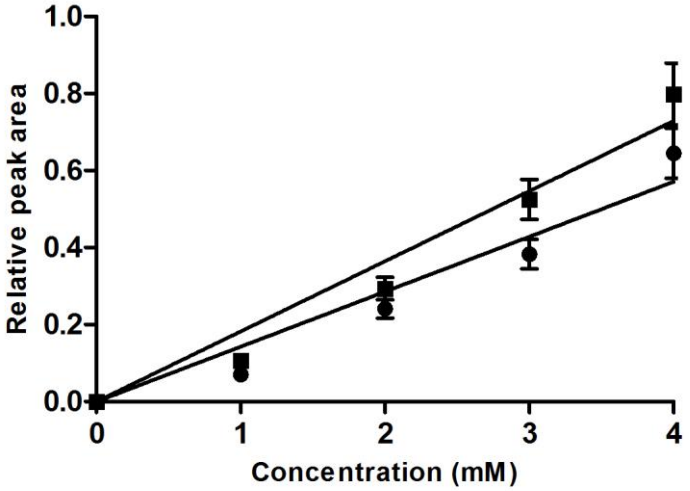

Fig. 8.17. Calibration curve for 4-chloroaniline (●) and 4-chloronitrobenzene (■) in the heptane phase.

5. Calibration curves for 2,4-dinitrotoluene and 2,4-diaminotoluene:

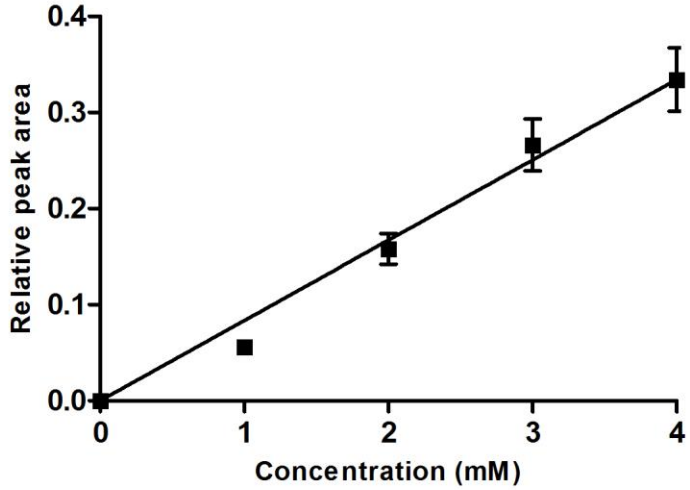

Fig. 8.18. Calibration curve for 2,4-dinitrotoluene (■) in the heptane phase.

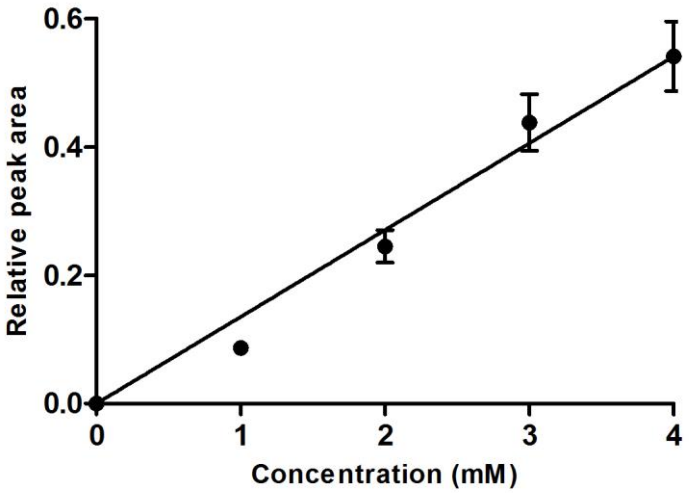

Fig. 8.19. Calibration curve for 2,4-diaminotoluene (●) in the aqueous phase.

6. Calibration curves for 1-chloro-2,4-dinitrobenzene and 4-chloro-1,3-diaminobenzene:

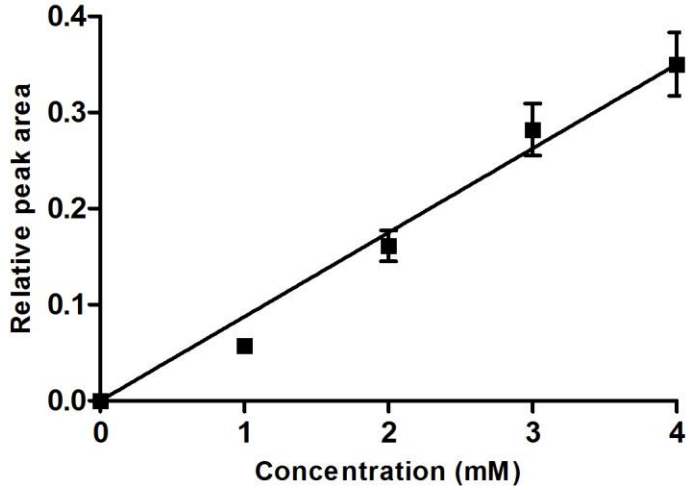

Fig. 8.20. Calibration curve for 1-chloro-2,4-dinitrobenzene (■) in the heptane phase.

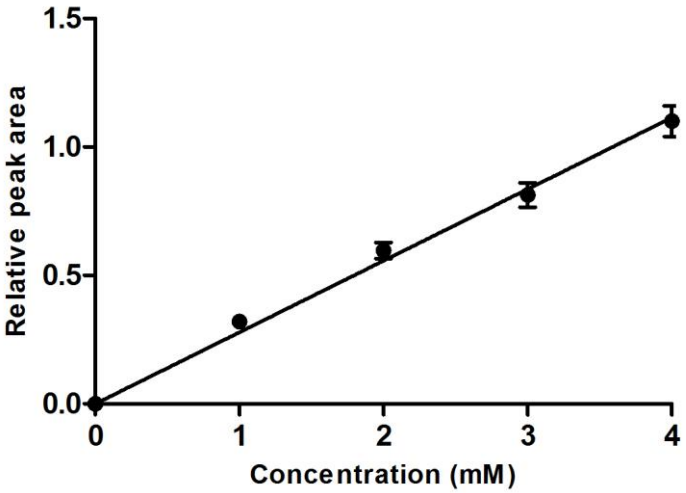

Fig. 8.21. Calibration curve for 4-chloro-1,3-diaminobenzene (●) in the aqueous phase.

7. Calibration curves for *p*-nitroanisole and *p*-anisidine:

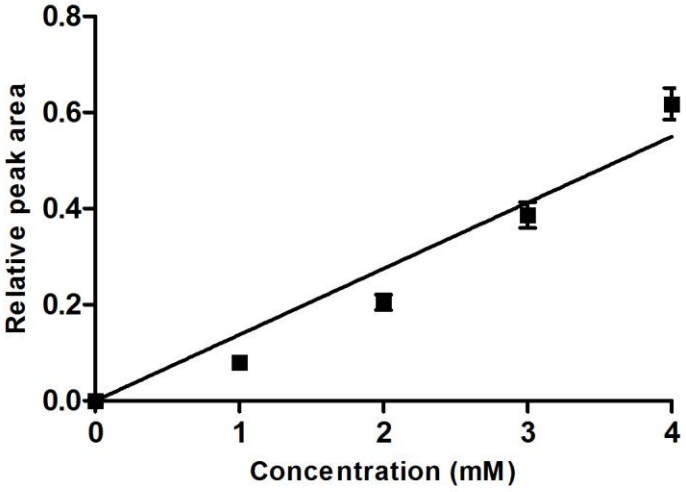

Fig. 8.22. Calibration curve for *p*-nitroanisole (■) in the heptane phase.

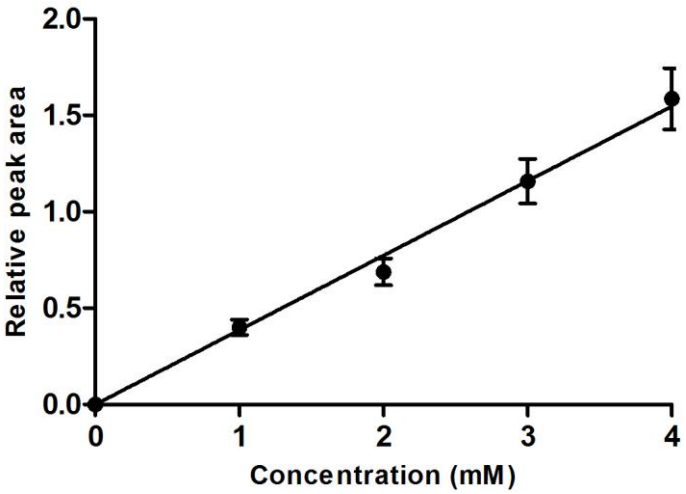

Fig. 8.23. Calibration curve for *p*-anisidine (●) in the aqueous phase.

8.7. Partial purification of *(E)*-2-nitro-1-phenylpropene reductase from *C. sporogenes*

An attempt to purify the reductase was performed by Dr. Karl Fisher, Manchester Interdisciplinary Biocentre, University of Manchester, UK. *C. sporogenes* DSM795 was grown in the Giesel medium (2 L) supplemented with 12.1 mM L-phenylalanine as the growth substrate. Overnight culture was harvested and cell-free protein extracts were prepared using a French press under a flow of nitrogen. To the extract solid ammonium sulphate was added until 56% saturation. The suspension was stored for 1 h and centrifuged at 26500 x g for 20 min. The supernatant was loaded onto DEAE-Sepharose column and the enzyme was separated by elution with the elution buffer (50 mM potassium phosphate buffer, pH 7.5; 0.1 mM EDTA; sucrose, 85.6 g/L; 0.01% v/v 2-mercaptoethanol). The protein fractions showing activity towards 2-nitro-1-phenylpropene in the spectrophotometric reduction assay were applied to hydrophobic interaction chromatography (HIC) column. The proteins were eluted using a gradient 0-100% B of elution buffers A (20 mM sodium phosphate, pH 7.0 + 1.0 M ammonium sulphate) and B (20 mM sodium phosphate, pH 7.0). Protein fractions (7.5 mL) from A1 to C12 were tested in the biphasic phenylnitroalkene reduction biotransformation (Fig. 8.24).

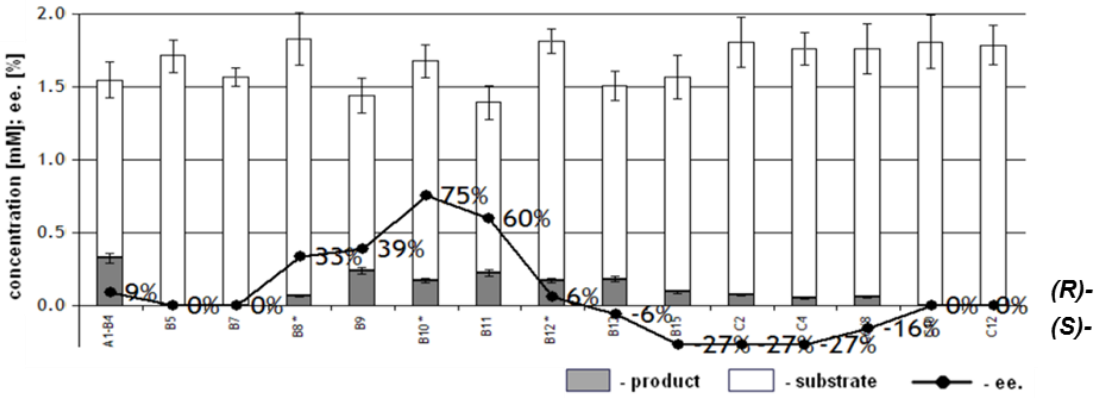

Fig. 8.24. Results of the phenylnitroalkene reduction biotransformation. Protein fractions from A1 to C12 were used to reduce 1.7 mM *(E)*-2-nitro-1-phenylpropene in the expense of NADH. Columns represent product and substrate concentrations after 24 h. The line represents the enantiomeric excess of the products.

Further steps of the 2-nitro-1-phenylpropene reductase purification and determination of its amino acid sequence were unsuccessful due to a loss of the enzymatic activity. However, results of the phenylnitroalkene biotransformation after purification using a HIC column showed that *C. sporogenes* DSM795 possessed multiple enzymes reducing 2-nitro-1-phenylpropene with different enantioselectivities.

8.8. Identification of *fldZ* gene in *C. sporogenes*

8.8.1. Fragment of *C. sporogenes* fldZ gene identified during creation of the fldZ enoate reductase mutant:

```
>Csp795.fldZ.949bp
TCCATGCATATGGATCAAAAATATTTTTACAATTATCAGCAGGCTTTGGTAGAGTAAGTATACCATCTATTGTAGG
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TAAGATTTTTGAAGATAAATATGATAAAGTTAGACCTTGTTTATCTTGTCAAGAAGGGTGTATGGGAAGATTACAG
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AGATTAAGAAAGTTCTTGTTGTAGGTGGCGGTGTTGC
```

8.8.2. Amino acid sequence encoded by Csp.fldZ.949bp DNA fragment:

```
>Csp795.fldZ.949bp
HAYGSKIFLQLSAGFGRVSIPSIVGKVAVAPSKIPHRFLPGVTCRELTTEEVKEYVKAFGESAEIAKKAGFDGVEI
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EKGRDIPEGIEAAKILVAAGYDALNGDVGSYDSWYWSHPPMYQKKGLYLPYNEILKKVVDVPIITAGRMEDPELSS
DAILSGKTDMIALGRPLLADAEIPNKIFEDKYDKVRPCLSCQEGCMGRLQNFATVSCAVNPACGREKEYGLKKAEQ
IKKVLVVGGGV
```

8.8.3. Amino acid sequence of fldZ enoate reductase from *C. sporogenes* ATCC15579 (CLOSPO_02780)

```
>Csp15579.fldZ
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LRYSIKSFIKDWCKGGLPDEEFEEKGRDIPEGIEAAKILVSAGYDALNGDVGSYDSWYWSHPPMYQKKGLYLPYNE
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TVSCAVNPACGREKEYGLKKAEQIKKVLIVGGGVAGMEAARVAAIRGHKVTLIEKNGYLGGNIVPGGVPDFKDDDR
ALVKWYEGILKDLGVEIKLNVDASKENIKEFGADEVLLATGSSPRTLTIEGADKVYSAEDVLMERKNVGEKVIVIG
GGLVGCETALWLKQQGKEVTIVEMQNDILQVGGPLCHANHDMLNDLIKFNKIDVKTSSYITKKTDKGFVLNTDGQE
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```

8.8.4. FldZ enoate reductase gene from *C. sporogenes* ATCC15579:

```
>Csp15579.fldZ
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```

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TGAAGTAGCTAAAAATATTTAA

8.8.5. Sequences of fldZ genes with flanking regions from *C. sporogenes* ATCC15579, *C. sporogenes* NCIMB10696 and *C. botulinum* ATCC3502:

>Csp10696.fldZ
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GTCATGCAAACCACGATATGCTTGTTGATTTAATTAAATTCAATAAGATTGATGTTAAGACAAGCTCCTATATCAG
CAAGAAAACAGATGAAGGATTTGTTTTAAATACAAATGGAGAAGAATCAATTATTAATGCTGATAGTGCTGTTGTA
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ATGCTAATAAAGTTCAAAATATTATGTATGCTATTTGGAGTGCATATGAAGTCGCTAAAAATATTTAAATAAACTT
TTGCAAAAAACTAGAATGGGTTTATATACATATCTAGTTTTTTGTTTTTTACTGAAATTCTATAAGTATATGAGTG
TTTGTTATATATTTATTATAAAATACTAATTATTTAAAAATTTATTTAATGAATCATTATCTTTAAATAGGTTTCT
TAAGTTCCCATATTTTTGTTCTAAATTTTTTCTATCATATTTATAGGATTTTTCTCCTATATTCTCTACATTAAAT
TTAATAATTTCAGCATTTGATATAAGATATAATAATACCACTGCATTCTTTTCTAAAAAATTGTTAGCTTTTTTAT
CATTCCAAAATTTATTATAATTTTCTTCTCCTAAATTTTCATTTGTTTTATAGTTAACGGTTATTTCATAAGGTTC
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TTATCACCTACATATGTGTCTTTATATTTTATCAAATCATATGTTTCAACCTTAGAACTATTCTTCGCATTCTCCT
TTAATTTTTTTTCAGAATTTACTCCACAACCTATTAAATTTAATGATAGGATGAGTACTAAAGGTACTAATATTTT
AGACTTGTTTTTCATATAATGCCTCCTGTTTTACAATTATATATTAGATTATGCTGTAAGTATAGTTGGATTATAA
CATTAATTACAATATAATGGTATCCATTTTTAGTGGTCATTTTGACATGGGTATTGAATATATTTACTTTTAATAA
AATTAATATTATTTTAGAATTAAGCAAAAGAAGAATATTTTATAATCTTTACGGTCAGTTTTAAAAATAGCAAAAT
AAGAACAGAAAAATATTTTGTTTTGTTATATCATAATAATAGCTTTATAAAGTAGGTGAGTATTTAATGAATTATA
GAAAAGATATAGAAAATTGTATTGATTATATAGAAGAGCATATTAAAGAGCCTCTTACACTTAAAGAAATTACACA
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AGAAAAAGGAGACTTTCTTTATCTACTATAGATTTATTAGAAGGTAAAAAAATAATAGATGTTGCCTTTAAGTGGG
GATTTGAAACTCCAAGTAGTTTTGCAAGAGCCTTTAGAAAAGAGTTTGGATGTAGTCCAAGTCAATATATTAAAAA
AATGAAATCCTATTACAAAAGTGAAAATAATTTAATCCTAAGTAAGTTTATGAATGACCCATATATTGTTAAAAAG
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TTTGGAGCAATTATAATGGAGAAAATTTAGAAAGTAAACTATATAAAATTTTATCACCATTAAAACATGGGGAAGT
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ACAGTTATGGATATTTATGTGCCAATAAAAAAGT

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CGTAGTTCACAAAAGATAATTCATGAATGGGTAAAAGCAATGCCAACAGTTCAGCTGGGGGTATTTGATTCAAGAA
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TAGTTGAGGTTGAAAAGGGAGCAAAATTACATCCATATATAGAATTGTGGATTTCGATAAAAATATAACAATATCC
TCTTGAAAGTAAAGCTGCTTTATGCTCTATACTGAAGGTGACAGGAAAATAAAAAAATACAAATGTGAATTCAGAA
AGGAGAAAGAAAATGAAGGATAAGTATAAGGTACTTTATGACCCAATTAAAATTGGAAAATTGGAGATTAAAAATA
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TTATGTAGAACGTGCAAAAGGCGGAACTGGATTAATTATGACAGGTGTAACAATGGTAGAAAATAATATTGAAAAA
TGTGCCCTGCCATCTATGCCATGTCCAACAATTAACCCTTTAAACTTTATTACAACAGGTAACGAAATGACAGAAA
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GAAGAAGTAAAAGAATATGTAAAAGCATTTGGTGAATCAGCAGAAATTGCAAAAAAAGCTGGATTTGATGGTGTGG
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TGGTGGATCATTAGAAAATCGTTTAAGATTTGCCTGTGAAGTTGTACAAGAGATTAAGAAATGTTGTGGACAAGAC
TTCCCAGTTTCACTTAGATACAGTATTAAGAGCTTCATTAAGGATTGGTGTAAGGGCGGCTTACCGGATGAAGAAT
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CCATACAATGAAATACTTAAAAAGGTAGTAGACGTACCTATTATTACAGCAGGAAGAATGGAAGATCCTGAACTAT
CAAGTGATGCAATTTTGTCAGGAAAAACAGATATGATTGCTTTAGGAAGACCACTTCTTGCAGATGCAGAAATTCC
AAATAAAATCTTTGAAGATAAATATGATAAAGTTAGACCTTGCTTATCTTGTCAGGAAGGATGTATGGGAAGATTA
CAGAATTTTGCAACAGTATCCTGTGCAGTAAATCCTGCCTGTGGACGTGAAAAGGAGTATGGACTAAAAAAAGCAG
AACAGATTAAGAAAGTTCTTATTGTAGGTGGCGGTGTTGCAGGAATGGAAGCCGCAAGAGTTGCAGCTATTCGTGG
ACATAAAGTAACATTGATTGAAAAGAATGGTTATCTTGGCGGAAATATTGTACCAGGAGGAGTTCCAGATTTCAAA
GATGACGATCGTGCACTTGTTAAGTGGTATGAAGGCATATTGAAAGATTTAGGTGTTGAAATAAAATTAAATGTGG
ATGCATCAAAGGAAAATATCAAAGAATTTGGAGCTGATGAAGTGCTTTTAGCAACAGGTTCTAGTCCAAGAACATT
GACTATTGAAGGAGCTGATAAGGTTTATTCAGCAGAAGATGTATTAATGGAAAGAAAAAATGTTGGTGAAAAGGTT
ATCGTAATTGGTGGAGGACTTGTTGGATGTGAAACAGCTCTTTGGTTAAAACAACAAGGTAAAGAGGTTACAATTG
TAGAGATGCAAAATGATATCCTGCAAGTAGGTGGACCTTTATGCCATGCAAACCATGATATGCTTAATGATTTAAT
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GATGGACAAGAATCAATTATTAATGCTGATAGTGCCGTTGTAGCTATTGGATATTTATCTGAAAAAGACTTATATA
GTGAAGTTAGATTTGATATTCCAAATGCAAGGCTGATTGGAGATGCTAATAAAGTTCAAAATATTATGTATGCTAT
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TTAAAAATTTATTTAGTGAGTCATTATCTTTAAATAAGTTTTTTAAGTTTCCATATTTTTGTTCTAAATTTTTTCT
ATCATATTTAAAGGATTCTTCTCCTATATTGTCTACATTAAACTTAACAATTTCAGCGTTTGGTATAAGAGATAAT
AGTACCACCGCAATCTTTTCTAAAAGCTTGTTAGCTTTTTTGTCACTCCAAAATTTATTATAATTTTCTTCACCTA
AATTTTCATTTGCTTTATAGTTAACAGTTATTTCATAGGGCTCTTTACTAGTTTGTAGACTAAATCCAGCACTATA
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CTAAATTTAATGATAGGATTATTATTAAAGGTATTAATATTTTAAGTTTGTTTTTCATATAATGCCTCCTATTTTG
CAATGTATTGTATCACAATCATACATTAGATTAAGGTGTAACTATAGTTGGATTATAGCATTAATTGCAATATAAT
GGTATCCATTTTTAGTTGTTATTTTCACATGGGTATTGAACATATTCATTTTTAATAAAATTAATATTATTTTAGA
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ACATTTTGTTTTGTTATATTATAATAATAGCTTTATAAAGTAGGTGAGTATTTAATGAATTATAGAAAAGATATAG
AAAATTGTATTGATTATATAGAAGAGCATATTAAAGAGCCCCTTACACTTAAAGAAATTACACAAGAAATAGGGTA
CTCATCATATCATTTCTGCAGAGTTTTTTCTTTTTTAAAGGGTATGCCCTTAATGGAATATGTAAGAAAAAGAAAA
CTTTCTTTATCTACTATAGATTTATTAGAAGGTAAAAAAATAATAGATGTTGCTTTAAAATGGGGATTTGAAACAC
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TTACAAAAGTGAAAATAATTTAATCCTAGGTAAGTTTATGAATGATCCATATATAGTTAAAAAGCATGGATTTAAA
GTTGCTGGATATGGTATTAAAACTAATGTGGCAGATAGTAAATACACAAAGGATGTAGCCTCCTTTTGGAGCAATT
ATAATGGAGAAAATCTAGAAAGTAAACTATACAAAATTCTATCACCCTTAAAACATGGGGAAGTTGGATTATGCAT
ACCTTGTTCTGATAGTGGGGATATAACCTATTTATTAGGGGTTATTGTTGAGGATTTTTCCAAGGTAACTTCTGAT
ATGATAACTGTTGAAGTACCAAAAGCCCAGTATGGAGTATTTACTACAAATCCTGTTAATACAGTAGATAGCAAAG
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CGAATACGATGAAGAAAAATTGGATTTTGAATTTTATGATGAAAGATGTCATTTAAGACCAGACACAGTTATGGAT
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ATGAGTTACTACATCCTCATGCACCAGAAGATGAGCTGCAATTTTATTTACAATATGCAAAAAAGGAGATGAAAAT
ACTAGAACCACTTTGTGGAAGTGGTAGATTTCTAGTACCTTTTTTAGAAAGAGGCTTT

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TAAATTATTTTAATAAAGCAGAAGGATAATCTTTTAACAAATACCTAAAGTGACTTTAGGCAGAAAGGAGAGAATT
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TACAATTGTAATGGCAGATGAGGACTTCACACAACAACCACAGAAGGTTTTTAAAAAGGCATCTGAATTTGCAATA
AAAAAAGGTCTTGAAATAGGCGAAATAGCTTGGGGAAAGATATTATTAGTTGAGGTTGAAAAGGGAGCAAAATTAC
ATCCATATATAGAATTGTGGATTTCGATAAAAATATAACAATATCCTCTTGAAAGTAAAGCTGCTTTATGCTCTAT
ACTGAAGGTGACAGGAAAATAAAAAAATACAAATGTGAATTCAGAAAGGAGAAATAAAATGAAGGATAAGTATAAG
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GAATGTGTAACGCCGATGGTAGTTTTAATAAAAGAGGAATTGAGTTTTATGTAGAACGTGCAAAAGGCGGAACTGG
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GGTGAGTCAGCAGAAATTGCAAAAAAAGCTGGATTTGATGGTGTAGAAATTCACGCAGTACATGAAGGATACTTAT
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TGCCTGTGAAGTTGTACAAGAGATTAAGAAACGTTGTGGACAAGACTTCCCAGTTTCACTTAGATACAGTATTAAG
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GTGGACCTTTATGTCATGCAAACCATGATATGCTTGTTGATTTAATTAAATTCAATAAGATTGATGTTAAGACAAG
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AGTGCTGTTGTAGCTATTGGATATTTATCTGAAAAAGACTTATATAGTGAAGTTAGATTTGATATCCCAAATGCAA
GACTTATTGGAGATGCTAATAAAGTTCAAAATATTATGTATGCTATTTGGAGTGCATATGAAGTAGCTAAAAATAT
TTAAATGAACCTTTGTAAAAACTAGAATGGATTTATATCCATATCTAGTTTTTGCTCTTTACTGAAATTCTATAAG
TATATGGGTGTTTGTTATGTACTATTTGTGTATATTTAAAGCTTTCCAAGTGAACTCAAATTCAAAGCCTATTCTA
AGCATTGATATATAAGGATTAGAATAGGTTTTTATTATGTATTTTTTAAAAAGCAGAATCACTTGGAAAAATTTCT
TTAAAACCTTGTAAAATCAATGGGTATATGATATTCTTAGAAATAAGGAATGCCTATTTTACTATGATTGAACAAT
AACATAGGATGTATTTAAATTCTTTATATAGATGCATATCTTCTAATACTCCACCTGTTGAACATTAACATGAGAT
GTATTTAAGCAGGGAATCCAAATTCCTGATTTGGTATGAGTCGCTTACTCCTTCGAAAAATAGAAGAAGTAGTTTT
ATTAATGAACATAGTGTATATATAATTAAATTTTTCCAAGTGAATTTAATCTTCAAACCTATCCTACCCATTGAAA
TATAAGGATTAGGATAGGTTTTTATTGTGTATTTATGAAAAAACAAAATCACTTGGAAAAATTTCTACAAAACCTT
GTAAAATCAATGGATATATGATATTCTTGTAAATAAAGAATGGCTATTTTACTATGGTTGAACAGTAACATAGGAT
GTATTTAAATATTTGTCCAAATAACAATATTTATCACCCCCTATTCGTGTTGAACAGTAACATAAGATGTATTTAA
ATTTTCTATTTTCTTTAAAAAATTGCCTATCTACATTTGTTGAACAGTAATATAGAATGTATTTAAATATAGATAA
ACAACTCTGAAGATATGCTTTGCTTACCAGTTGAACCTTAACATGATATGTATTTAAGCAGAGAACCCAAATCTGT
GATTTGGTGTGAATCGCTTACTCCTTCGAGAAGAGGAGAAGGAGTTTCATTAAATAATAATTAGACACTTAGTATT
TTAAGCTTAAGTATGATCTGAAAAGGTTGAACATTAACATGAGATGTATTTAAATTATTTACATTCTGTTATACCA
CAACATTTACAACATGTTGAACAATAACATAAGATGTATTTAAGCAGAGAACTCAACTTTACGATTTGGAGTAAGT
TGTTTATTTCCTGTAGAGGAGAAGTATGAGACTCATTAATGAGCATAGTATATATATCATATCTAATTAAATTTTT
CCAAGTGAATTTAAATTTAAAACCTATTCTAGCCATCGATATGTAAGGATTAGGGTAGATTTTTATTATGTATTTA

TGAAAAATAAGAATCGGTTGGGAAAATTTCTTTAAAACCTTGTAAAATCAATGTGTATATGCTATTCTTAAAAATA
AGGAATGGCTATTTTACTATGGTTGAACATTAACATGAGATGTATTTAAATTCTAAATCTTTTGCACTTGCTAATT
TACCTTTTTCGTTGAACATTAACATGAAATGTATTTAAATTTAATATATTTACCAAGGTTTTATATTATCATACTT
AAGTTGAACATTAACATGAGATGTATTTAAATACTACTGCAGTAACGGAATGTGGTGGCAAACCTAATGTTGAACA
TTAACATGAGATGTATTTAAATAAGGGTTCTCCACACTCTAGTTCTTTCATAAGTTCAGTTGAACATTAACATGAG
ATGTACTTAAGCAAGAAATGCAATCATCTGATTTAGAATAATCATTTATACATGGCAATAATTTTTTGCTTTATTA
CTTCTTGAGTTAAATTGTGGGATTTTATTGACTAAAATATTTTTAAAATATAGTTTTAATTTTGGTTGTGCTTTAA
AGTAGTGTTGGAATTTTAAACCCCATTAAAAGAAGTTAAATATTTTCTTTTAATGGGGTTTATATAAATTGTAAGA
TACTAATTATTTAAAAATTTATTTAATGAATCATTATCTTTAAATAAGTCTTTTAAGTTACCGTATTTTTGTCCTA
AATTTTTTCTATCATATTTATAGGATTC

8.8.6. Consensus sequence of fldZ-ORF product (of 5 PCR products that were sequenced):

>fldZ-ORF
GAAGATCCATGGCTATGAAGGATAAGTATAAGGTACTTTATGACCCAATTAAAATTGGAAAATTGGAGATTAAAAA
TAGATATGTTCTTGCTCCAATGGGACCAGGAGGAATGTGTAACGCCGATGGCAGTTTTAATAAAAGAGGAATTGAG
TTTTATGTAGAACGTGCAAAAGGCGGAACTGGATTAATTATGACAGGTGTAACAATGGTGGAAAACAATATTGAAA
AATGTGCCCTGCCATCCATGCCATGTCCAACAATTAACCCTCTAAACTTTATTACAACAGGTAATGAAATGACAGA
AAGAGTTCATGCATATGGATCAAAAATATTTTTACAATTATCAGCAGGCTTTGGTAGAGTAAGTATACCATCTATT
GTAGGAAAAGTGGCAGTAGCACCTTCTAAAATTCCACATAGATTTTTACCAGGAGTAACTTGTCGTGAGTTAACTA
CAGAAGAAGTAAAAGAATATGTAAAAGCCTTTGGTGAATCAGCAGAAATTGCAAAAAAAGCCGGATTTGATGGTGT
AGAAATTCATGCAGTACATGAAGGATACTTATTAGATCAATTTGCTATTTCTTTCTTTAACCATCGTACTGATGAA
TATGGTGGATCATTAGAAAATCGTTTAAGATTTGCCTGTGAGGTTGTACAAGAAATTAAGAAACGTTGTGGACAAG
ACTTCCCAGTTTCACTTAGATACAGTATAAAAAGCTTCATTAAGGATTGGTGTAAGGGTGGCTTACCAGATGAAGA
ATTTGAAGAAAAAGGAAGAGATATTCCAGAAGGAATTGAAGCTGCTAAAATACTTGTTGCAGCAGGATATGATGCT
TTAAATGGAGATGTTGGGTCTTATGATTCATGGTATTGGAGTCATCCACCAATGTATCAAAAGAAGGGATTATACC
TTCCATACAATGAAATACTTAAAAAGGTAGTAGATGTACCTATTATTACAGCAGGAAGAATGGAAGACCCTGAACT
ATCAAGTGATGCAATTTTGTCAGGAAAAACAGATATGATTGCTTTAGGAAGGCCACTTCTTGCAGATGCAGAAATT
CCAAATAAGATTTTTGAAGATAAATATGATAAAGTTAGACCTTGTTTATCTTGTCAAGAAGGGTGTATGGGAAGAT
TACAGAATTTTGCAACAGTATCCTGTGCAGTAAATCCTGCCTGTGGACGTGAAAAAGAGTATGGACTAAAAAAAGC
AGAACAGATTAAGAAAGTTCTTGTTGTAGGTGGCGGTGTTGCAGGAATGGAAGCTGCAAGAGTTACAGCTGTTCGT
GGACACAAAGTAACATTGATTGAAAAGAATGGTTATCTTGGTGGAAATATTGTACCAGGAGGAATTCCAGATTTCA
AAGATGATGATCGTGCACTTGTTAAATGGTATGAAGGAATATTGAAAGATTTAGGTGTTGAAATAAAATTAAATGT
GGGTGCATCAAAGGAAAATATCAAAGAATTTGGAGCGGATGAAGTGCTTTTAGCAACAGGCTCTAGTCCAAGAACA
TTGACTATTGAAGGAGCAGATAAGGTTTATTCAGCAGAAGATGTGTTAATGGAAAGAAAAACTGTTGGTGAAAAAG
TTATTGTGATTGGTGGAGGACTTGTTGGATGTGAAACAGCTCTTTGGTTAAAACAACAAGGTAAAGAGATTACAAT
TGTAGAGATGCAAAATGATATCCTGCAAGTAGGTGGACCTTTATGTCATGCAAACCACGATATGCTTGTTGATTTA
ATTAAATTCAATAAGATTGATGTTAAGACAAGCTCCTATATCAGCAAGAAAACAGATGAAGGATTTGTTTTAAATA
CAAATGGAGAAGAATCAATTATTAATGCTGATAGTGCTGTTGTAGCTATTGGATATTTATCTGAAAAAGACTTATA
TAGTGAAGTTAGATTTGATATTCCAAATGCAAGACTAATTGGAGATGCTAATAAAGTTCAAAATATTATGTATGCT
ATTTGGAGTGCATATGAAGTAGCTAAAAATATTGCGGCCGCTTATCTTGCTGAAA

Consensus sequence of fldZ-5’ product (of 3 PCR products that were sequenced):

>fldZ-5’
GATGGATGTACTACAAGTGCTATTCACTATTTTGAAAAAGAACATTTAATTGAAGTGGAAAAAGGAAAAAATGGTC
ATCGTTATTATAATGTTGTAGATGTATTTCGATTGCTTTCTTATACAAAGTATCGTTCTATGGAAATACCTATGAA
AACTATTATTGCACAATTTGGCGGGGAAGAAAATAATTATAAATTAATAGAAAAAAGAGAAACAATGTATCAATTA
GAGGCCTTAAAAAGAGCCCAATATTATATGAATTTAGCAGATGCTATTGGGGAACATCTTGTTAGCATAAGAAGAA
TTGAAGAACTATTGAATAAGTATGAATTTGCAAAGTCACCTGAAGTAACGATTATGTGTGATGATGAGTGTGGGTG
GCTTTCAAAAAAGCGTAGTTCACAAAAGATAATTCATGAATGGGTAAAAGCAATGCCAACAGTGCAGCTAGGGGTA
TTTGATTCAAGAATGGGAATGAGTAACTTTGGATATTTGGTAAAAACTAAAAAGCGAGAAGAATTAGAACTTCCGC
TAGGGTTATATGCCAAAGAGCTAAAAAGTACATCTTGTATACATACAATTGTAATGGCAGATGAAGATTTCACACA
ACAACCACAGAAGGTTTTTAAAAAGGCTTCTGAATTTGCAATAAAAAAAGGTCTTGAAATAGGTGAAATAGCCTGG
GGAAAGATATTATTAGTTGAGGTTGAAAAGGGAGCTAAATTGCATCCATACATAGAATTATGGATTTCGATAAAAA
TATAACAATATCCTCTTGAAAGTAAAGCTACTTTATGCTCTATACTGAAGGTAACAGGAAAATAAAAAAATACAAA
TGTGAATTCAGAAAGGAGAAAGAAAATGAAGGATAAGTATAAGGTACTTTACGACCCAATTAAAATTGGAAAATTG
GAGATTAAAAATAGATATGTTCTTGCTCCAATGGGACCAGGAGGAATGTGTAACGCCGATGGCAGTTTTAATAAAA
GAGGAATTGAGTTTTATGTAGAACGTGCAAAAGGCGGAACTGGATTAATTATGACAGGTGTAACAATGGTGGAAAA
CAATATTGAAAAATGTGCCCTGCCATCCATGCCATGTCCAACAATTAACCCTCTAAACTTTATTACAACAGGTAAT
GAAATGACAGAAAGAGTTCATGCATATGGATCAAAAATATTTTTACAATTATCAGCAGGCTTTGGTAGAGTAAGTA
TACCATCTATTGTAGGATCGTCTAGA

Consensus sequence of fldZ-3’ product (of 3 PCR products that were sequenced):

>fldZ-3’
GATCTATTATTACAGCAGGAAGAATGGAAGACCCTGAACTATCAAGTGATGCAATTTTGTCAGGAAAAACAGATAT
GATTGCTTTAGGAAGGCCACTTCTTGCAGATGCAGAAATTCCAAATAAGATTTTTGAAGATAAATATGATAAAGTT
AGACCTTGTTTATCTTGTCAAGAAGGGTGTATGGGAAGATTACAGAATTTTGCAACAGTATCCTGTGCAGTAAATC
CTGCCTGTGGACGTGAAAAAGAGTATGGACTAAAAAAAGCAGAACAGATTAAGAAAGTTCTTGTTGTAGGTGGCGG
TGTTGCAGGAATGGAAGCTGCAAGAGTTACAGCTGTTCGTGGACACAAAGTAACATTGATTGAAAAGAATGGTTAT
CTTGGTGGAAATATTGTACCAGGAGGAATTCCAGATTTCAAAGATGATGATCGTGCACTTGTTAAATGGTATGAAG
GAATATTGAAAGATTTAGGTGTTGAAATAAAATTAAATGTGGGTGCATCAAAGGAAAATATCAAAGAATTTGGAGC
GGATGAAGTGCTTTTAGCAACAGGCTCTAGTCCAAGAACATTGACTATTGAAGGAGCAGATAAGGTTTATTCAGCA
GAAGATGTGTTAATGGAAAGAAAAACTGTTGGTGAAAAAGTTATTGTGATTGGTGGAGGACTTGTTGGATGTGAAA
CAGCTCTTTGGTTAAAACAACAAGGTAAAGAGATTACAATTGTAGAGATGCAAAATGATATCCTGCAAGTAGGTGG
ACCTTTATGTCATGCAAACCACGATATGCTTGTTGATTTAATTAAATTCAATAAGATTGATGTTAAGACAAGCTCC
TATATCAGCAAGAAAACAGATGAAGGATTTGTTTTAAATACAAATGGAGAAGAATCAATTATTAATGCTGATAGTG
CTGTTGTAGCTATTGGATATTTATCTGAAAAAGACTTATATAGTGAAGTTAGATTTGATATTCCAAATGCAAGACT
AATTGGAGATGCTAATAAAGTTCAAAATATTATGTATGCTATTTGGAGTGCATATGAAGTCGCTAAAAATATTTAA
ATAAACTTTTGCAAAAAACTAGAATGGGTTTATATACATATCTAGTTTTTTGTTTTTTACTGAAATTCTATAAGTA
TATGAGTGTTTGTTATATATTTATTATAAAATACTAATTATTTAAAAATTTATTTAATGAATCATTATCTTTAAAT
AGGTTTCTTAAGTTCCCATATTTTTGTTCTAAATTTTTTCTATCATATTTATAGGATTTTTCTCCTATATTCTCTA
CATTAAATTTAATAATTTCAGCATTTGATATAAGATATAATAATACCACTGCATTCTTTTCTAAAAAATTGTTAGC
TTTTTTATCATTCCAAAATTTATTATAATTTTCTTCTCCTAAATTTTCATTTGTTTTATAGTTAACGGTTATTTCA
TAAGGTTCTTTAGTAGTTTGTAGACTAAATCCAGCACTATCTTCT

8.8.7. *C. sporogenes* DSM795 *fldZ* gene:

>Csp795.fldZ-full.sequence
GGATGTACTACAAGTGCTATTCACTATTTTGAAAAAGAACATTTAATTGAAGTGGAAAAAGGAAAAAATGGTCATC
GTTATTATAATGTTGTAGATGTATTTCGATTGCTTTCTTATACAAAGTATCGTTCTATGGAAATACCTATGAAAAC
TATTATTGCACAATTTGGCGGGGAAGAAAATAATTATAAATTAATAGAAAAAAGAGAAACAATGTATCAATTAGAG
GCCTTAAAAAGAGCCCAATATTATATGAATTTAGCAGATGCTATTGGGGAACATCTTGTTAGCATAAGAAGAATTG
AAGAACTATTGAATAAGTATGAATTTGCAAAGTCACCTGAAGTAACGATTATGTGTGATGATGAGTGTGGGTGGCT
TTCAAAAAAGCGTAGTTCACAAAAGATAATTCATGAATGGGTAAAAGCAATGCCAACAGTGCAGCTAGGGGTATTT
GATTCAAGAATGGGAATGAGTAACTTTGGATATTTGGTAAAAACTAAAAAGCGAGAAGAATTAGAACTTCCGCTAG
GGTTATATGCCAAAGAGCTAAAAAGTACATCTTGTATACATACAATTGTAATGGCAGATGAAGATTTCACACAACA
ACCACAGAAGGTTTTTAAAAAGGCTTCTGAATTTGCAATAAAAAAAGGTCTTGAAATAGGTGAAATAGCCTGGGGA
AAGATATTATTAGTTGAGGTTGAAAAGGGAGCTAAATTGCATCCATACATAGAATTATGGATTTCGATAAAAATAT
AACAATATCCTCTTGAAAGTAAAGCTACTTTATGCTCTATACTGAAGGTAACAGGAAAATAAAAAAATACAAATGT
GAATTCAGAAAGGAGAAAGAAAATGAAGGATAAGTATAAGGTACTTTATGACCCAATTAAAATTGGAAAATTGGAG
ATTAAAAATAGATATGTTCTTGCTCCAATGGGACCAGGAGGAATGTGTAACGCCGATGGCAGTTTTAATAAAAGAG
GAATTGAGTTTTATGTAGAACGTGCAAAAGGCGGAACTGGATTAATTATGACAGGTGTAACAATGGTGGAAAACAA
TATTGAAAAATGTGCCCTGCCATCCATGCCATGTCCAACAATTAACCCTCTAAACTTTATTACAACAGGTAATGAA
ATGACAGAAAGAGTTCATGCATATGGATCAAAAATATTTTTACAATTATCAGCAGGCTTTGGTAGAGTAAGTATAC
CATCTATTGTAGGAAAAGTGGCAGTAGCACCTTCTAAAATTCCACATAGATTTTTACCAGGAGTAACTTGTCGTGA
GTTAACTACAGAAGAAGTAAAAGAATATGTAAAAGCCTTTGGTGAATCAGCAGAAATTGCAAAAAAAGCCGGATTT
GATGGTGTAGAAATTCATGCAGTACATGAAGGATACTTATTAGATCAATTTGCTATTTCTTTCTTTAACCATCGTA
CTGATGAATATGGTGGATCATTAGAAAATCGTTTAAGATTTGCCTGTGAGGTTGTACAAGAAATTAAGAAACGTTG
TGGACAAGACTTCCCAGTTTCACTTAGATACAGTATAAAAAGCTTCATTAAGGATTGGTGTAAGGGTGGCTTACCA
GATGAAGAATTTGAAGAAAAAGGAAGAGATATTCCAGAAGGAATTGAAGCTGCTAAAATACTTGTTGCAGCAGGAT
ATGATGCTTTAAATGGAGATGTTGGGTCTTATGATTCATGGTATTGGAGTCATCCACCAATGTATCAAAAGAAGGG
ATTATACCTTCCATACAATGAAATACTTAAAAAGGTAGTAGATGTACCTATTATTACAGCAGGAAGAATGGAAGAC
CCTGAACTATCAAGTGATGCAATTTTGTCAGGAAAAACAGATATGATTGCTTTAGGAAGGCCACTTCTTGCAGATG
CAGAAATTCCAAATAAGATTTTTGAAGATAAATATGATAAAGTTAGACCTTGTTTATCTTGTCAAGAAGGGTGTAT
GGGAAGATTACAGAATTTTGCAACAGTATCCTGTGCAGTAAATCCTGCCTGTGGACGTGAAAAAGAGTATGGACTA
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CTGTTCGTGGACACAAAGTAACATTGATTGAAAAGAATGGTTATCTTGGTGGAAATATTGTACCAGGAGGAATTCC
AGATTTCAAAGATGATGATCGTGCACTTGTTAAATGGTATGAAGGAATATTGAAAGATTTAGGTGTTGAAATAAAA
TTAAATGTGGGTGCATCAAAGGAAAATATCAAAGAATTTGGAGCGGATGAAGTGCTTTTAGCAACAGGCTCTAGTC
CAAGAACATTGACTATTGAAGGAGCAGATAAGGTTTATTCAGCAGAAGATGTGTTAATGGAAAGAAAAACTGTTGG
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ATTACAATTGTAGAGATGCAAAATGATATCCTGCAAGTAGGTGGACCTTTATGTCATGCAAACCACGATATGCTTG
TTGATTTAATTAAATTCAATAAGATTGATGTTAAGACAAGCTCCTATATCAGCAAGAAAACAGATGAAGGATTTGT
TTTAAATACAAATGGAGAAGAATCAATTATTAATGCTGATAGTGCTGTTGTAGCTATTGGATATTTATCTGAAAAA
GACTTATATAGTGAAGTTAGATTTGATATTCCAAATGCAAGACTAATTGGAGATGCTAATAAAGTTCAAAATATTA
TGTATGCTATTTGGAGTGCATATGAAGTAGCTAAAAATATTTAAATAAACTTTTGCAAAAAACTAGAATGGGTTTA
TATACATATCTAGTTTTTTGTTTTTTACTGAAATTCTATAAGTATATGAGTGTTTGTTATATATTTATTATAAAAT

ACTAATTATTTAAAAATTTATTTAATGAATCATTATCTTTAAATAGGTTTCTTAAGTTCCCATATTTTTGTTCTAA
ATTTTTTCTATCATATTTATAGGATTTTTCTCCTATATTCTCTACATTAAATTTAATAATTTCAGCATTTGATATA
AGATATAATAATACCACTGCATTCTTTTCTAAAAAATTGTTAGCTTTTTTATCATTCCAAAATTTATTATAATTTT
CTTCTCCTAAATTTTCATTTGTTTTATAGTTAACGGTTATTTCATAAGGTTCTTTAGTAGTTTGTAGACTAAATCC
AGCACT

8.8.8. *C. sporogenes* DSM795 fldZ reductase amino acid sequence:

>Csp795.fldZ-full.sequence
MKDKYKVLYDPIKIGKLEIKNRYVLAPMGPGGMCNADGSFNKRGIEFYVERAKGGTGLIMTGVTMVENNIEKCALP
SMPCPTINPLNFITTGNEMTERVHAYGSKIFLQLSAGFGRVSIPSIVGKVAVAPSKIPHRFLPGVTCRELTTEEVK
EYVKAFGESAEIAKKAGFDGVEIHAVHEGYLLDQFAISFFNHRTDEYGGSLENRLRFACEVVQEIKKRCGQDFPVS
LRYSIKSFIKDWCKGGLPDEEFEEKGRDIPEGIEAAKILVAAGYDALNGDVGSYDSWYWSHPPMYQKKGLYLPYNE
ILKKVVDVPIITAGRMEDPELSSDAILSGKTDMIALGRPLLADAEIPNKIFEDKYDKVRPCLSCQEGCMGRLQNFA
TVSCAVNPACGREKEYGLKKAEQIKKVLVVGGGVAGMEAARVTAVRGHKVTLIEKNGYLGGNIVPGGIPDFKDDDR
ALVKWYEGILKDLGVEIKLNVGASKENIKEFGADEVLLATGSSPRTLTIEGADKVYSAEDVLMERKTVGEKVIVIG
GGLVGCETALWLKQQGKEITIVEMQNDILQVGGPLCHANHDMLVDLIKFNKIDVKTSSYISKKTDEGFVLNTNGEE
SIINADSAVVAIGYLSEKDLYSEVRFDIPNARLIGDANKVQNIMYAIWSAYEVAKNI

8.9. Identification of the OYE-like reductase gene in *C. sporogenes*

8.9.1. CLOSPO_03444 amino acid sequence:

```
>Csp15579.OYE-like.reductase
MKSLFDKTCIKTMELKNRFVRSATWEGMATEEGHITERLLNLYEELAKGGVGLIITSYTTIFDYDKPSLRILGIYD
DSFIEEYKLLTDTIHKCGAKVLMQIVLGENYINNKTGSEFYGLSENMPEDDIKAIVKSFAEAAKRAKESGFDGIQI
HGAHGYFLSRTLSPLFNKRKDKYGGSVEKRGALILEVYDKIRKTVGEDFHISIKINCSDFEEGGATFKECEFVCRE
LSKKGIDSIEISGGGTIWTETNKKESIYKEYASKIAEQVDTPIILVGMNRSYNNMDQILNNSKIEYFSMARPFIRE
PDLINKFEKDENRKAKCISCGKCYGENGIRCIFNM
```

8.9.2. *C. sporogenes* ATCC15579 gene encoding CLOSPO_03444 reductase:

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>Csp15579.OYE-like.reductase
TTAAATATTAGAATTTTCAACTCTCTCATCATTTTTATATTCACCAATGAAAATTTCACCATTAGAGTGAATGTAC
ATACCTTTTCCATGTCTTAAATCATTTACCCATTGACCAATATATTTATCTCCATGGTTGCAAACATAAACTCCAT
GTCCATGACGAAAATCACAAATCCAATCACCAGTATAAACATCACCATCGGACCAAGTATAAATACCCTGACCGGA
TTTTAAATCATTTTCCCAATATCCAACATAACTTTCTCCATCAGGCCAAGTATATATGCCATATCCATGTTTTTCA
TCATCTTTCCAACTACCAGTATATTTTTCACCAGAAGCCCAAATTAAAACACCTTCACCATGCATCATGTTTTCTT
TCCAGTAACCTACATATTTAGTTCCGTTAGTGTAAGTATATGTACCAAAACCATGCATTTTCCCATCTTTTCTCTC
ACCTTCATATACTCCACTATGACTATGATGATGTTCATTAGAAATAACAGCTTCAGATGCTGTAGCTTTAACACAT
ATAACATTCAATTCATGATTTTTAGGATTTTCTATTTTAAATTTATTCATATTATATACCTTACCTTTCTAATATT
AGTTATATTTATCTAATTTTTTAATATATTTATATAAATGATTTTTCATTTCATTTAATAATGAATAGCAAGTATA
AAATATTATATCATAAAATATTGAAAACAAGATACTAAAAATCAATAATTTTATTGATTTTTTAGTATCTGAAAAT
CTTATTTAATATATTCTGTATGTTTGTTTTAAGAAGCTATTTTATTTTAATAAGTAAGTAAAAATTAAGTTCATTA
ATATTAATATAATTTAAGATTTTTGTATATGAAAACAGGCTAATCATAATCATAATTTCTAATATTGGGTACAATA
AATAAATTTTAACAATAGAATCTTTTAAAAAATAGAGGAAGAGTTAATATATTTAAAGTAATATATAAGAGATAGT
TAAAAAATCTGTTTTAACTCCTTTGCTGAAAGTTCAACATTAAATCAAACTATGCTAATATAATAATATAGAATTG
AAATAAAACAGGGGGAACTTTTATGAAAAGTTTATTTGATAAAACCTGCATAAAAACAATGGAACTTAAAAATAGA
TTTGTTCGTTCAGCCACCTGGGAGGGCATGGCCACAGAAGAAGGACATATCACGGAGAGATTACTTAATTTATATG
AAGAATTAGCAAAGGGTGGAGTTGGATTAATAATAACTAGTTACACTACTATATTTGATTATGATAAGCCAAGTCT
TAGAATCCTTGGAATTTATGATGACAGTTTTATTGAAGAATATAAGCTTTTAACAGATACAATTCATAAATGTGGG
GCTAAAGTATTAATGCAAATAGTCTTAGGCGAGAATTATATTAATAATAAAACTGGTAGTGAATTTTATGGACTAA
GTGAAAATATGCCAGAAGATGATATAAAGGCTATAGTAAAATCTTTTGCTGAGGCAGCTAAAAGGGCTAAAGAGTC
AGGTTTTGATGGAATTCAAATTCATGGAGCACATGGATATTTTTTAAGTAGAACATTAAGTCCTCTTTTTAATAAA
AGAAAGGATAAATACGGTGGTTCAGTGGAAAAGAGAGGTGCATTAATACTAGAAGTTTATGATAAGATAAGAAAAA
CAGTAGGAGAGGATTTTCATATATCCATAAAAATTAATTGTTCTGATTTTGAAGAAGGAGGAGCAACCTTTAAGGA
ATGTGAATTTGTTTGCAGGGAACTTTCTAAGAAAGGTATAGATTCTATAGAAATCAGCGGTGGAGGAACAATTTGG
ACAGAAACTAATAAAAAGGAATCTATATATAAAGAGTATGCTTCCAAAATAGCAGAACAAGTAGATACTCCTATAA
TTTTAGTTGGTATGAATAGAAGTTATAATAATATGGATCAAATATTAAACAATAGTAAGATAGAATATTTTTCAAT
GGCTAGACCTTTTATAAGGGAACCAGATTTAATAAATAAATTTGAAAAAGATGAAAATAGAAAAGCTAAATGTATA
TCCTGTGGGAAATGTTATGGTGAAAATGGAATAAGATGTATATTTAATATGTAAAATAAATATAAGGATGGAGAAT
TCAAGTGAAATTCTTCATCCTTTATTTATGTAAACATTATTTTAAAACATAGCAAATTAATATTTTAAAAACTAAA
GGGTGATAATTACTAAAGTATTAAACAAATACTATAAAAAATTGATATTTTATTAAAAAATATATTGACTTAATGA
TAAAATTACGATAATATCTAATATATAATTAATAAATTGATTTTGGATATAATAATCATAATGTTAGTACTAATAG
GTAATTATTTAATCCTAGATATAAGACTATAGGGTTTATGTAAAATAATTTTGAAGAAAGTTTTAGGAGGGAAACA
TATGATAAAATTTAAAGATAGATTAATGATACCTGAAGGATATGAATCCACATTAGGAATAAGAGAAACAGAGGTT
GCAATAAAAAAGGTTAAAGATTTTTTTGAAAGAACTTTAGCAGAAAAGTTGAACCTTACAAGAGTTTCAGCACCTT
TATTTGTAAGAAAAAATACAGGTATGAATGATAACTTAAATGGAGTAGAAAGACCAGTAGCTTTTGATATGAAAGA
TCTAAAGGAAGAAATGATAGAAATAGTACATTCTTTAGCTAAGTGGAAAAGAATGGCTTTACACAGATATGATTTT
AAAGTTGGTGAAGGTCTTTATACAGATATGAATGCCATAAGAAGAGATGAGGATTTAGATAACCTTCATTCTATAT
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CAAAATATATAGTGTATTTAAGGAAACAGAGAGAGAGATTTGTAGCCAATATGAGGGATTCAATGAAATATTACCA
GAGGAAATATGTTTTATAACTTCTCAAGAACTCGAAGATACTTATCCGGATTTAGATTCAAAGGAAAGAGAAGATG
CTATAACTAAAGAAAAGGGAGCAGTATTTTTAATGGAAATAGGGGGAGTTTTAGCCTCAGGAGAAAAGCATGATGG
AAGAGCTCCAGATTATGATGACTGGACATTGAACGGAGATATATTATTTTGGAATCCAGTATTAGAAAGAGCCTTT
GAATTATCTTCTATGGGGATAAGAGTAGATGAAGAATCTCTAGAAAAACAACTTAAAATAGCAGGTTGTGAAGATA
GAAAAAATCTAGAATTTCATAGATTGCTTTTAGAGGGTAAATTGCCATATACCGTTGGTGGTGGAATTGGACAGTC
AAGAATATGTATGTATTTTTTAAGAAAAGCACATATAGGGGAAGTTCAAGCATCCATATGGCCAGATTTAATGATA
GAGGATTGTGAAAAAGCAAAAATCAACTTGCTATAG
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8.9.3. *C. sporogenes* DSM795 gene encoding CLOSPO_03444 reductase:

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>Csp795.OYE-like.reductase
CATTAAATCAAATTATGCTAATATAATAATATAGAATTGAAATAAAACAGGGGGAACTTTTATGAAAAGTTTATTT
GATAAAACCTGCATAAAAACAATGGAACTTAAAAATAGATTTGTTCGTTCAGCTACTTGGGAGGGTATGGCAACAG
AAGAAGGACATATCACGGAGAGATTACTTAATTTATATGAAGAATTAGCAAAGGGTGGAGTTGGATTAATAATAAC
TAGTTACACTACTATATTTGATTATGATAAACCAAGTCTTAGAATCCTTGGAATTTATGATGACAGTTTTATTAAA
GAATATAAGCTTTTGACAGATACAATTCATAAATATGGAGCTAAAGTATTAATGCAAATAGTCTTAGGAGAAAATT
ATATAAATAATGAAACCGGTAGTGAATTTTACGGATTAAGTGAAAATATGCCAGAAGATGATATAAAGGCTATAGT
GAAATCTTTTGCCGAAGCAGCTAAAAGGGCTAAAGAGTCAGGTTTTGATGGAATTCAAATTCATGGAGCACATGGA
TATTTTTTAAGTAGAACATTAAGTCCTCTTTTTAATAAAAGAAAGGATAAATACGGTGGTTCAGTAGAAAAGAGAG
GTGCATTAATACTAGAGGTTTATGATGAGATAAGAAAAGCAGTGGGAAAAGATTTTCATATATCCATAAAAATTAA
TTGTTCTGATTTTGAAGAGGGAGGAGCAACCTTTAAGGAATGTGAATTTGTTTGCAGGGAACTCTCTAAGAAAGGT
ATAGATTCTATAGAAATCAGCGGTGGAGGAACAATTTGGACAGAAACTAATAAAAAGGAATCTATATATATAGAGT
ATGCTTCCAAAATAGCTGAAGAAGTAGATACTCCCATAATTTTAGTTGGTATGAATAGAAGTTATGACAATATGAA
TGAAATATTAAATAATAGTAAGATCGAATATTTTTCAATGGCTAGACCTTTTATAAGGGAACCGGATTTGATAAAT
AAATTTGAAAAAGGTGAGGATAGAAAAGCTAAATGTATATCCTGTGGAAAATGTTATGGTGAAAATGGAATAAGGT
GCATATTTAATATGTAAAATATATATAAGGATGGAGAATTTAAGTGAAATTCTTCATCCTTTATTTATGTAAACAT
TATTTTAAAACATAGCAAATTAATATTTTAAAAATTAAGGGATGATAATTACTAAAGTATTAAACAAATACTATAA
AAAATTGATATTTTATTAAAAAATATATTGACTTAATGATAAAATTACGATAATATCTAATATATAATTAATAAAT
TGATTTTGGATATAATAATCATAATGTTAGTACTAATAGGTAATTATTTAATCCTATAGGATTTATGTAAAATAAT
TTTGAAGAAAGTTTTAGGAGGGAAACATATGATAAAATTTAAAGATAGATTAATGATACCTGAAGGATATGAATCC
ACATTAGGAATAAGAGAAACAGAGGTTG
```

8.9.4. *C. sporogenes* OYE-like reductase (CLOSPO_03444):

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>Csp795.OYE-like.reductase (CLOSPO_03444)
MKSLFDKTCIKTMELKNRFVRSATWEGMATEEGHITERLLNLYEELAKGGVGLIITSYTTIFDYDKPSLRILGIYD
DSFIKEYKLLTDTIHKYGAKVLMQIVLGENYINNETGSEFYGLSENMPEDDIKAIVKSFAEAAKRAKESGFDGIQI
HGAHGYFLSRTLSPLFNKRKDKYGGSVEKRGALILEVYDEIRKAVGKDFHISIKINCSDFEEGGATFKECEFVCRE
LSKKGIDSIEISGGGTIWTETNKKESIYIEYASKIAEEVDTPIILVGMNRSYDNMNEILNNSKIEYFSMARPFIRE
PDLINKFEKGEDRKAKCISCGKCYGENGIRCIFNM
```

8.10. Identification of the hypothetical cinnamic acid reductase

8.10.1. CLOSPO_00312 acyl-CoA dehydrogenase ATCC15579:

>Csp15579.CLOSPO_00312
MFFTEQHELIRKLARDFAEQEIEPIADEVDKTAEFPKEIVKKMAQNGFFGIKMPKEYGGAGADNRAYVTIMEEISR
ASGVAGIYLSSPNSLLGTPFLLVGTDEQKEKYLKPMIRGEKTLAFALTEPGAGSDAGAVATTAREEGDYYILNGRK
TFITGAPISDNIIVFAKTDMSKGTKGITTFIVDSKQEGVSFGKPEDKMGMIGCPTSDIILENVKVHKSDILGELNK
GFITAMKTLSVGRIGVAAQALGIAQAAVDEAVKYAKQRKQFNRPIAKFQAIQFKLANMETKLNAAKLLVYNAAYKM
DCGEKADKEASMAKYFAAESAIQIVNDALQIHGGYGYIKDYKIERLYRDVRVIAIYEGTSEVQQMVIASNLLK

8.10.2. CLOSPO_00312 acyl-CoA dehydrogenase ATCC15579:

> Csp15579.CLOSPO_00312
ATGTTTTTCACAGAACAACATGAACTTATTAGAAAATTAGCAAGAGATTTTGCAGAGCAGGAAATAGAGCCTATTG
CAGATGAAGTAGATAAAACTGCCGAGTTCCCTAAAGAAATTGTGAAAAAAATGGCCCAAAATGGTTTCTTTGGAAT
AAAAATGCCTAAAGAATATGGTGGAGCTGGAGCAGATAATAGAGCCTATGTAACTATAATGGAAGAGATATCAAGA
GCATCTGGTGTAGCTGGTATATATTTATCATCACCAAATTCATTGCTTGGCACACCATTCCTACTAGTTGGTACAG
ATGAACAAAAGGAAAAATATTTAAAACCAATGATTAGAGGAGAAAAGACATTAGCTTTTGCTTTAACAGAGCCAGG
TGCAGGTTCAGATGCAGGAGCAGTTGCAACAACTGCTAGAGAAGAAGGGGACTATTATATATTAAATGGAAGAAAA
ACTTTTATTACAGGAGCTCCAATATCTGATAATATCATAGTGTTCGCAAAAACAGATATGAGCAAAGGAACAAAGG
GAATTACTACATTTATAGTAGATAGTAAACAAGAAGGTGTATCTTTTGGTAAGCCTGAAGACAAGATGGGGATGAT
TGGTTGCCCTACTAGTGATATTATTTTAGAAAATGTAAAGGTCCATAAATCTGATATACTTGGAGAATTAAATAAA
GGATTTATAACAGCAATGAAGACTTTAAGTGTTGGCCGTATAGGAGTTGCTGCGCAAGCTTTAGGAATAGCACAAG
CTGCTGTAGATGAAGCTGTTAAATATGCAAAACAAAGAAAGCAATTTAATAGACCTATAGCAAAATTCCAAGCTAT
TCAATTTAAATTGGCTAATATGGAAACAAAATTAAATGCAGCAAAATTATTAGTGTATAATGCTGCTTATAAAATG
GATTGTGGAGAAAAGGCAGATAAAGAAGCTTCTATGGCAAAATATTTTGCTGCTGAATCTGCTATACAAATCGTAA
ATGACGCACTACAAATTCATGGTGGTTATGGATACATAAAAGATTATAAAATAGAAAGATTATATAGAGATGTTAG
AGTAATTGCTATATATGAAGGAACTTCTGAAGTTCAACAAATGGTAATCGCATCAAATCTTTTAAAGTAA

8.10.3. CLOSPO_00312 acyl-CoA dehydrogenase DSM795:

> Csp795.CLOSPO_00312
ATGTTTTTCACAGAACAACATGAACTTATTAGAAAATTAGCAAGAGATTTTGCAGAGCAGGAAATAGAGCCTATTG
CAGATGAAGTAGATAAAACTGCCGAGTTCCCTAAAGAAATTGTGAAAAAAATGGCCCAAAATGGTTTTTTTGGAAT
AAAAATGCCTAAAGAATATGGTGGAGCTGGAGCAGATAATAGAGCCTATGTAACTATAATGGAAGAGATATCAAGA
GCATCTGGTGTAGCTGGTATATATTTATCATCACCAAATTCATTGCTTGGCACACCGTTCCTACTAGTTGGTACAG
ATGAACAAAAGGAAAAATATCTAAAACCAATGATTAGAGGAGAAAAAACATTAGCTTTTGCTTTAACAGAACCAGG
TGCAGGTTCAGACGCAGGAGCACTTGCAACAACTGCTAGAGAAGAAGGGGACTATTATATATTAAATGGAAGAAAA
ACTTTTATTACAGGAGCTCCAATATCTGATAATATCATAGTGTTCGCAAAAACAGATATGAGCAAAGGAACAAAGG
GAATTACTACATTTATAGTAGATAGTAAACAGGAAGGTGTATCTTTTGGTAAACCTGAAGACAAGATGGGTATGAT
TGGTTGCCCTACTAGTGATATTATTTTAGAGAATGTAAAGGTTCATAAATCTGATATACTTGGAGAAGTAAATAAA
GGCTTTATAACAGCAATGAAGACTTTAAGTGTTGGTCGTATAGGAGTTGCTTCTCAAGCTTTAGGAATAGCACAAG
CTGCTGTAGATGAAGCTGTTAAATATGCAAAACAAAGAAAACAATTTAATAGACCTATAGCAAAATTTCAAGCTAT
TCAATTTAAATTGGCTAACATGGAAACAAAATTAAATGCGGCAAAATTATTAGTGTATAATGCTGCTTATAAAATG
GATTGTGGAGAAAAGGCAGATAAAGAAGCTTCTATGGCAAAATATTTTGCTGCTGAATCTGCTATACAAATCGTAA
ATGACGCACTACAAATCCATGGTGGTTATGGATACATAAAAGATTATAAAATAGAAAGATTATATAGAGATGTTAG
AGTAATTGCTATATATGAAGGAACTTCTGAAGTTCAACAAATGGTAATCGCATCAAATCTTTTAAAGTAA

8.10.4. CLOSPO_00312 acyl-CoA dehydrogenase DSM795:

> Csp795.CLOSPO_00312
MFFTEQHELIRKLARDFAEQEIEPIADEVDKTAEFPKEIVKKMAQNGFFGIKMPKEYGGAGADNRAYVTIMEEISR
ASGVAGIYLSSPNSLLGTPFLLVGTDEQKEKYLKPMIRGEKTLAFALTEPGAGSDAGALATTAREEGDYYILNGRK
TFITGAPISDNIIVFAKTDMSKGTKGITTFIVDSKQEGVSFGKPEDKMGMIGCPTSDIILENVKVHKSDILGEVNK
GFITAMKTLSVGRIGVASQALGIAQAAVDEAVKYAKQRKQFNRPIAKFQAIQFKLANMETKLNAAKLLVYNAAYKM
DCGEKADKEASMAKYFAAESAIQIVNDALQIHGGYGYIKDYKIERLYRDVRVIAIYEGTSEVQQMVIASNLLK

8.11. Sequencing of f*ldZ* expression constructs

>fldZ
ATGAAAGACAAATACAAAGTGCTGTATGACCCGATTAAAATCGGCAAATTGGAAATCAAGAATCGCTATGTCCTGG
CACCTATGGGGCCAGGCGGGATGTGCAATGCAGATGGGTCCTTTAACAAACGTGGGATCGAATTCTATGTTGAACG
CGCAAAAGGCGGTACTGGCCTGATTATGACGGGTGTCACGATGGTGGAAAACAACATTGAGAAATGTGCGCTCCCT
AGCATGCCCTGTCCCACTATCAACCCGCTGAATTTCATTACCACCGGGAACGAAATGACCGAACGTGTGCACGCGT
ACGGTTCGAAAATTTTCCTGCAACTTTCAGCGGGCTTTGGCCGCGTATCCATTCCAAGTATCGTTGGGAAAGTAGC
GGTGGCACCCTCGAAGATCCCGCATCGCTTTCTGCCAGGTGTCACCTGCCGTGAACTGACCACCGAAGAAGTGAAA
GAGTACGTTAAGGCGTTTGGTGAGTCTGCCGAAATCGCGAAGAAAGCGGGTTTTGATGGAGTGGAAATTCATGCCG
TCCACGAAGGCTATCTGCTGGATCAGTTTGCTATCTCTTTCTTTAACCACCGTACCGATGAATATGGCGGTTCTCT
GGAAAACCGCTTACGTTTTGCGTGCGAGGTGGTTCAAGAGATTAAAAAGCGCTGTGGTCAGGACTTCCCGGTTTCT
CTGCGCTACAGCATTAAGAGCTTTATCAAGGACTGGTGCAAAGGAGGCTTACCGGACGAAGAATTCGAAGAGAAAG
GCCGGGATATTCCAGAAGGTATTGAGGCCGCCAAAATTCTGGTGGCTGCGGGGTATGATGCCCTGAACGGCGACGT
GGGCAGTTATGATAGCTGGTATTGGTCGCATCCTCCGATGTACCAGAAAAAAGGGCTCTACTTACCGTATAACGAG
ATTCTGAAAAAGGTGGTGGATGTCCCGATTATCACCGCAGGCCGTATGGAGGATCCAGAACTGAGCAGCGATGCCA
TTCTTTCTGGCAAAACGGACATGATTGCGCTTGGACGCCCGCTCTTGGCTGATGCGGAGATTCCGAATAAAATCTT
TGAAGATAAGTACGACAAAGTCCGCCCGTGCCTTTCATGCCAGGAAGGCTGTATGGGTCGTCTGCAGAACTTTGCT
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AAGTACTGGTGGTTGGCGGCGGTGTAGCGGGTATGGAAGCTGCCCGCGTCACAGCCGTTCGTGGCCACAAAGTGAC
CCTGATTGAAAAGAACGGCTATTTAGGCGGGAATATCGTACCGGGAGGCATTCCGGACTTTAAAGACGATGATCGT
GCACTGGTTAAATGGTATGAAGGCATTCTGAAAGATCTGGGCGTCGAAATCAAACTGAATGTGGGTGCGAGCAAAG
AGAACATCAAAGAGTTCGGGGCTGACGAAGTGCTCTTAGCTACAGGAAGCAGTCCGCGCACATTGACGATTGAGGG
TGCGGACAAAGTTTATTCGGCCGAAGATGTGTTAATGGAACGGAAAACGGTCGGTGAGAAAGTGATCGTTATTGGT
GGCGGCTTGGTTGGTTGCGAAACTGCACTTTGGCTGAAACAACAGGGCAAAGAAATCACCATTGTGGAAATGCAAA
ACGATATCCTGCAAGTGGGTGGACCGCTCTGTCATGCAAACCATGACATGTTGGTTGACTTGATTAAATTTAACAA
AATTGATGTCAAAACTTCCTCGTACATTTCCAAAAAAACGGATGAAGGCTTCGTCTTGAATACGAATGGTGAGGAA
AGCATTATCAATGCCGATAGTGCTGTGGTTGCCATCGGATACCTGTCAGAGAAAGATCTGTACTCCGAGGTACGCT
TCGATATCCCCAATGCCCGTCTGATTGGTGATGCGAATAAGGTCCAGAACATCATGTATGCGATTTGGTCAGCCTA
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>fldZ:pelB
ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGAAAGACA
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GCCAGGCGGGATGTGCAATGCAGATGGGTCCTTTAACAAACGTGGGATCGAATTCTATGTTGAACGCGCAAAAGGC
GGTACTGGCCTGATTATGACGGGTGTCACGATGGTGGAAAACAACATTGAGAAATGTGCGCTCCCTAGCATGCCCT
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>fldZ:NtH
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>fldZ:CtH
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8.12. FldZ expression variants alignment (ClustalW)

```
fldZ            ----------------------MKDKYKVLYDPIKIGKLEIKNRYVLAPMGPGGMCNADG 38
fldZ_NtH        --MGSSHHHHHHSSGLVPRGSHMKDKYKVLYDPIKIGKLEIKNRYVLAPMGPGGMCNADG 58
fldZ_CtH        ----------------------MKDKYKVLYDPIKIGKLEIKNRYVLAPMGPGGMCNADG 38
fldZ_pelB       MKYLLPTAAAGLLLLAAQPAMAMKDKYKVLYDPIKIGKLEIKNRYVLAPMGPGGMCNADG 60
                                       ************************************

fldZ            SFNKRGIEFYVERAKGGTGLIMTGVTMVENNIEKCALPSMPCPTINPLNFITTGNEMTER 98
fldZ_NtH        SFNKRGIEFYVERAKGGTGLIMTGVTMVENNIEKCALPSMPCPTINPLNFITTGNEMTER 118
fldZ_CtH        SFNKRGIEFYVERAKGGTGLIMTGVTMVENNIEKCALPSMPCPTINPLNFITTGNEMTER 98
fldZ_pelB       SFNKRGIEFYVERAKGGTGLIMTGVTMVENNIEKCALPSMPCPTINPLNFITTGNEMTER 120
                ************************************************************

fldZ            VHAYGSKIFLQLSAGFGRVSIPSIVGKVAVAPSKIPHRFLPGVTCRELTTEEVKEYVKAF 158
fldZ_NtH        VHAYGSKIFLQLSAGFGRVSIPSIVGKVAVAPSKIPHRFLPGVTCRELTTEEVKEYVKAF 178
fldZ_CtH        VHAYGSKIFLQLSAGFGRVSIPSIVGKVAVAPSKIPHRFLPGVTCRELTTEEVKEYVKAF 158
fldZ_pelB       VHAYGSKIFLQLSAGFGRVSIPSIVGKVAVAPSKIPHRFLPGVTCRELTTEEVKEYVKAF 180
                ************************************************************

fldZ            GESAEIAKKAGFDGVEIHAVHEGYLLDQFAISFFNHRTDEYGGSLENRLRFACEVVQEIK 218
fldZ_NtH        GESAEIAKKAGFDGVEIHAVHEGYLLDQFAISFFNHRTDEYGGSLENRLRFACEVVQEIK 238
fldZ_CtH        GESAEIAKKAGFDGVEIHAVHEGYLLDQFAISFFNHRTDEYGGSLENRLRFACEVVQEIK 218
fldZ_pelB       GESAEIAKKAGFDGVEIHAVHEGYLLDQFAISFFNHRTDEYGGSLENRLRFACEVVQEIK 240
                ************************************************************

fldZ            KRCGQDFPVSLRYSIKSFIKDWCKGGLPDEEFEEKGRDIPEGIEAAKILVAAGYDALNGD 278
fldZ_NtH        KRCGQDFPVSLRYSIKSFIKDWCKGGLPDEEFEEKGRDIPEGIEAAKILVAAGYDALNGD 298
fldZ_CtH        KRCGQDFPVSLRYSIKSFIKDWCKGGLPDEEFEEKGRDIPEGIEAAKILVAAGYDALNGD 278
fldZ_pelB       KRCGQDFPVSLRYSIKSFIKDWCKGGLPDEEFEEKGRDIPEGIEAAKILVAAGYDALNGD 300
                ************************************************************

fldZ            VGSYDSWYWSHPPMYQKKGLYLPYNEILKKVVDVPIITAGRMEDPELSSDAILSGKTDMI 338
fldZ_NtH        VGSYDSWYWSHPPMYQKKGLYLPYNEILKKVVDVPIITAGRMEDPELSSDAILSGKTDMI 358
fldZ_CtH        VGSYDSWYWSHPPMYQKKGLYLPYNEILKKVVDVPIITAGRMEDPELSSDAILSGKTDMI 338
fldZ_pelB       VGSYDSWYWSHPPMYQKKGLYLPYNEILKKVVDVPIITAGRMEDPELSSDAILSGKTDMI 360
                ************************************************************

fldZ            ALGRPLLADAEIPNKIFEDKYDKVRPCLSCQEGCMGRLQNFATVSCAVNPACGREKEYGL 398
fldZ_NtH        ALGRPLLADAEIPNKIFEDKYDKVRPCLSCQEGCMGRLQNFATVSCAVNPACGREKEYGL 418
fldZ_CtH        ALGRPLLADAEIPNKIFEDKYDKVRPCLSCQEGCMGRLQNFATVSCAVNPACGREKEYGL 398
fldZ_pelB       ALGRPLLADAEIPNKIFEDKYDKVRPCLSCQEGCMGRLQNFATVSCAVNPACGREKEYGL 420
                ************************************************************

fldZ            KKAEQIKKVLVVGGGVAGMEAARVTAVRGHKVTLIEKNGYLGGNIVPGGIPDFKDDDRAL 458
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fldZ_CtH        KKAEQIKKVLVVGGGVAGMEAARVTAVRGHKVTLIEKNGYLGGNIVPGGIPDFKDDDRAL 458
fldZ_pelB       KKAEQIKKVLVVGGGVAGMEAARVTAVRGHKVTLIEKNGYLGGNIVPGGIPDFKDDDRAL 480
                ************************************************************

fldZ            VKWYEGILKDLGVEIKLNVGASKENIKEFGADEVLLATGSSPRTLTIEGADKVYSAEDVL 518
fldZ_NtH        VKWYEGILKDLGVEIKLNVGASKENIKEFGADEVLLATGSSPRTLTIEGADKVYSAEDVL 538
fldZ_CtH        VKWYEGILKDLGVEIKLNVGASKENIKEFGADEVLLATGSSPRTLTIEGADKVYSAEDVL 518
fldZ_pelB       VKWYEGILKDLGVEIKLNVGASKENIKEFGADEVLLATGSSPRTLTIEGADKVYSAEDVL 540
                ************************************************************

fldZ            MERKTVGEKVIVIGGGLVGCETALWLKQQGKEITIVEMQNDILQVGGPLCHANHDMLVDL 578
fldZ_NtH        MERKTVGEKVIVIGGGLVGCETALWLKQQGKEITIVEMQNDILQVGGPLCHANHDMLVDL 598
fldZ_CtH        MERKTVGEKVIVIGGGLVGCETALWLKQQGKEITIVEMQNDILQVGGPLCHANHDMLVDL 578
fldZ_pelB       MERKTVGEKVIVIGGGLVGCETALWLKQQGKEITIVEMQNDILQVGGPLCHANHDMLVDL 600
                ************************************************************

fldZ            IKFNKIDVKTSSYISKKTDEGFVLNTNGEESIINADSAVVAIGYLSEKDLYSEVRFDIPN 638
fldZ_NtH        IKFNKIDVKTSSYISKKTDEGFVLNTNGEESIINADSAVVAIGYLSEKDLYSEVRFDIPN 658
fldZ_CtH        IKFNKIDVKTSSYISKKTDEGFVLNTNGEESIINADSAVVAIGYLSEKDLYSEVRFDIPN 638
fldZ_pelB       IKFNKIDVKTSSYISKKTDEGFVLNTNGEESIINADSAVVAIGYLSEKDLYSEVRFDIPN 660
                ************************************************************

fldZ            ARLIGDANKVQNIMYAIWSAYEVAKNI----------- 665
fldZ_NtH        ARLIGDANKVQNIMYAIWSAYEVAKNI----------- 685
fldZ_CtH        ARLIGDANKVQNIMYAIWSAYEVAKNIAAALEHHHHHH 676
fldZ_pelB       ARLIGDANKVQNIMYAIWSAYEVAKNIAAALEHHHHHH 698
                ***************************
```

8.13. Spectral changes during the cinnamic acid spectrophotometric assay

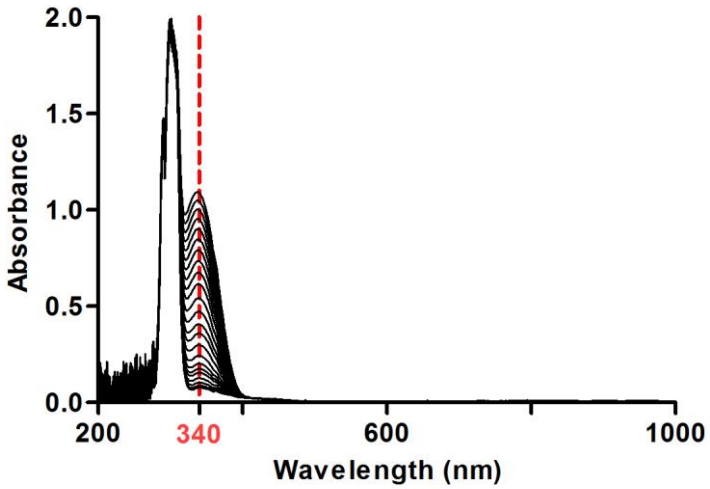

Fig. 8.25. Spectral changes in the cinnamic acid reduction assay using the protein cell-free extract of *E. coli* expressing the wild type fldZ.

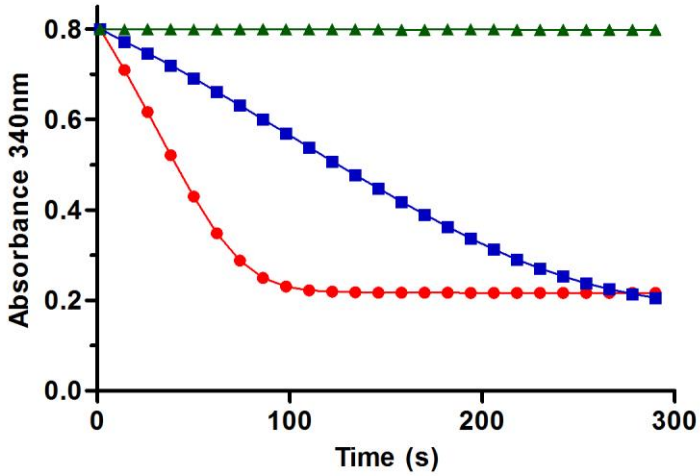

Fig. 8.26. Traces of the cinnamic acid spectrophotometric assay using the protein cell-free extract of *E. coli* expressing fldZ (●), fldZ:NtH (■) and pET20b(+) control (▲).

8.14. Traces of the fldZ activity assay at different temperature and pH

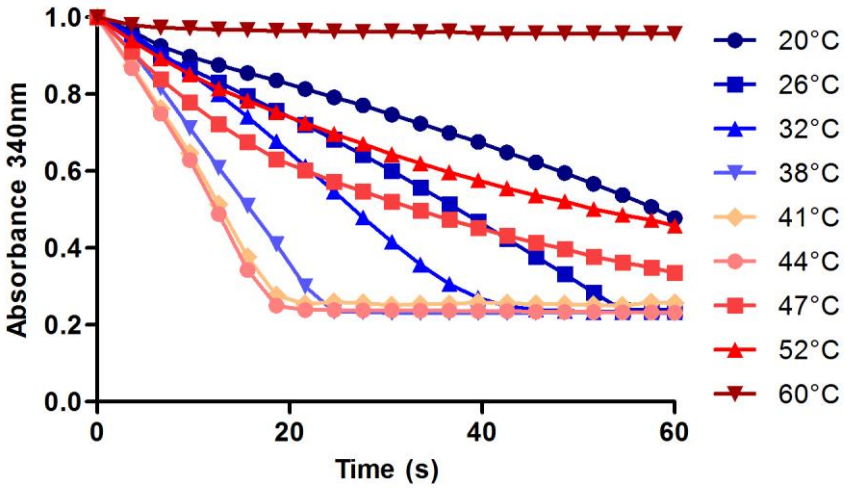

Fig. 8.27. Traces of the cinnamic acid spectrophotometric assay using the protein cell-free extract of *E. coli* expressing fldZ at a range of temperatures. The NADH concentration in the reaction mixture was increased from 0.125 mM to 0.157 mM.

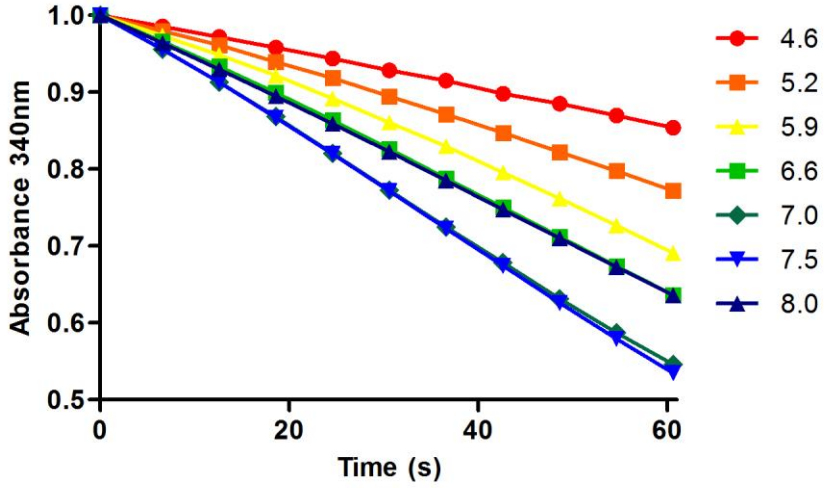

Fig. 8.28. Traces of the cinnamic acid spectrophotometric assay using the protein cell-free extract of *E. coli* expressing fldZ at a range of pH. The NADH concentration in the reaction mixture was increased from 0.125 mM to 0.157 mM.

8.15. Gas chromatograms and mass spectra for biotransformation using fldZ

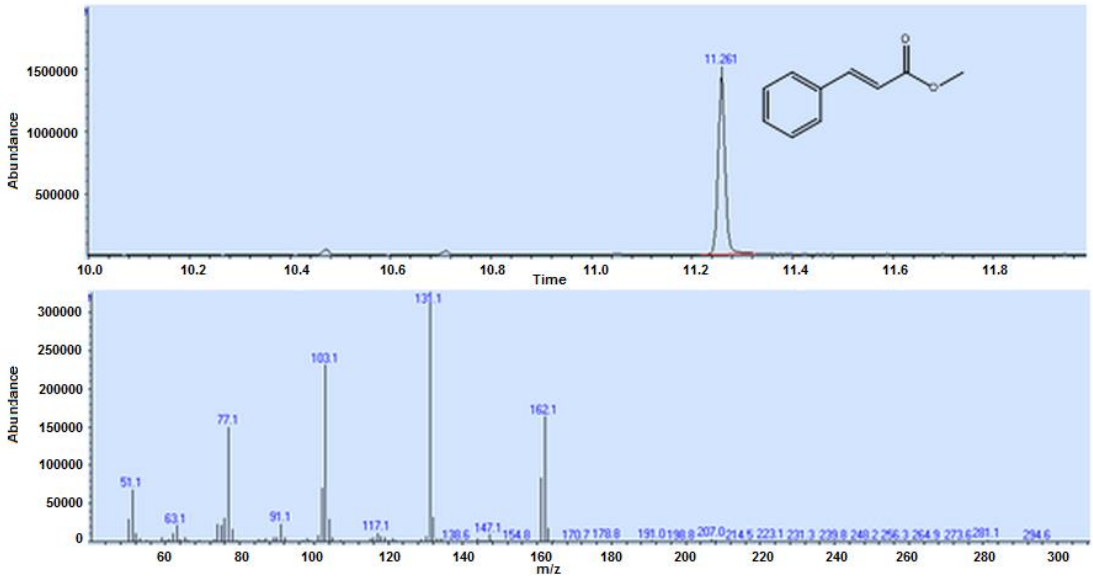

Fig. 8.29. Gas chromatogram of the control pET20b(+) sample collected after 24 h and derivatized with trimethylsilyldiazomethane. Mass spectrum of the cinnamic acid methyl ester peak at 11.261 min.

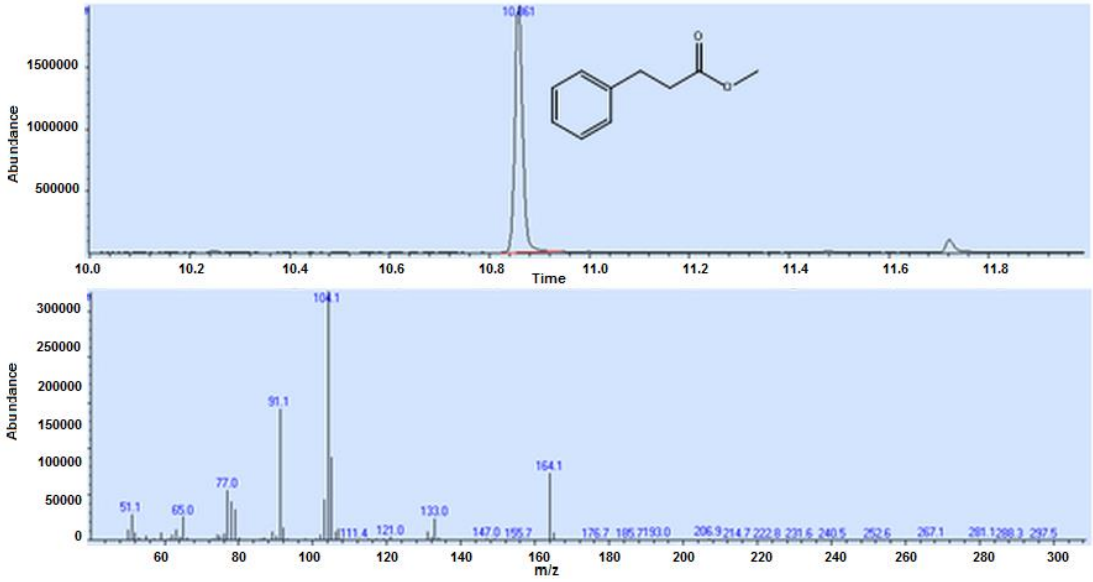

Fig. 8.30. Gas chromatogram of the sample containing the fldZ reductase collected after 24 h and derivatized with trimethylsilyldiazomethane, mass spectrum of the 3-phenylpropionic acid methyl ester peak at 10.861.

8.16. Sequencing of OYE-like reductase expression constructs

8.16.1. OYE nucleotide and amino acid sequences:

```
>OYE
ATGAAAAGTTTATTTGATAAAACCTGCATAAAAACAATGGAACTTAAAAATAGATTTGTTCGTTCAGCTACTTGGG
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AAAATGGAATAAGGTGCATATTTAATATGTAA

>OYE
MKSLFDKTCIKTMELKNRFVRSATWEGMATEEGHITERLLNLYEELAKGGVGLIITSYTTIFDYDKPSLRILGIYD
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HGAHGYFLSRTLSPLFNKRKDKYGGSVEKRGALILEVYDEIRKAVGKDFHISIKINCSDFEEGGATFKECEFVCRE
LSKKGIDSIEISGGGTIWTETNKKESIYIEYASKIAEEVDTPIILVGMNRSYDNMNEILNNSKIEYFSMARPFIRE
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```

8.16.2. OYE:CtH nucleotide and amino acid sequences:

```
>OYE:CtH
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>OYE:CtH
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```

8.17. Identification and overexpression of nitroreductases

8.17.1. *Cs*NTR1 (CLOSPO_01010)

>Csp795.CsNTR1
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AATGGTAA

>*Cs*NTR1
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VADIRSRIKKDIELSLNENSPQLELKQIIRDTSIAIEHLVLSAEKFGLGTCWVAWFTQEEIRPILNIPSDKYVVSI
ITLGYSNELPKDRPHKKLEDIIHYEKW

>CsNTR1-adapted
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AGCTGATCGAAAGTGCCCGTATGGCTCCTTCAGGGAGCAATACCCAACCGTGGCATTTCATCGTGGTCAAATCCGA
CACGACTCGCGAGAAATTGGCGAAAGTGAGCCATAATCAGGGTTGGATGATGGGTGCACCAGTATTCATTGTCTGC
GTTGCAGACATTCGCTCTCGCATTAAGAAAGATATCGAACTGTCGCTGAATGAAAACAGTCCGCAACTGGAGCTCA
AGCAGATTATCCGCGATACGTCTATCGCGATTGAACATCTGGTGTTAAGCGCCGAAAAGTTTGGCTTAGGAACCTG
TTGGGTAGCGTGGTTTACACAGGAAGAAATTCGTCCGATCTTGAACATTCCGAGCGACAAATACGTTGTGTCGATT
ATTACCCTGGGCTATTCCAACGAACTGCCCAAAGATCGTCCACACAAGAAACTTGAAGATATTATCCACTATGAGA
AATGGTAA

8.17.2. *Cs*NTR3 (CLOSPO_01572)

>Csp795.CsNTR3
ATGGATTTTTTAGACTTAGCTAAAGAAAGATATTCTGTAAGAAATTTTGATACTAAAAAGATCGAACAAGAAAAAC
TAGATTTAATTTTAAAAGCAGGACAATTAGCCCCAACTGCTGTAAATTATCAGCCTCAAAGAATTTTAGTAATTGA
AAGTAATGATGCTCTTGCAAAACTAAAAACTTGCACAATTTATCACTTCAATGCACCCATGGCTCTACTTATATGC
GCGGACAAAGATGAAGCCTGGAAACGTAGTTATGACGGAAAAAGCCATACTGATATTGATGGGAGTATTGTTGCTA
CACATATGATGCTACAAGCTGCTGAACTTGGCTTGGGTACAACATGGGTTGGTCATTTTGATCCAAGTGCTATTCG
TAATGCTTTTAGTATACCAGCAAATCTTGAACCAATATGCCTTTTGCCAGTAGGTTACCCAAGTAAGGATGCAAAA
CCAAATCCTAATCACAAAAAAAGAAAAGATATTTCCCAAACTGTTTTTTATAATCATTTTTAA

>*Cs*NTR3
MDFLDLAKERYSVRNFDTKKIEQEKLDLILKAGQLAPTAVNYQPQRILVIESNDALAKLKTCTIYHFNAPMALLIC
ADKDEAWKRSYDGKSHTDIDGSIVATHMMLQAAELGLGTTWVGHFDPSAIRNAFSIPANLEPICLLPVGYPSKDAK
PNPNHKKRKDISQTVFYNHF

>CsNTR3-adapted
ATGGATTTTCTGGATCTGGCCAAAGAGCGTTACAGCGTACGTAACTTCGACACCAAGAAAATCGAACAGGAGAAAC
TGGATCTGATCCTGAAAGCGGGACAATTGGCACCCACCGCAGTGAACTATCAGCCACAGCGCATTCTCGTGATCGA
ATCGAATGATGCGCTTGCTAAACTGAAAACGTGCACCATTTACCACTTCAATGCCCCTATGGCGCTGCTGATTTGT
GCCGATAAAGACGAAGCGTGGAAACGCTCGTATGATGGCAAGAGCCATACGGATATTGACGGTTCAATTGTTGCGA
CCCACATGATGCTGCAAGCTGCTGAACTCGGCTTAGGGACAACTTGGGTTGGCCATTTTGACCCGAGTGCAATCCG
GAATGCATTCAGCATTCCGGCGAACTTGGAACCGATTTGCCTTTTACCAGTCGGTTATCCGTCCAAAGATGCCAAA
CCTAATCCGAACCATAAGAAGCGCAAAGACATCTCTCAGACTGTGTTTTACAACCACTTTTAA

8.17.3. *Cs*NTR4 (CLOSPO_01641)

>Csp795.CsNTR4
ATGTTAGATTTATTAAAACAAAGAAGAAGTATAAGAAAGTTTCAGCAAAAGCCAATAGAAGAGGAAAAAGTAGAAG
CTCTAAAAAAAGGCATTACTTTTAGCCCCATCCTCTAGAAATATAAAACCTTTAGAGTTTATATTTTTAGAAGATAA
GGAAATTTTAAAGGATATTTCAAACTGTAAATCTCATGGAGGAACTTTTATAAAGGATGCAGCCTTAGCAGTAGTT
ATATTAGGGGATGAGGATAAAAGTGATGTATGGGTAGAGGATGCCTCTATAGCCTCTATAATACTACAACTTGAAG
CAGAATCTCTAGGATTAGGATCTTGCTGGTCACAAATAAGAAATAGAAGCTATGATGAAAATAAAATGGCGGAGGA
TTATATAAAAGAAAAACTAAACATAAAAGAGAATTATAAAGTAGAATCTATAATAGCCATAGGCTACAAAGATGAA
GTTAAGGAACCTATAAGTGAAGAAAAATTAGATTTTGATAAAATTCATGTTAACAAATTTATTTAA

>*Cs*NTR4
MLDLLKQRRSIRKFQQKPIEEEKVEALKKALLLAPSSRNIKPLEFIFLEDKEILKDISNCKSHGGTFIKDAALAVV
ILGDEDKSDVWVEDASIASIILQLEAESLGLGSCWSQIRNRSYDENKMAEDYIKEKLNIKENYKVESIIAIGYKDE
VKEPISEEKLDFDKIHVNKFI

>CsNTR4-adapted
ATGCTGGATCTGCTGAAACAACGTCGCAGTATTCGCAAATTCCAGCAAAAGCCGATTGAAGAAGAGAAAGTCGAAG
CACTCAAGAAAGCGTTACTGCTGGCACCAAGTTCCCGCAATATTAAGCCTTTGGAATTTATCTTTCTGGAGGACAA
AGAGATCCTGAAAGACATCTCGAATTGCAAATCTCATGGTGGAACCTTCATCAAAGATGCCGCTTTGGCTGTTGTG
ATTCTGGGCGATGAAGATAAAAGCGATGTATGGGTTGAGGATGCCTCGATTGCGAGCATTATTCTTCAGCTGGAAG
CCGAATCTTTAGGTCTTGGCAGCTGTTGGTCACAGATTCGGAATCGTTCCTATGACGAAAACAAAATGGCGGAAGA
TTACATCAAAGAGAAACTCAACATCAAAGAGAACTACAAAGTCGAATCAATCATTGCGATCGGGTATAAAGATGAA
GTGAAGGAACCGATTAGCGAAGAAAAGCTGGACTTTGACAAAATTCACGTGAACAAATTCATTTAA

8.17.4. *Cs*NTR5 (CLOSPO_01855)

>Csp795.CsNTR5
ATGGATTTTTATGATGTTATTGAAGATAGAAAAAGTATAAGAAAATTTAAAAATGGAGAGATTTGTAAAGAAAAGA
TGGCAAGAGTAATAAATGCAGCTATGAGATCACCTTCTTGGAAGAACGAAACTTCTTATAAATTTATAATAGTACA
AGATGGAACTAAAAGAATGGAATTAGCTAGTGCTATAATAAATAAATCTGATGAAGCTTCAGAAGCTATAAGAGTA
GCACCTGTAACAGCTGTGGTTGTTGCAGATCCAGATAAATCGGGTACTATAGAAAACAAACAATATTATCTAGTAG
ATAGTGCTATAGCTATGGAGCATTTTATATTAGCAGCTACAGCAGAAGGCTACGGTACTTGTTGGATAGGAGCTTT
TGATGAAGATAAAGTAAAAGGTGTATTAGATATACCTCAAAATTATAAAGTAGTTGGCATGACTCCTGTAGGAGAA
TCAAATGAAAATAAAGAAAGTCATCCTAAAAAAGATGTGAGAGAATATGTGTTTTTAGATAAATGGCATAATTCAT
ATACAGAAAATATATAA

>*Cs*NTR5
MDFYDVIEDRKSIRKFKNGEICKEKMARVINAAMRSPSWKNETSYKFIIVQDGTKRMELASAIINKSDEASEAIRV
APVTAVVVADPDKSGTIENKQYYLVDSAIAMEHFILAATAEGYGTCWIGAFDEDKVKGVLDIPQNYKVVGMTPVGE
SNENKESHPKKDVREYVFLDKWHNSYTENI

>CsNTR5-adapted
ATGGACTTTTATGATGTGATTGAGGATCGCAAATCCATTCGCAAATTCAAGAATGGCGAAATTTGCAAAGAGAAAA
TGGCACGTGTGATCAATGCCGCAATGCGTTCACCTTCGTGGAAGAATGAAACGAGCTACAAATTCATCATTGTCCA
AGATGGCACGAAACGGATGGAATTAGCGTCTGCGATCATCAACAAATCGGACGAAGCGAGTGAAGCCATTCGCGTT
GCTCCAGTTACAGCGGTAGTCGTTGCTGATCCGGACAAAAGTGGCACCATCGAAAACAAACAGTACTATCTGGTTG
ATAGCGCGATTGCTATGGAACACTTCATTTTGGCCGCAACTGCCGAAGGCTATGGTACCTGTTGGATTGGAGCGTT
TGATGAGGACAAAGTGAAAGGGGTCCTGGATATTCCGCAGAATTACAAAGTGGTAGGTATGACCCCGGTAGGTGAA
AGCAACGAAAACAAGGAGTCTCATCCCAAGAAAGATGTGCGTGAGTATGTGTTTCTGGACAAATGGCATAACTCCT
ATACCGAAAACATCTAA

8.17.5. *Cs*NTR6 (CLOSPO_02936)

>Csp795.CsNTR6
ATGAATGCTATATTAAAGAGAAGAAGTATAAGAAAATACAAGGATAAAAAGATAAGTGATGATATTGTTGAAGAAT
TATTAAGAGCAGGAATGGCTGCACCTTCTGCTGTAAATGAACAACCTTGGCAATTTATAGTGTTAAGAGATAAAGA
AACTATGAAAAAGATAACTAAGGTGCATGAGTATTCAAAGATGTTATTAGAGGCAGATGTTGCAATTGTAGTTTGT
GGAGATAAATCAAAAGAGTTAGTAGATGATTTTTGGGTACAAGATTGTTCAGCAGCTACAGAGAACATATTAATAG
AGGCACAAGATAAAGGATTAGGCGCAGTATGGTTAGGTGTTTATCCAATAAAAGAGAGAGTAGATGGAATAAAAGA
AATTCTAAATTTACCAGAAGGTATAACTCCTTTATCTGTAATACCTATAGGATATCCAGATGAAAAAAAGGAGCCA
GCAGATAGATCTAATAAAGAAAGAGTACATTATGATAAATGGTAATAA

>*Cs*NTR6
MNAILKRRSIRKYKDKKISDDIVEELLRAGMAAPSAVNEQPWQFIVLRDKETMKKITKVHEYSKMLLEADVAIVVC
GDKSKELVDDFWVQDCSAATENILIEAQDKGLGAVWLGVYPIKERVDGIKEILNLPEGITPLSVIPIGYPDEKKEP
ADRSNKERVHYDKW

>CsNTR6-adapted
ATGAATGCCATCCTCAAACGCCGGTCAATCCGCAAATACAAAGACAAGAAAATCTCCGATGATATTGTCGAAGAAC
TGTTACGTGCCGGTATGGCTGCTCCATCTGCGGTAAACGAACAGCCTTGGCAGTTCATTGTGCTGCGTGACAAAGA
AACCATGAAGAAGATTACCAAAGTGCATGAGTACAGCAAAATGCTGCTTGAAGCGGATGTTGCGATTGTCGTTTGT
GGCGATAAATCGAAAGAATTGGTTGACGACTTTTGGGTCCAAGATTGCAGTGCAGCAACGGAAAACATTCTGATTG
AGGCCCAGGATAAAGGCTTAGGTGCGGTTTGGTTGGGTGTGTATCCGATCAAAGAGCGTGTGGATGGGATCAAAGA
GATTCTGAATCTGCCAGAAGGAATCACTCCGCTGAGCGTGATTCCGATTGGCTATCCGGATGAGAAGAAAGAACCC
GCAGATCGCTCGAACAAAGAACGCGTACACTATGACAAATGGTAA

8.17.6. *Cs*NTR7 (CLOSPO_02936)

>Csp795.CsNTR7
ATGAATACTGTATTACAAACTATAAAAAATAGAAGAAGTATAAGAGGTTATAAATCTGAGCAAATAAAAGAAGAAG
AACTTCAATGTATATTAGAAGCTGGAATCTATGCTCCTTCTGGTTGTAATCATCAATCCTGGCATTTTACTGTTAT
TCAAAACAGAGAATTAATTACAACTATGAGCAATGTGGCTAAAGAAAAACTTAAAGATTCACCTAATGAAAATTTT
AGAAATATGGGTAATAATGAAAAACTTGATTTAACTCATGGTGCACCTACTTTAATTGTAGTATCAGGGAAAGAAG
GTAACTATTCCCCTTTAGTTGATTGTTCTGCTGCCATAGAAAATATGCTAATTGCTGCTGAAAGCTTAAATATAGG
TTCTTTATGGATAGGCCTTATTTCACTAGCTTTTGAAAATAAAGAAATCATGGAAAAATTAAATATACCTAAGGGA
TATAAACCTTATTTTGGCATAGCTTTAGGATATAAAAACGTAAGTGAAGCTAAAGCTCCTAAAAGAAATGAAAATG
TAATAACTTACATAAGATAA

>*Cs*NTR7
MNTVLQTIKNRRSIRGYKSEQIKEEELQCILEAGIYAPSGCNHQSWHFTVIQNRELITTMSNVAKEKLKDSPNENF
RNMGNNEKLDLTHGAPTLIVVSGKEGNYSPLVDCSAAIENMLIAAESLNIGSLWIGLISLAFENKEIMEKLNIPKG
YKPYFGIALGYKNVSEAKAPKRNENVITYIR

>CsNTR7-adapted
ATGAATACCGTACTGCAAACGATCAAGAACCGTCGTAGCATTCGTGGCTACAAAAGCGAACAGATCAAAGAGGAGG
AACTGCAGTGCATTCTGGAAGCGGGTATCTATGCGCCATCTGGCTGCAATCACCAAAGCTGGCATTTTACCGTCAT
TCAGAATCGCGAACTGATCACCACCATGTCTAATGTGGCGAAAGAGAAACTGAAAGATTCCCCGAATGAAAACTTC
CGCAATATGGGCAACAATGAGAAACTGGATCTTACTCATGGAGCACCTACACTCATCGTCGTTTCGGGTAAAGAAG
GGAACTATTCGCCGTTGGTTGACTGTAGTGCTGCCATTGAAAACATGTTGATTGCTGCAGAAAGCCTGAACATTGG
TTCCTTATGGATCGGCTTAATCTCACTTGCGTTCGAAAACAAAGAAATTATGGAGAAACTCAACATTCCGAAGGGG
TATAAGCCCTACTTTGGTATTGCACTGGGCTATAAGAACGTGAGTGAAGCCAAAGCCCCGAAACGCAATGAAAACG
TGATCACGTACATTCGGTAA

8.18. List of protein sequences used for the phylogenetic analysis

Q99190 enoyl reductase *Saccharomyces cerevisiae*;

YP_002987388 NADH:flavin oxidoreductase *Dickeya dadantii*;

YP_878535 reductase *Clostridium novyi*;

CAA76082 2-enoate reductase *Moorella thermoacetica*;

CAA76083 2-enoate reductase *Clostridium kluyveri*;

CAA71086 2-enoate reductase *Clostridium tyrobutyricum*;

YP_001254661 2-enoate reductase *Clostridium botulinum* str. A;

fldZ 2-enoate reductase CLOSPO_02780 *Clostridium sporogenes* (this study);

A6ZRN6 2,4-dienoyl-CoA reductase *Saccharomyces cerevisiae*;

OYE-like hypothetical protein CLOSPO_03444 *Clostridium sporogenes* (this study);

ZP_06424320 NADH oxidase *Peptostreptococcus anaerobius*;

P54550 *Bacillus subtilis*;

Q9FEW9 OPR3 *Lycopersicon esculentum*;

Q9XG54 OPR1 *Lycopersicon esculentum*;

E7KDJ6 Oye2p *Saccharomyces cerevisiae*;

YP_003226383 NADH:flavin oxidoreductase *Zymomonas mobilis*;

3KFT_A PETNR *Enterobacter cloacae*.

8.19. Similarities in the structure between clostridial 2-enoate reductases and OYEs

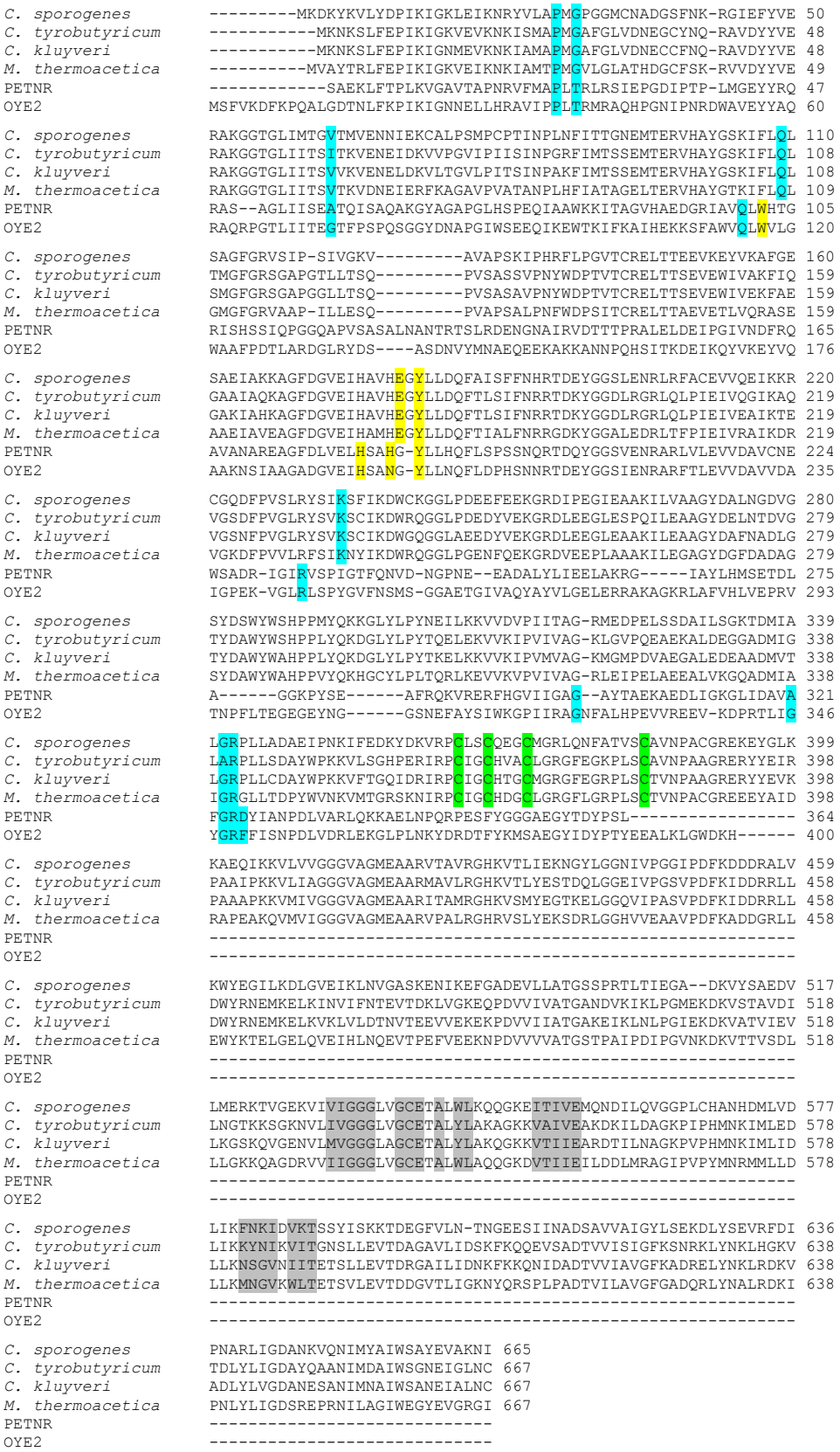

Fig. 8.31. Alignment showing homology of clostridial 2-enoate reductases and OYE-reductases. Functional residues are highlighted: GREEN - 4Fe-4S cluster binding site; BLUE - FMN binding site; YELLOW - substrate binding and active site; GRAY - FAD binding domain.