DEVELOPMENT OF RECOMBINANT ENZYMES TOWARDS THE PRODUCTION OF PHARMACEUTICAL INTERMEDIATES USING BIOTRANSFORMATIONS

By

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Based on research carried out under the supervision of

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Caio Roberto Souza Braganç.

ID No.: W20060661

Date: 12/09/2022

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"You are so faithful
My Lord, there is none like you
I want to thank you everyday
For your love and truth
Don't let me ever forget
That all that I am, all that I have
All that I'll ever be
Comes from your heart to me."
(Diante do trono)

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"(…) enquanto eu descanso em Deus, Ele me dá tudo o que eu preciso"

(Diante do trono)

ABSTRACT

BRAGANÇA, Caio Roberto Soares, South East Technological University, May 2022. **Development of recombinant enzymes towards the production of pharmaceutical intermediates using biotransformations**. Supervisor: Lee Coffey. Co-Supervisor: Catherine O'Reilly.

Nitrile compounds are versatile and can be converted into amides, amines, imines, oximes, carboxylic acids, esters and alcohols, encompassing a large group of economically important synthetic intermediates. The pharmaceutical industry particularly requires amides and acids for use as intermediates in the manufacture of many drugs and chemicals. The biotransformation of nitriles mediated by microorganisms has therefore attracted considerable attention in academia and industry as a sustainable alternative to the conventional chemical reactions that require drastic conditions of pH, temperature and pressure, use of metal catalysts, high-energy consumption and low selectivity in the process. Certain bacterial cells contain a nitrile-metabolizing gene; when the corresponding enzyme is incubated in a reaction-mixture containing a nitrile, the nitrile-metabolizing enzyme catalyses the conversion of the nitrile to the corresponding amide or acid. The amide or acid may then be extracted from the reaction mixture. This biological conversion is referred to as a biotransformation and is considered "Green Chemistry". As a result, the search for microorganisms which contain the enzymes responsible for these biotransformations (nitrilases, NHase and amidases) is crucial.

The main goals of this research were to; isolate bacteria with activity towards three pharmaceutically relevant β-hydroxynitriles from environmental samples collected worldwide; develop a high throughput screening strategy for filamentous fungi with potential for nitrile biotransformation; apply functional metagenomics to search for novel nitrile hydrolyzing enzymes using environmental samples collected in Ireland; and to formulate an appropriate production medium using statistical optimization that can substantially increase nitrilase production.

In this study, we have found three promising bacterial isolates which are source of genes for nitrile-degrading enzymes including, *Nocardia coeliaca* strain DSM, *Klebsiella oxytoca* strain JCM1665 and *R. erythropolis* PR4. The three strains presented with enantiomeric excess of >90 % towards 3-hydroxybutyronitrile (3HBN). Of the three promising isolates, one showed exceptional >99.99 ee % towards 3HBN,

indicating that the bacterial isolate is highly enantioselective and possibly enantiospecific with 100 % ee of (S)-acid. In contrast, while most industrial nitrilase enzymes are derived from bacterial sources, the potential of filamentous fungi was explored with a view to industrial use. The fungus *Fusarium solani* strain F3 was isolated and exhibited exceptional enantioselectivity towards 3-phenylpropionitrile with >99.99 % ee and enantioselectivity towards 3-hydroxybutyronitrile with 98.03 %, indicating the presence of a highly enantioselective enzyme using the whole mycelial cells.

Once an enzyme is characterized and chosen for its desired properties, large scale production in heterologous hosts often becomes essential, with growth conditions requiring optimisation even before scale-up can begin. Acknowledging this frequent need, providing an approach that addresses these technical challenges was demonstrated in this work by integrating two science fields: statistics with microbiology laboratory experiments to optimize the fermentation process parameters. Our results showed that an average of 5.54 mmol/L of nitrilase activity was attained using whole cells in the validation experiment under optimized conditions, which was 66 % higher than the prior yield of 3.33 mmol/L.

ABBREVIATIONS

2PPN	2-phenylpropionitrile
3HBN	3-hydroxybutyronitrile
3HGN	3-hydroxyglutaronitrile
3HPPN	3-hydroxy-3-phenylpropionitrile
4HPAN	4-hydroxyphenylacetonitrile
BLAST	Basic Local Alignment and Search Tool
bp	Base pairs
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOE	Design of experiments
EDTA	Ethylenediaminetetraacetic acid
ee	Enantiomeric excess
g	gram
GC	Gas chromatography
HPLC	High performance liquid chromatography
IPTG	Isopropyl-Beta-D-thiogalactopyranoside
KCl	Potassium chloride
KDa	Kilodaltons
L	Liter
LB	Luria-Bertani
LC-MS	Liquid chromatography-mass spectrometry
mg	Milligrams

$MgCl_2$	Magnesium chloride
mL	Milliliter
mM	Milimolar
MN	Mandelonitrile
ng	Nanogram
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
Ph	Phenyl
rpm	revolutions per minute
rt	Room temperature
Taq	*Thermus aquaticus*
TP	Total protein
WIT	Waterford Institute of Technology
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

LIST OF SYMBOLS

%	Percentage
^{o}C	Degrees Celsius
≥	Greater than or equal to
≤	Less than or equal to
±	More or less

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CHAPTER 1
INTRODUCTION AND BACKGROUND INFORMATION

1.1 Introduction

The use of natural catalysts – enzymes – for the conversion of a series of natural and non-natural chemicals is not at all new: they have been used for more than 100 years, employed either as whole cells or isolated enzymes (Neidleman, 1990; Roberts *et al.,* 1995). Certainly, the object of most of the early research was very different from that of the present day. The elucidation of biochemical pathways and enzyme mechanisms was the main reason for research several decades ago (Grunwald, 2017). It was mainly in the steep rise of asymmetric synthesis during the 1980s, that the enormous potential of applying natural catalysts to transform non-natural organic compounds was recognized. What started as an academic curiosity in the late 1970s became a hot topic in synthetic organic chemistry in the 1990s. Since then, the impact of this field has only increased, especially in the pharmaceutical arena, which has seen investment of billions of dollars in this area (Liese *et al.,* 2006; Faber, 2011; Chen *et al.,* 2019).

Worldwide, about 90 % of all chemical processes are performed via catalysis driving to an annual product value of around USD 3 trillion. As a result, biocatalytic methods represent the main pillar of applied biotechnology, which has been classified as “White” Biotechnology by EuropaBio 2003, and which stands for the application of nature’s toolset to sustainable industrial production (Faber, 2011). Other sectors of biotechnology have been defined as ‘Red’ (biotechnology in healthcare), ‘Green’ (biotechnology for food and agriculture) and ‘Blue’ (marine biotechnology) (Gaskell *et al.,* 2000).

A number of products that can be used as pharmaceutical intermediates have been obtained by enzymatic reactions. Both enzyme systems that convert nitriles from microorganisms are potential biocatalysts for the production of carboxylic acids and amides of interest for industrial applications (Chen *et al.,* 2009; Santoshkumar *et al.,* 2010; Coady *et al.,* 2013; Kim, 2015). However, to establish an effective biotransformation process, a detailed analysis of the factors that influence the development and optimization of the integrated biotechnological process is required, which depends on: a) the biocatalyst, b) the bioconversion and c) the isolation and purification of the product (Oliveira *et al*. 2015). In this way, with careful selection of biocatalytic systems, it is possible to produce enantiomeric compounds; amides and carboxylic acids with high enantiopurity, making the isolation of microorganisms as “nitrile biocatalysts” a large but potentially rewarding challenge.

In fact, microbial cells are excellent sources of enzymes and represent about 90 % of the total biotransformation market. More than half of the enzymes used industrially are sourced from fungi and bacteria, with the remainder derived from animals and plants (Bhalla *et al.,* 2018). In addition, new microorganisms are constantly being screened to obtain new enzyme sources with catalytic specificity. Once discovered, these biocatalysts can be optimized using robust enzymatic engineering tools (Truppo, 2017).

The use of the biodiverse environment in the search for novel catalysts by microbiological selection techniques is the traditional method for discovery of new enzymes in the development of biocatalysts for different industrial sectors. The use of microorganisms has a particular interest due to the short period of cultivation, the wide variety of metabolic processes/enzymes involved and due to an unlimited number of microorganisms in the environment which are very different among themselves, thus providing the discovery of enzymes with different applications (Adrio and Demain, 2014; Bragança *et al.,* 2017).

There are two main different approaches in the isolation of microorganisms from the environment including culture-dependent and -independent methods. Both allow the characterization of and access to some of the diverse microbiome within a sample and therefore are considered complementary. It has been estimated that 1 % of the microorganisms are detected on plates with culture medium due to selective conditions, depending on the composition of the culture media (Torsvik *et al.,* 1990; Whitman *et al.,* 1998). On the other hand, the culture-independent methods indicate a predominance of many uncultivated species (Schloss *et al.,* 2004). One example of a culture-independent method is metagenomics, which consists of direct extraction of nucleic acids from environmental samples, followed by cloning into a suitable vector, transformation into host cells and screening for the genes and/or functions of interest (Handelsman, 2004), without going through prior DNA amplification steps.

Currently, culture enrichment techniques have allowed microorganisms with nitrile-hydrolyzing enzymes to be isolated (Coady *et al.,* 2013). In addition, uncultured environmental samples can be explored by the metagenomic approach (Daniel, 2005). Most of the well-characterized NHases, amidases and nitrilases, which are largely of bacterial origin, have been obtained by selection methods allowing only the positive strains to grow on a minimal medium with a nitrile as the sole nitrogen source (Martínková

et al., 2008; Coffey *et al.,* 2009, 2010; Coady *et al.,* 2013). On the other hand, filamentous fungi seem to be a rich but so far little exploited source of nitrile-hydrolysis enzymes.

1.1.1 Common Prejudices Against Enzymes

The following prejudices are frequently encountered when enzymes are commonly used in biotransformations:

(1) Enzymes are sensitive molecules: most enzymes are sensitive to the conditions at which they work, i.e, changes in pH and temperature. They often have a narrow range of conditions under which they work properly, but that also holds for most organic reagents. However, enzymes can become remarkably stable when certain strategies are used (Baross and Deming, 1983; Zhang *et al.,* 2016). Some candidates are active at temperatures as high as 110 °C and operate at a very low acidic pH (Vieille and Zeikus, 2001).

(2) Enzymes are expensive: which means that the cost of the biocatalyst isolation and purification is higher compared to traditional catalysts (Homaei *et al.,* 2013). In fact, the higher cost of the enzyme production is due to the level of its purification. On the other hand, the level of purification is less important when many bulk industrial enzymes are considered. Most enzymes for industrial purposes today are sold as inexpensive of overall process cost, including as crude preparations of cell extracts or whole cells (Robinson, 2015). In addition, costs for enzyme production are constantly dropping due to the rapid advances in applied biotechnology field (Ravindran and Jaiswal, 2016).

(3) Enzymes are only active and work only in their natural substrates and environment: This statement is not true for the majority of them. Most enzymes can function in nonaqueous media, such as organic solvents (Koskinen and Klibanov, 1996; Gutman and Shapira, 2006), and are capable of catalyzing side reactions in addition to their main reaction - sometimes called promiscuous, which allows organisms the ability to adapt to varying environmental conditions (evolutionary pressure) (Leveson-Gower *et al.,* 2019; Singla and Bhardwaj, 2019). In addition to its catalytic promiscuity, the enzymes can still be divided into “substrate promiscuity” (conversion of a nonnatural substrate) (Bornscheuer and

Kazlauskas, 2004), and “condition promiscuity” (catalysis occurring in a nonnatural environment) (Hult and Berglund, 2007; Babtie and Hollfelder, 2010; Copley, 2015). For this reason, this statement implies that man-made organic compounds could be considered as substrates, which has opened up huge possibilities of using enzymes in biotransformations (Peretó *et al.,* 2017). An interesting example was described by Kern *et al.,* (2010); they have described for the first time an example of a catalytic chemo-promiscuous activity using the enzyme alcohol dehydrogenase (ADH) from *R. erythropolis* (RE-ADH) to perform the reduction of aldoxime to valuable intermediates.

1.1.2 Advantages and Disadvantages of Biocatalysts

Enzyme-catalyzed reactions are environmentally friendly and can be very efficient catalysts. Typically, the rates of enzyme-mediated processes under optimum conditions are faster by a factor of approximately 10^{12} than chemical catalyzed processes (Nelson and Cox, 2017), and in some cases, can even accelerate changes in its substrate by exceeding a factor of 10^{17} times as fast as the uncatalyzed one as demonstrated by Radzicka and Wolfenden (1995). As a result enzymes are considered as potent catalysts, which evidently makes them more effective by some orders of magnitude (Table 1.1).

Table 1.1 Catalytic efficiency of representative enzymes (Adapted from Nelson and Cox, 2017).

Enzyme	Reaction catalyzed	TON
Carbonic Anhydrase	Hydration of CO_2	600.000
3-Ketosteroid isomerase	Isomerization	280.000
Catalase	Oxidation	93.000
Acetylcholinesterase	Ester hydrolysis	25.000
Penicillinase	Amide hydrolysis	2.000
Lactate dehydrogenase	Carbonyl reduction	1.000
Mandelate racemase	Racemisation	1.000
β–galactosidase	Transgalactosylation	200
Chymotrypsin	Amide hydrolysis	100

DNA polymerase I	Exonuclease activity	15
Tryptophan synthetase	Condensation	5
Tyrosinase	Hydro-lyase	1
Lysozyme	Glycosidases	0.5

TON = turnover number (number of substrate molecules that can be converted to product by a single enzyme molecule per second).

Probably the most attractive property of a biocatalyst is its high selectivity, which provides an advantage in biotransformation studies. In this context, it is good to mention that three major types of selectivities are displayed by the enzymes and considered highly important in all types of catalysis (Abdelraheem *et al.*, 2019). First, chemoselectivity, which the purpose of an enzyme is to distinguish different functional groups in the presence of others, is the main challenge in biocatalysis. As a result, side reactions and thus purifications steps could be reduced by its selectivity (Faber, 2004). Second, some enzymes exhibit interesting product site-selectivity (regioselectivity). It means that the catalyst may distinguish selectively between functional groups at one of several possible positions within the same substrate molecule, which means if the reaction is unselective, a mixture of products will arise (e.g., enantiomers and isomers) (Wang *et al.,* 2017). Third, enantioselectivity – chemical reaction in which one new chiral product (enantiomer) is preferentially formed in a substrate molecule. As a consequence, products in unequal amounts are produced. For this reason, the development of chiral enzymes for enantioselective synthesis plays an important role in fine chemicals and drug synthesis in the field of pharmaceuticals (Agranat *et al.,* 2002).

In fact, enzymes govern all the main biochemical events taking place within an organism (Berg *et al.,* 2011). Since most of them are highly selective with respect to distinguishing between the two enantiomers of a chiral substrate, it is noticeable that the enantiomers of a given compound will cause undesired side effects, e.g., on biological organisms (Han and Yao, 2019). In that case, they must be considered as two different species. The isomer with higher activity is called the eutomer, whereas the one possessing less, toxic or unwanted activities is called the distomer (Nelson and Cox, 2017; Shilpa and Varalakshmi, 2018). The ratio of activity between the two enantiomers is defined as the

eudismic ratio. In addition, biological effects of enantiomers are often very different, as shown in Figure 1.1.

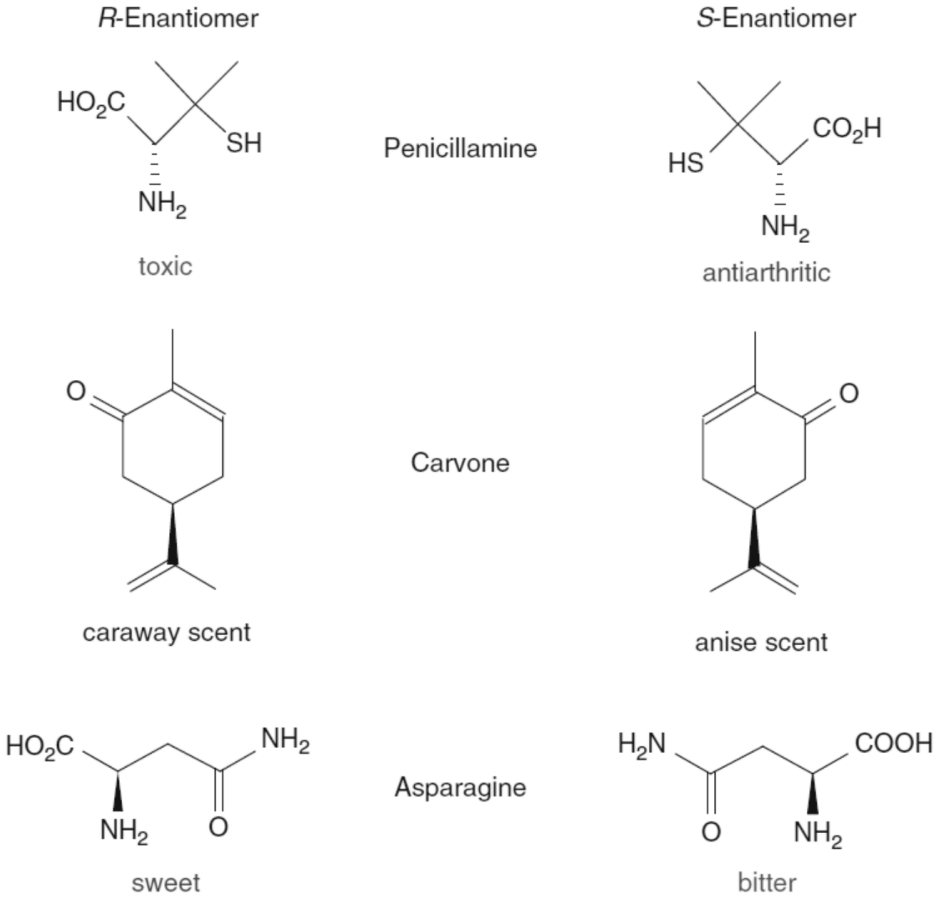

Fig. 1.1 Biological effects of enantiomers by Faber (2011).

Markedly, one of the most well-known examples in the practical application of drugs in the healthcare sector is thalidomide, which has a tragic history in 1957. It was introduced in Germany as a sedative and hypnotic and was marketed over the counter largely as a drug for treating morning sickness in pregnant women. Thalidomide exists in two mirror-image forms: it is a racemic mixture of (R)- and (S)-enantiomers. The (R)-enantiomer has sedative effects, on the other hand, the (S)-isomer is highly teratogenic. Under those circumstances, infants were born with phocomelia, or limb malformation (Sih and Wu,

2007; Vargesson, 2015). For this reason, the thalidomide tragedy forced pharmaceutical industries to focus on single enantiomers in drug development (Brooks *et al*., 2017).

In 1992, a policy statement containing guidelines for the development of chiral drugs was issued by the FDA (US Food and Drug Administration). According to these guidelines, the FDA recommends the evaluation of pharmacological and toxicological effects of each enantiomer for racemic drugs in biological systems, and encourages pharmaceutical companies to switch existing marketed racemic medicines to a single-enantiomer drug, known as chiral switching (Hubbard, 2003; Eman M. M. Abdelraheem *et al.*, 2019). Currently, the development of racemates is not prohibited according to the regulatory guidelines. However, the properties of each enantiomer should be rigorously tested before the pharmaceutical companies introduce it to the market (Farina *et al.*, 2006; Calcaterra and D'Acquarica, 2018).

Curiously, most chiral drugs in life science were introduced annually as racemates, before 1985. In recent years, however, the situation has changed dramatically, with racemic form being introduced only rarely to the market. In 2015, for example, all the new chiral drugs approved (45 in the world) were enantiomerically pure compounds. In addition, by 2024 the Global Chiral Chemicals Market is predicted to grow from $7 billion to $10 billion, predominantly driven by nearly 95 % of enantiopure drugs (Calcaterra and D'Acquarica, 2018; Eman M. M. Abdelraheem *et al.*, 2019).

1.1.3 Isolated Enzymes vs. Whole Cell Biocatalysis

Due to its high selectivity, environmental friendliness, low toxicity and the reaction ability under mild conditions, biocatalysis has been a widely applied method as an alternative to conventional chemical approaches in chiral synthesis, which includes isolated enzymes from biological sources or whole cells containing the desired enzyme to speed up chemical reactions (Carvalho, 2017; Paul *et al.,* 2019). In general, many factors must be considered to the final decision as to whether one should use whole microbial cells or isolated enzymes, including the reaction type, cofactor recycling and the scale-up production, as demonstrated in Table 1.2.

Table 1.2 Pros and cons of using isolated enzymes vs. whole cell systems (Faber, 2011).

Biocatalyst	Form	Pros	Cons
Isolated enzymes	Any	Simple apparatus, simple workup, better productivity due to higher concentration tolerance	Limited enzyme stabilities
	Dissolved in water	High enzyme activities	Side reactions possible, lipophilic substrates insoluble, workup requires extraction
	Suspended in organic solvents	Easy to perform, easy workup, lipophilic substrates soluble, enzyme recovery easy	Reduced activities
	Immobilized	Enzyme recovery easy	Loss of activity during immobilization
Whole cells	Any	No cofactor recycling necessary, no enzyme purification required	Expensive equipment, tedious workup due to large volumes, low productivity due to lower concentration tolerance, low tolerance of organic solvents, side reactions likely due to uncontrolled metabolism
	Growing culture	Higher activities	Large biomass, enhanced metabolism, more byproducts, process control difficult
	Resting cells	Workup easier, reduced metabolism, fewer byproducts	Lower activities
	Immobilized cells	Cell reuse possible	Lower activities

Notably, whole-cell biocatalysis approaches are of great importance in the academic and industrial fields, and can generally be separated into biotransformation and fermentation bioprocesses (Schmid *et al.,* 2001). In fermentations, the growing microbial cells (fermenting cells) are used in the synthesis of products from growth substrates via its native metabolism, which naturally results in the high amounts of biomass and by-product formation. On the other hand, in biotransformation, the substrates are converted to the desired products by using nongrowing but metabolically active microbial cells (resting cells), which can be of high interest to increase product yields on carbon and energy sources and reduce biomass (Julsing *et al.*, 2012; Lin and Tao, 2017; Nor Suhaila *et al.*, 2019). The characteristics of each process using fermenting and resting cells are outlined in Table 1.3 (Faber, 2011).

Table 1.3 Characteristics of resting vs. fermenting cells.

	Resting cells	Fermenting cells
Microbial cells	Resting	Growing
Reaction type	Short, catalytic	Long, life process
Number of reaction steps	Few	Many
Number of enzymes active	Few	Many
Starting material	Substrate	C + N source
Product	Natural or nonnatural	Only natural
Concentration tolerance	High	Low
Product isolation	Easy	Tedious
Byproducts	Few	Many

In particular, most publications on biocatalysis in organic transformations focus on isolated enzymes obtained by overexpression in genetically engineered bacteria and fungi (Garzón-Posse *et al.*, 2018; Janssen and Stucki, 2020). Until recently, only enzymes that were abundantly produced by cells could be used in industrial applications. Facilitated by recent advances in molecular biology techniques and their relevance to biotransformation research, the application of recombinant microbial cell factories expressing the gene of interest is rapidly growing. On the other hand, the use of wild-type

microorganisms from natural environments which often show lower protein expression levels is constantly decreasing (Madigan *et al.,* 2017). Notably, several steps are involved in the design of the recombinant microbial cell factories for biotransformation applications, which includes, the identification of the desired product; selection of a host cell; and genetic/physiological engineering approaches to allow the formation of the desired products (de Carvalho, 2017).

1.2 Nitriles and their occurrence

The pharmaceutical industry requires amides and acids for use as intermediates in the manufacture of many drugs and chemicals (Thuku *et al*. 2009). The nitrile compounds are versatile and can not only be converted into amides, but also amines, imines, oximes, carboxylic acids, esters and alcohols that are a group of economically important synthetic intermediates (Faber 2011). The biotransformation of nitriles mediated by microorganisms has attracted considerable attention in the academic and industry environment to be a sustainable alternative to the conventional chemical reactions ahead that require drastic conditions of pH, temperature and pressure, use of metal catalysts, high-energy consumption and low selectivity in the process (Gong *et al*. 2012). As a result, the search for microorganisms, which contain the enzymes responsible for these biotransformations (nitrilases, NHase and amidases) and that, carried out with high chemo-, regio- and stereoselective is crucial (Chen *et al*. 2009).

In addition to this synthetic value, these enzymes play a key role in terms of environmental protection; they have been successfully employed in biodegradation and bioremediation of contaminated areas with organonitriles from anthropogenic origin (Prasad and Bhalla 2010).

Environmentally occurring nitriles are due to both biological and industrial practices. Plants and microorganisms hydrolyse nitriles that are naturally occurring as either cyanogenic glycosides or cyanolipids (Graham *et al.,* 2000).

During plant biosynthesis of cyanoglycosides (CNG's) and cyanolipids, nitriles are produced as intermediates (Kobayashi and Shimizu, 2000). Cyanoglycosides are amino-acid-derived secondary plant products and are found in more than 2000 species. Upon

tissue injury, the cyanoglycosides are hydrolysed to either sugar, keto or aldehyde compound or HCN (Howden and Preston, 2009).

Biotransformation with nitrile and amide metabolising enzymes has become a focal point for biopharmaceuticals. An *Acinetobacter* sp. strain AK226 was used in the biotransformation of S-(+)-ibuprofen from racemic 2-(4'-isobutylphenyl) propionitrile (Yamamoto *et al*. 1990). An immobilised and soluble NHases from *R. erythropolis* A4 has been used for the biotransformation of nitriles such as 3-oxonitriles,3-hydroxy-2-methylenenitriles, 4-hydroxy-2-methylenenitriles, 3-hydroxynitriles and 3-acyloxynitrile into amides. Iminodiacetic acid (IDA) is widely used for the production of glyphosate herbicides, electroplating solutions, chelating resin, surfactants, and anticancer drugs. An *Alcaligenes faecalis* mutant isolated by (Zhang, Liu and Zheng, 2013) exhibited excellent catalytic activitity for the conversion of iminodiacetonitrile to iminodiacetic acid. A culture of *R. rhodococcus* J1 cells displayed NHase activity against isonicotinamide catalyzing the production of isonicotinic acid which is used for in the industrial production of a tuberculostatic (Hashimoto *et al.,* 2005).

Another example is the nitrilase produced by *Rhodococcus* sp. NDB 1165 which has been used in the production of nicotinic acids employed in pharmaceutical formulations (Velankar *et al.,* 2010). The nitriles exhibited high enzyme substrate specificity for aromatic unsaturated aliphatic nitriles (Prasad and Bhalla, 2010). In fact, a number of nitrile-related enzymes have been found and screened over the past years for use in synthetic applications (Kobayashi and Shimizu, 2000).

The overuse of nitrile herbicides and pesticides led to their abundant occurrence in the environment. Herbicides such as Casoron (2,6-dichlorobenzonitrile) and Buctril (4-(octanoyloxy)-3,5-dibromobenzonitrile) are widely used. (Kobayashi and Shimizu, 2000) and pose a threat to ecological systems. On the other hand, bioremediation has turned out to be a successful option for the ‘clean-up’ of these polluted sites. NHases are present in the environment at high levels due to their ability to degrade benzonitriles to benzamides. The metabolites produced by the degradation of these compounds such as herbicides or pesticides are often more prevalent in the environment as they can be less susceptible to degradation. (Holtze *et al.,* 2008).

In addition, most nitriles are highly toxic, mutagenic and carcinogenic in nature and humans can be affected by nitrile toxicity by either dietary intake or physical contact (Grogan *et al.,* 1992). Aliphatic nitriles have been thought to manifest their toxicity by the discharge of cyanide (Ahmed and Farooqui, 1982a). The most common symptom of the toxicities of nitriles in humans is gastric problems, nausea, respiratory distress, convulsions, coma or skeletal deformities (Sharma *et al*., 2009a).

1.3 Microbial Hydrolysis of Nitriles to High-Value Acids or Amides

Cyano group-containing organic compounds occur naturally in the environment with different structural types, which are synthesized by plants, fungi, bacteria, seaweed, sponges and insects. Both plants and micro-organisms are able to produce nitriles of aliphatic and aromatic types such as cyanoglycosides, cyanolipids, ricinine, phenyl acetonitrile, among others (Fig. 1.2) (Howden and Preston, 2009). These compounds can serve not only as a nitrogen storage, but also as protecting agents against attack by hungry predators (Faber, 2011).

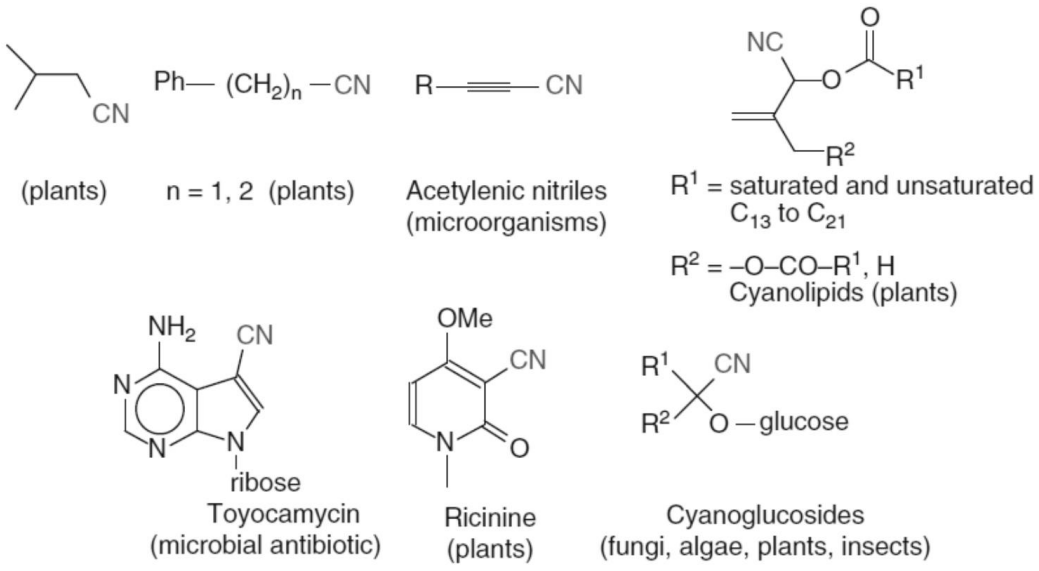

Fig. 1.2 Naturally occurring organic nitriles (Faber 2011).

Chemical hydrolysis of nitriles was extensively applied to synthesize amides and acids previously; however, these applications may not be suitable for the hydrolysis of nitriles

in the presence of sensitive groups. On the other hand, enzymatic hydrolysis of nitriles could alleviate this problem ascribed to the mild reaction conditions. Besides, three classes of nitrile-amide converting enzymes, namely NHase (EC 4.2.1.84), nitrilase (EC 3.5.5.1) and amidase (EC 3.5.1.4) involved in the transformation of nitriles or amides exhibit great potential of enantioselective and regioselective synthesis (Martínková and Kren, 2010).

The hydrolysis is the most common pathway for the metabolism of microbial nitriles which occurs by two enzymatic routes: (i) by nitrilase (NL) that hydrolyzes the nitrile to the corresponding carboxylic acid directly via addition of two molecules of water; (ii) by NHase that converts the nitrile to the corresponding amide via the addition of one molecule of water, which by action of the amidase is converted to the corresponding carboxylic acid via the addition of another molecule of water (Kiełbasiński *et al.,* 2008) as shown in Figure 1.3.

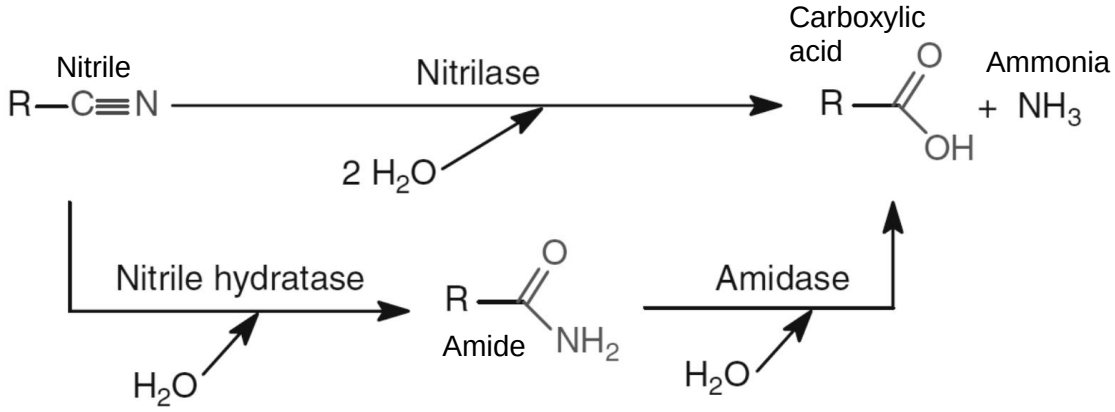

Fig. 1.3 General pathways of the enzymatic hydrolysis of nitriles (Faber 2011).

The reactions mediated by biocatalysts that convert nitriles are most active at pH neutral or slightly alkaline (Martínková *et al.*, 2008). Under extremely acidic or alkaline environment, these enzymes exhibit low activity (Chen *et al.,* 2009). However, there are some exceptions, for example, hydrolysis of 2-phenyl acetonitrile and phenyl proprionitrile by acid-tolerant *Exophiala oligosperma* R1 yeast took place in a pH range varying from 1.5 to 9.0 (Rustler and Stolz, 2007a).

To date, various nitrile-amide converting organisms isolated from bacteria, fungi and plant have been described (Brenner, 2002; Song *et al.,* 2008; Coffey *et al.,* 2009; Coady *et al.,* 2013). Most of them have been derived from bacterial species by enrichment strategies using nitriles as sole nitrogen source (Layh *et al.,* 1997). Some reactions mediated by nitrile-converting enzymes have been applied on a large scale in industry. Productions of acrylamide (Kobayashi, Nagasawa and Yamada, 1992) and nicotinic acid (Mathew *et al.,* 1988) on an industrial scale have proved the commercial value of these enzymes.

1.3.1 Description of Three Classes of Nitrile-Amide Converting Enzymes

The nitrilases and amidases belong to the class of hydrolase enzymes, whereas NHases enzymes belong to the class of lyases (Brady *et al.,* 2004; Winkler, Glieder and Klempier, 2006).

1.3.1.1 Nitrilases

Since their discovery in plants (1958) and in bacteria (1964), more than 30 nitrilases have been reported in many organisms, including bacteria, fungi as well as plants (Table 1.4), which are generally isolated using enrichments from environmental samples. (Howden and Preston, 2009). The first purified bacterial nitrilase was isolated and partially characterized from *Pseudomonas* sp., which catalyzed N-methyl-3-cyano-4-methoxy-2-pyridone-N-methyl-3-carboxyl-4-methoxy-2-pyridone (O'Reilly and Turner, 2003).

Table 1.4 Some reported prokaryotic and eukaryotic organisms with nitrilase activity [Adapted from Gong *et al.*, (2012) and Chen *et al.*, (2019)].

Bacteria	Fungi	Plants
Acidovorax facilis 72W (Gavagan *et al.,* 1999)	*Fusarium solani* IMI196840 (Harper, 1977b)	*Arabidopsis thaliana* (Piotrowski, *et al.,* 2001)
Bacillus pallidus Dac521 (Almatawah, Cramp and Cowan, 1999)	*Fusarium oxysporum* (Goldlust and Bohak, 1989)	*Barley* (Mahadevan and Thimann, 1964)
Alcaligenes faecalis JM3 (Nagasawa *et al.,* 1990)	*Cryptococcus* sp. UFMG-Y28 (Rezende *et al.,* 2000)	*Chinese cabbage* (Nagasawa *et al.,* 1990)
Alcaligenes faecalis ATCC8750 (Yamamoto *et al.,* 1992a)	*Aspergillus niger* K10 (Vejvoda *et al.,* 2006)	Brassica rapa (Ishiawa *et al.,* 2007)
Rhodococcus rhodochrous J-1 (Kobayashi *et al.,* 1989)	*Penicillium multicolor* (Ondřej Kaplan *et al.,* 2006)	
Rhodococcus rhodochrous NCIMB 11216 (Harper, 1985)	*Exophiala oligosperma* R1 (Rustler and Stolz, 2007b)	
Rhodococcus rhodochrous PA-34 (Bhalla *et al.,* 1992)		
Rhodococcus rhodochrous K22 (Kobayashi *et al.,* 1990)		
Comamonas testosterone (Lévy-Schil *et al.,* 1995)		
Pseudomonas fluorescens DSM 7155 (Layh, Parratt and Willetts, 1998)		
Rhodococcus rubber (Hughes, Armitage and Symes, 1998)		
Acinetobacter sp. AK 226 (Yamamoto and Komatsu, 1991)		
Klebsiella ozaenae (Stalker, Malyj and McBride, 1988)		
Arthrobacter sp. J1 (Bandyopadhyay *et al.,* 1986)		
Streptomyces sp. MTCC 7546 (Khandelwal *et al.,* 2007)		
Bacillus subtilis ZJB-063 (Zheng *et al.,* 2008)		

Most of the characterized nitrilases are of bacterial origin and were obtained by selective methods that allowed only the Gram-positive strains to develop in a culture medium in the presence of nitrile as sole nitrogen sources (Veselá *et al.,* 2012). The cultivation of microorganisms in selective media is an efficient method for isolating microorganisms

capable of hydrolyzing nitriles. As amidases and nitrilases convert their substrates (containing cyano group) to ammonia, which is used as a nitrogen source, the microorganisms that produce the enzymes of interest can grow in the presence of nitriles or amides as the sole source of nutrition. In some instances carboxylates derived from hydrolysis of nitrile or amide are also utilized by microorganisms, thereby promoting the substrate degradation (Martínková *et al*. 2008).

Bacteria of the genera *Rhodococcus, Alcaligenes, Bacillus, Pseudomonas, Acinetobacter, Corynebacterium, Arthrobacter* and *Nocardia* are sources of various nitrilases with different specificities for substrates, which enzymes were purified and characterized (Banerjee *et al*. 2002; Martínková *et al*. 2009; O'Reilly and Turner 2003). In relation to the fungal nitrilases, the first was isolated from *Aspergillus niger*, which has been biochemically characterized and sequenced (Kaplan *et al.,* 2006; Martínková and Kren, 2010). Other genera were known to produce these enzymes, such as *Fusarium, Gibberella, Pichia, Neurospora, Trichoderma, Macrophomina and Penicillum* (Martínková, 2019).

Nitrilases have all the earmarks of being rare in bacteria. From over 150 sequenced bacterial genomes, only 10 contain nitrilase genes (Podar *et al.,* 2005). Likewise, Sharma *et al.*, (2017) demonstrated that only 138 nitrilases among 2000 bacterial genomes have been predicted by genome mining data which means that only 6.9 % of bacteria express nitrilases. In bacteria, those genes are often organized in clusters, such as operons and regulons, that reflect involvement in a common metabolic process or association in a supramolecular complex (Lathe *et al.,* 2000; Rogozin *et al.,* 2002).

On the basis of their amino acid sequence and catalytic activity, the nitrilase superfamily can be classified into 13 distinct branches, of which most have one or more member of known substrate specificity and function. 9 of 13 branches have known or deduced specificity for specific reaction of hydrolysis of nitrile or amide, or condensation reactions to form amides. Besides, a nitrilase- related domain is fused to at least one additional conserved domain in 7 branches of the nitrilase superfamily, as shown in the Figure 1.4 (Pace and Brenner 2001).

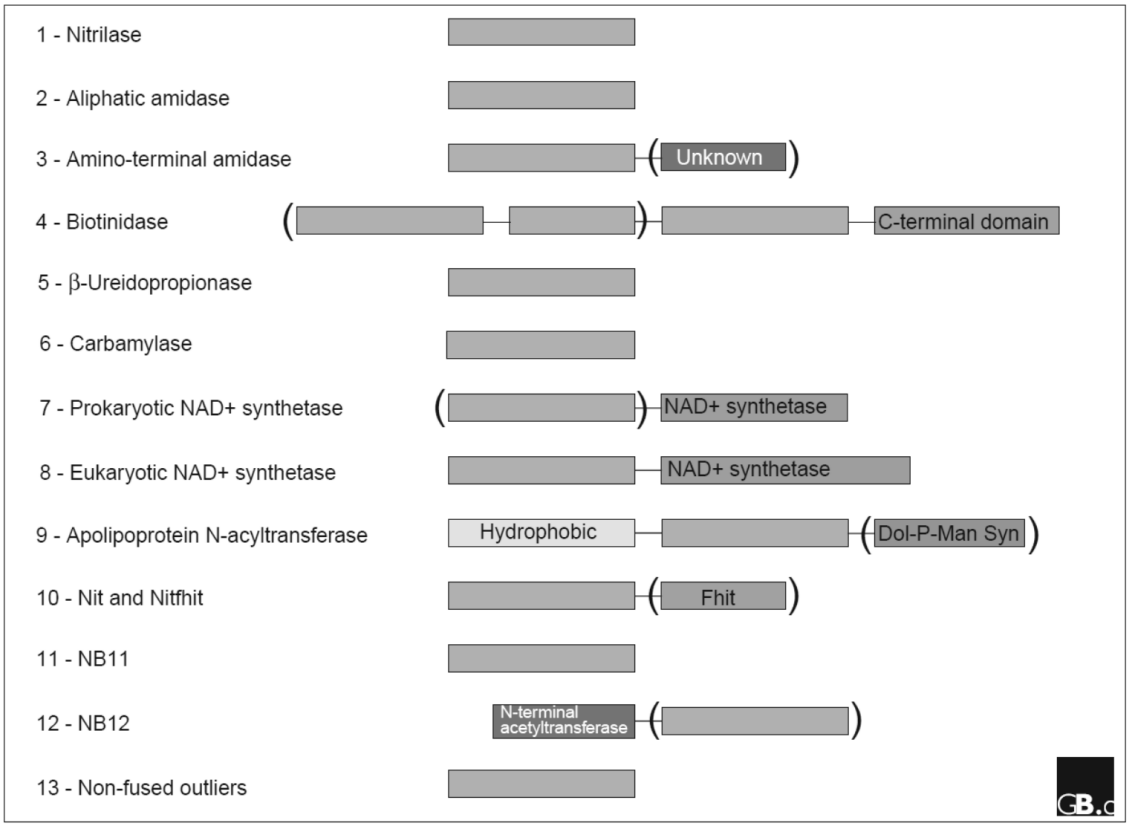

Fig. 1.4 Domain structures for 13 branches of the nitrilase superfamily. Parentheses denote domains found in only some members of the branch (Pace and Brenner 2001).

Members of the nitrilase branch are found in plants, animals (*Caenorhabditis elegans*), fungi (*Saccharomyces cerevisiae*), and many types of bacteria. The best evidence that nitrilase functions *in vivo* to convert indoleacetonitrile to the plant growth factor indole-3-acetic acid (auxin) comes from *Arabidopsis*, in which it was shown that recessive mutations in a nitrilase gene resulted in reduced sensitivity to the auxin-like effects of indoleacetonitrile and that overexpression of a nitrilase caused increased sensitivity to indoleacetonitrile (Normanly *et al.,* 1997). It is not clear, however, whether bacterial nitrilases mainly function in ecological relationships with plants or whether they benefit isolated microbes (Pace and Brenner, 2017).

The biocatalysis reactions of the nitrilases are widely reported by several groups. The results achieved by Mathew *et al*. (1988), showed that all nitrilases are classed as sulfhydryl enzymes and they are divided into three categories according to their substrate specificity such as (1) those that hydrolyse aromatic or heterocyclic nitriles, (2) those that

hydrolyse aliphatic nitriles, and (3) those that hydrolyse arylacetonitriles (Banerjee, Sharma and Banerjee, 2002).

Unlike NHases, the nitrilases do not show the presence of any metal co-factor or prosthetic group. Kobayashi *et al*. (1992) showed that cysteine residues are fundamental in the function of its active site and improves the nitrilase performance (Gong, Lu, *et al.*, 2012), such as Cys-165 in *R. rhodochrous* J1 or Cys-163 from *Alcaligenes faecalis* JM3 (Kobayashi *et al*. 1993).

Likewise, nitrilases are commonly inducible enzymes composed of one or two types of subunits (Banerjee, Sharma and Banerjee, 2002). Most nitrilases possess a single polypeptide from 30 kDa to 45 kDa. Also, to increase their catalytic activity they must form heterocomplexes (Gong, Lu, *et al.*, 2012). The main form of nitrilase enzymes seems to be a large aggregate of 6-26 subunits (O'Reilly and Turner, 2003).

In 2004, Robertson *et al*., showed that 137 unique nitrilases were discovered after screening more than 600 environmental cDNA libraries which shows their extreme and diverse presence in several environments. In the phylogenetic classification by Robertson *et al*. (2004), the nitrilases were grouped into six subfamilies based on enzymatic and biochemical properties and associated gene clusters. In addition, the genes neighbouring nitrilase were studied in order to investigate their metabolic roles (Podar *et al*. 2005). In this way, it was found that all nitrilase genes of subfamily one showed any particular associations with a conserved cluster Nit1C as shown in Figure 1.5.

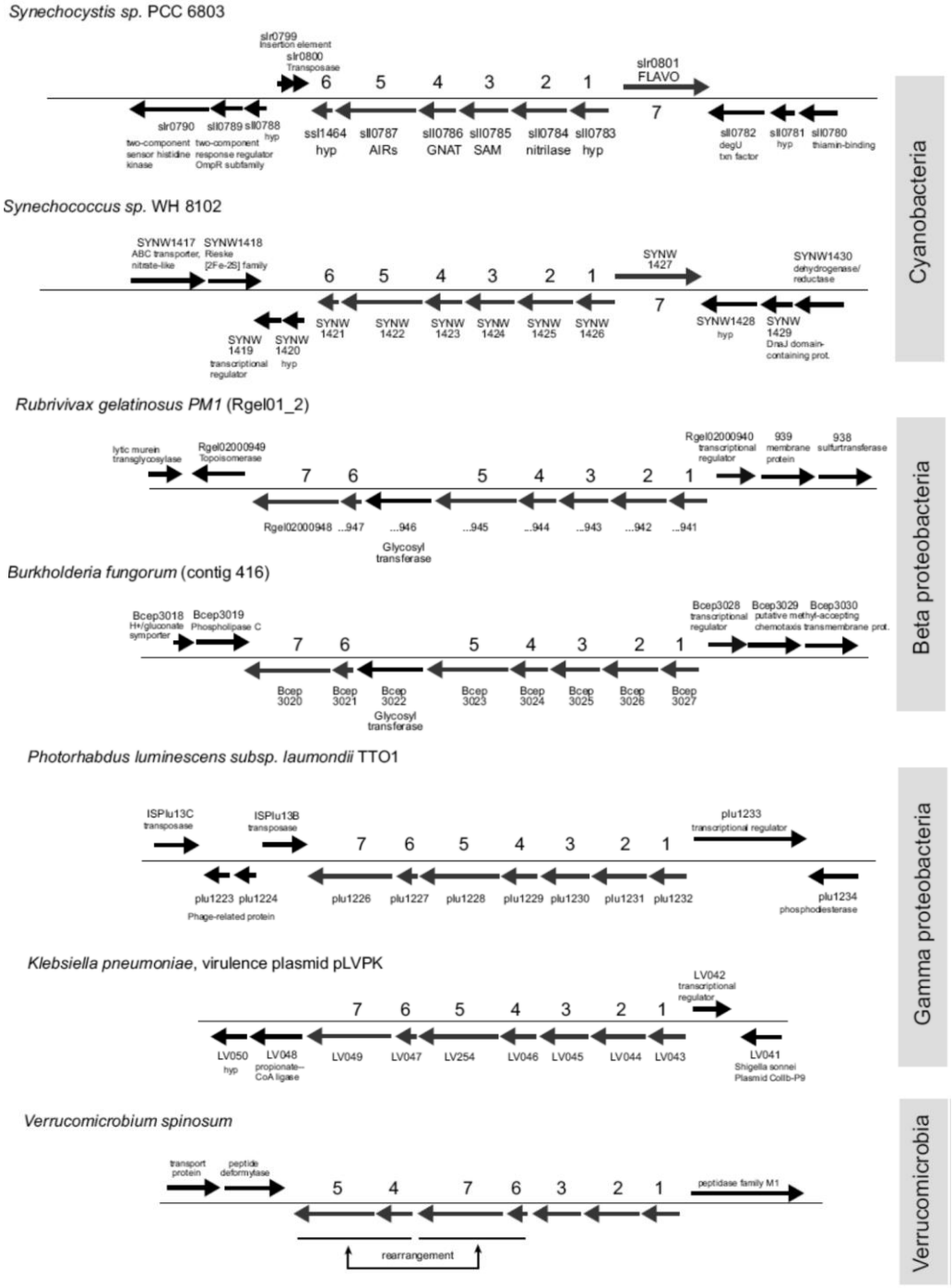

Fig. 1.5 Organization of gene clusters around the subfamily 1 nitrilases in sequenced bacterial genomes. The highly conserved gene cluster Nit1C is flanked by unrelated genomic neighbourhoods in the different species. Gene names are based on the available genomic annotation (Podar et al. 2005).

Other than Nit1C, there is no conservation between the predicted ORFs of the different species or the metabolic functions encoded by the gene clusters. However, the nitrilase genes of *Bacillus* sp. and *Pseudomonas syringae*, members of sub-family two, are apparently co-transcribed with a phenylacetaldoxime (PAOx) dehydratase gene and an araC transcription factor (Podar *et al*. 2005) which regulates expression of the *ara* genes which are responsible for allowing the bacteria to grow on L-arabinose as its sole source of carbon and energy (Beverin *et al*. 1971).

The idea that bacteria cells are capable of utilizing nitriles as N source from the environment, and, consequently, harboring genes encoding nitrilases is supported by evidence that these microorganisms are able to utilize nitriles as sole nitrogen source (Brady *et al.,* 2004). Sakashita *et al*. (2008) showed that *Pseudomonas chlororaphis* B23 expressed many NHases when methacrylamide was added into the media as the sole nitrogen source. It was also reported with regards to the number of genes belonging to NHase gene cluster, including oxdA, amiA, nhpA, nhpB, nhpC, nhpS, and acsA. Moreover, it was shown that the regulation of 4 enzymes expression resulting from 8 genes (nhpR, acsA, nhpS, nhpC, nhpB, nhpA, amiA and oxdA) was positively regulated by nhpR upstream of the NHase gene sequence cluster after the inducer (methacrylamide) was added to a minimal medium (Figure 1.6). Surprisingly, this study revealed that there are differences between the regulation mechanism and the gene cluster organization encoutered in this strain from those encoding the NHase and nitrilase (Komeda *et al*. 1996) in *R. rhodochrous* J1.

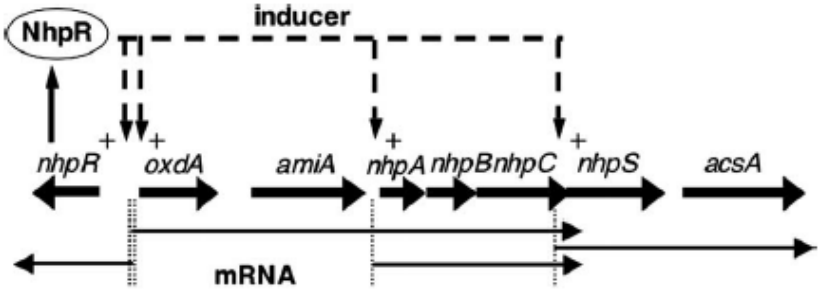

Fig. 1.6 The model for transcription of the NHase gene cluster caused by NhpR in *P. chlororaphis* B23. mRNA transcribed from the cluster is indicated by arrows at the bottom. +, stimulation of transcription (Sakashita *et al.,* 2008).

Nitrilase expression in microorganisms can best be initiated/optimised starting with the selection of a suitable inducer (substrate), since the nitrilases both of bacterial and fungal origin are usually induced in the presence of specific substrates (Martínková, Vejvoda, *et al.,* 2009). For instance, the nitrilase of the bacterium *Nocardia* sp. was induced using benzonitrile. Meanwhile, acetonitrile was used to induce the expression of the nitrilase in *Fusarium oxysporum* (Banerjee *et al*. 2002).

Nitrilases from filamentous fungi have shown low activity and thus the purification of these enzymes becomes difficult. However, several studies have demonstrated active nitrilases in filamentous fungi by using inducers, for example, 2-cyanopyridine and 3-cyanopyridine (Ondrej *et al*. 2006; Ondrej *et al*. 2011; Shaw *et al*. 2003). The 2-cyanopyridine was selected to be an excellent inducer of these enzymes in various genera of fungi (*Aspergillus, Fusarium* and *Penicillium*), since the resulting activity was two to three times higher than the activity with 3-cyanopyridine in culture media containing these substrates as inducers (Martínková *et al*. 2008). According to Martínková *et al*. (2009) 2-cyanopyridine can be regarded as a universal inducer of aromatic nitrilases in filamentous fungi. In addition, the nitrilases of filamentous fungi are generally classified as aromatic nitrilases due to high activity towards hetero-aromatic nitriles, however, these enzymes also catalyze the hydrolysis of a variety of aliphatic nitriles (Martínková *et al.,* 2009).

Recently, over 10 new nitrilases in Ascomycota (largest phylum of fungi) were reported (Gong, Li, *et al.*, 2012; Kaplan *et al.*, 2013; Veselá *et al.*, 2016; Rucká *et al.*, 2019), mainly in *Fusarium*, and it was revealed that phenylacetonitrile or mandelonitrile have been the main substrates for nitrilase activity. Nitrilases in Basidiomycota (second largest phylum of fungi), however, have been underexplored. Rucká *et al.*, (2019) reported overexpression of a novel nitrilase (NitAg) from mushroom-forming fungi in *E. coli* for the first time with excellent activity towards fumaronitrile.

1.3.1.2 Nitrile Hydratase (NHase)

NHases, which transforms nitriles to the corresponding amides is a vital enzyme in the bienzymatic hydrolysis of nitriles to acids. In 1980, acetonitrile hydratase was attributed to aliphatic nitrile degradation. The first occurrence of NHases was described in *R. rhodochrous* J1, this bacterium was initially identified as *Arthrobacter* sp. J1, which degraded acetonitrile to acetamide on an industrial scale (Asano *et al*. 1980).

The NHases are enzymes that have metal ions, either Fe^{3+} or Co^{2+}, in their active sites. Thus, the NHases can be classified into two groups: (i) iron-dependent NHases and (ii) cobalt-dependent NHases (Banerjee, Sharma and Banerjee, 2002). For instance, NHases from *Pseudomonas chlororaphis* B23, *Brevibacterium* sp. R312 and *Rhodococcus* sp. require iron, while the NHases from *R. rhodochrous* J1 and *Pseudomonas putida* NRRL 18668 require cobalt (Bornscheuer and Kazlauskas, 2005). NHases from *Agrobacterium tumefaciens*, however, require both metal ions. There are two main reasons for the presence of the metal ion in the active site of the enzyme. First, because the metal ions are optimal catalysts for hydration of the binding-CN. Second, the metal ions are essential to increase the flexibility (folding) and stability of the polypeptidic chain subunit (α and β) of NHases. So far, a considerable number of microorganisms were successfully screened as shown in Table 1.5 (Bhalla *et al.*, 2018; Chhiba-Govindjee *et al.*, 2019).

Table 1.5 Some previously reported microorganisms with NHases activity (Adapted from Bhalla *et al.*, (2018) and Chhiba-Govindjee et al., (2019).

Microorganisms	Substrates specificity	References
Agrobacterium tumefaciens d3	Arylnitriles, arylalkylnitriles, acrylonitrile	Bauer et al. (1998)
Arthrobacter sp. J-1	Alipatic nitriles	Asano et al. (1982)
Bacillus cereus	Acrylonitrile	Saroja et al. (2000)
Pseudomonas chlororaphis B23	Alkylnitrile	Hann et al. (1999)
Pseudomonas putida	Acetonitrile	Chapatwala et al. (1995)
Rhodococcus rhodochrous J-1	Alkylnitrile, heterocyclic nitriles, arylnitriles	Yamada & Kobayashi (1996)
Rhodococcus rhodochrous LL 100-21	Alkylnitriles, acrylonitrile, arylalkylnitriles, 3-cyanopyridine	Dadd et al. (2000)
Rhodococcus sp. AJ270	Wide spectrum nitrile hydratase	Meth-Cohn & Wang (1997)
Candida famata	Alkylnitriles	Linardi et al. (1996)
Cryptococcus flavus UFMG-Y61	Isobutyronitrile	Rezende et al. (1999)

The NHases were initially considered to be specific to aliphatic nitriles, however, activities of these enzymes were reported towards hetero-aromatic nitriles (Martínková *et al.,* 2008).

NHase expression in the wild type organisms of certain genera of gram-positive bacteria, such as *Rhodochrous* and *Nocardia* is generally inducible, which is regulated by its substrates or products like cyclohexanecarboxamide, butyronitrile, phenylacetonitrile, propionitrile, etc. However, some NHases are constitutive, as shown in the thermophilic bacterium *Bacillus pallidus* DSM 2349 by Cramp and Cowan (1999).

In general, only one type of NHase is produced by one organism. On the other hand, *R. rhodochrous* J1 produces two types of NHases [low-molecular weight NHase (L-NHase) and high-molecular weight NHase (H-NHase)] and their expression is regulated by supplementation of a specific inducer for each in the culture media. Both high and low molecular weight NHases (530 kDa and 130 kDa respectively), contain α- and β-subunits and are Co-type metalloenzymes. When cyclohexanecarboxamide is added to the culture media, the L-NHase is induced, which shows higher activity with aromatic and heterocyclic nitriles e.g. benzonitrile, cyanopyridines and cyanopyrazine (Wieser, Heinzmann and Kiener, 1997), while urea induced H-NHase exhibits higher specificity for aliphatic nitriles particularly acrylonitrile (Nagasawa *et al.,* 1991). These inducers seems to act at the transcriptional level to induce the expression of NHase genes and also to regulate the assembly of α- and β-subunits of H-NHase to acquire higher oligomeric state which is essential for its activity (Mizunashi *et al.,* 1998).

The characterization of the gene cluster of H-NHase induced by its reaction product in *R. rhodochrous* Jl was investigated by Komeda *et al*., (1996). Sequence analysis of the 4.6-kb region upstream from the gene encoding a cobalt-containing and amide-induced H-NHase from *R. rhodochrous* Jl revealed to be required for the expression of the H-NHase gene. It was reported that there are at least five open reading frames (*nhhC*, *nhhD*, *nhhE*, *nhhF* and *nhhG*) in addition to H-NHase α- and β-subunits genes (*nhhB* and *nhhA*, respectively). Two regulatory proteins (NhhC and NhhD) can be found in the H-NHase operon. Deletion of these proteins resulted in a decrease of NHase activity, suggesting in this way, a positive regulatory role of both ORFs in the H-NHase expression (Sun *et al.*, 2019). It is important to point out that the AmiC (regulatory protein involved in amidase expression regulation) in *Pseudomonas aeruginosa* showed significant sequence similarity that would allow one to infer homology to the NhhC from *R. rhodochrous* Jl (Sakashita *et al.*, 2008). Moreover, the H-NHase gene expression, which was determinated by H-NHase mRNA levels and also H-NHase activity in *R. rhodochrous* J1 has indicated that is regulated by an amide at the transcriptional level. Such findings corroborated for the participation of *nhhF* (IS1164, a member of IS256 family of insertion sequences) in the organization of the H-NHase gene cluster (Komeda *et al*., 1996).

The insertion of the IS1164 sequence represents a mobile DNA element and may be the basis for the lack of the amidase gene in this cluster, regardless of the fact H-NHase is induced by the presence of amides. *NhhE* is not required for NHase activity and its function still remains unclear. It is suggested that *nhhG* functions a self-subunit swapping chaperone but also as a metallochaperone in a subunit specific reaction (Zhou *et al.,* 2010).

The genetic structure of the L-NHase gene cluster was also determined by Komeda *et al*. (1996) in a *Rhodococcus* strain. The 1.4-kb downstream region from a nitrilase gene (*nitA*), was found to be required for the isovaleronitrile-dependent induction of nitrilase synthesis. Sequence analysis of the 1.4-kb region revealed the existence of an open reading frame (*nitR*) of 957 bp, which would encode a protein with a molecular mass of 35,100 Da.

The NHase from *Rhodococcus* sp. N-771 is the best characterised Fe-type NHase, possessing two different subunits (molecular masses: subunit α, 28,500 Da; subunit β, 29,000 Da) (Endo and Watanabe, 1989). The structure of its NHase gene cluster has been determined by Nojiri *et al*. (1999). The NHases operon consists of six genes encoding NHase regulator 2, NHase regulator 1, amidase, NHase α- and β-subunits and NHase activator, which differs considerably from the L-NHase and H-Nhase gene clusters from *R. rhodochrous* J1.

Another example was well studied by O'Mahony *et al*. (2005), the aim of which this study was to determine the structure of the NHases gene cluster in *R. erythropolis* AJ270. The NHase gene clusters of this strain are very similar in structure to those of *Rhodococcus* sp. N-771. On the other hand, the principle difference was the insertion of a complete copy of the insertion sequence IS1166 in the *nhr2* gene (*nhr2/IS*) Figure 1.7. The *nhr2* product plays a role in the photosensitivity of the NHases. The studies indicate that the NHases in these strains is an Fe-type enzyme and that this enzyme is inducible and is not photosensitive.

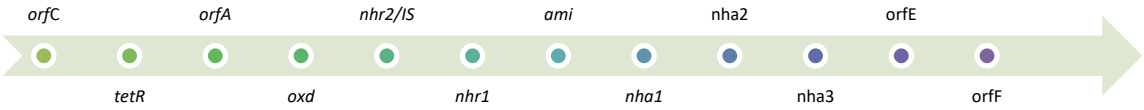

Fig. 1.7 The NHase/amidase gene cluster of *R. erythropolis* AJ270 [O'Mahony et al. (2005)].

The NHase/amidase genes of AJ270 display 100 % sequence identity to the corresponding genes in other *Rhodococcus* such as *R. erythropolis* N771. OrfE and *nha3* from AJ270 display a 93 % DNA sequence homology to the corresponding gene found in *Rhodococcus* sp. N771, however, the function of this gene is unknown. The NHase and amidase of AJ270 are inducible with a different repression/induction patterns (O'Mahony *et al.,* 2005).

Another interesting feature of NHase is its intracellular expression, consequently, recovery of whole cell from production media is essential and the harvested cells need to be suspended in buffer at pH approx. 7. The cells in buffer are known as resting cells which are used as a source of NHase for biocatalysis of nitrile to corresponding amides or for extraction of NHase (Prasad and Bhalla, 2010). Also, most of the NHases are thermolabile and their operational temperature has an optima range from 20 to 35 °C. However, few NHases exhibit maximum activity at 40 °C, 50 °C and 60 °C (Sharma, Sharma and Bhalla, 2009a).

Recently, a novel NHases was characterized of a bacterial plant pathogen - *Ralstonia* sp.ZA96, isolated from soil polluted with oil (Heidari and Asoodeh, 2019). The optimum temperature for its activity was 55 °C and at basic pH (8.5). It was evidenced that at 10 mM Zn^{2+} the NHases activity was reduced. In contrast, Ca^{2+} ion showed to increase this activity. Furthermore, the study presented that NHase can act on a broad range of substrates (either aliphatic or aromatic nitriles). Also, it has a potential to be used in the bioremediation of pollutants.

1.3.1.3 Amidase

Amidases are enzymes that catalyze the hydrolysis of amides to form carboxylic acids and ammonia. In addition, amidases catalyze the amide to hydroxamic acid (RCONHOH) in the presence of hydroxylamine (NH_2OH) as shown in Figure 1.8. Importantly, a majority of amidases bear enantio-selectivity, which contributes to the synthesis of chiral carboxylic acid via NHases and amidase, therefore, providing optically pure compounds of pharmaceutical interest such as amino acids or 2-arylpropionic acids (Chen *et al.*, 2009; Bhalla *et al.*, 2018).

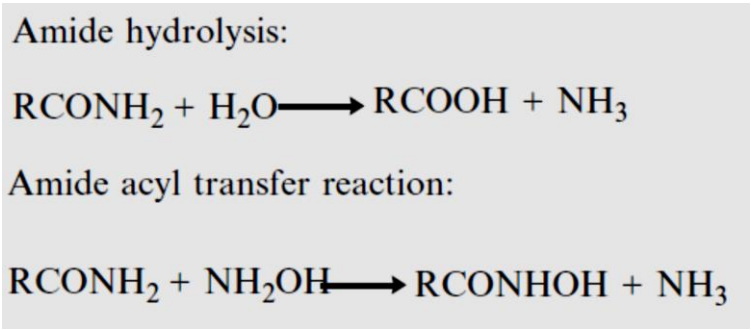

Fig. 1.8 The pathways of hydrolysis and transfer of amide. Amide hydrolysis and amide acyl transfer reaction (Chen *et al.*, 2009).

Accordingly, special attention has turned to isolation of organisms that produce amidases, including bacteria, yeasts, fungi, plants and animals (Martínková *et al.,* 2008; Martínková *et al.,* 2009; Winkler *et al.,* 2009; Santoshkumar *et al.,* 2010; Kaplan *et al.,* 2011; Veselá *et al.,* 2012). Characteristics of amidases from different microorganisms are given in Table 1.6 adapted from Martínková *et al.*, (2009) and Bhalla *et al.*, (2018).

Table 1.6 Biochemical properties of some amidases from different microorganisms.

Microorganisms	Formation type	MW (kDa)	Nº. of subunits and MW (kDa)	Reference
Arthrobacter sp. J1	Inducible	320	8(39)	Asano et al. (1982)
Brevibacterium sp. R132	Inducible	120	2(46)	Mayaux et al. (1990)
Klebsiella pneumonia NCTR1	Inducible	62	Monomer	Nawaz et al. (1996)
Pseudomonas chlororaphis B23	Inducible	105	2(54)	Ciskanik et al. (1995)
Ochrobactrum anthropi SV3	Inducible	40	--	Komeda & Asano (2000)
Agrobacterium tumefaciens d3	Inducible	490	-(63)	Trott et al. (2001)
Bacillus stearothermophillus BR388	Inducible	-	-	Cheong & Oriel (2000)
Rhodococcus sp.	Inducible	118	2(-)	Mayaux et al. (1991)
Rhodococcus sp.	Constitutive	360	-(44.5)	Nawaz et al. (1994)
Rhodococcus erythropolis MP50	Inducible	480	-(-)	Hirrlinger et al. (1996)
Rhodococcus rhodochrous M8	Constitutive	150	4(43)	Hirrlinger et al. (1996)

Recently a number of products that can be used as intermediates for pharmaceuticals, chemicals, food additives and waste material treatment has been obtained by enzymatic reactions. Both enzymes that convert nitriles (nitrilases and / or NHases and amidases) are potential catalysts for the production of carboxylic acids and amides of interest for biotechnological and industrial purposes (Gong *et al.*, 2012; Abdelraheem *et al.*, 2019; Chen *et al.*, 2019; Egelkamp *et al.*, 2019; Shen *et al.*, 2020).

Significantly, around 100 microbial species have been reported to have amidase. In contrast, only 40 microbes showed nitrilase activity against more than 60 possessing NHases (Bhalla *et al.*, 2018). Among bacterial enzymes, amidases from *Brevibacterium* sp. R312, *R. rhodochrous* J1, and *P. aeruginosa* are the best studied enzymes of this type. Native enzymes consist of a few identical subunits and are obviously related to the class of sulfhydryl enzymes (Ciskanik *et al.,* 1995). They exhibit two types of activity – (1) amidohydrolase and (2) amidotransferase. Contrasting with NHases, the association of amidases with metals such as iron or cobalt is reported only in *K. pneumoniae*, as demonstrated by Nawaz *et al*. (1996).

The substrate specificity and biological functions of these enzymes vary widely including carbon/nitrogen metabolism in prokaryotes through hydrolysis of amides (Cravatt *et al.,* 1996). The proteins structures revealed that aliphatic amidases share the typical α/β hydrolase fold (like those found in nitrilase superfamily), where signature amidases are evolutionary related to aspartic proteinases (Sharma *et al.,* 2009b).

Amidases hydrolyse a wide variety of amides (short chain aliphatic amides, mid-chain amides, arylamides, α-aminoamides and α-hydroxyamides) and can be grouped on the basis of their catalytic site and preferred substrate (Sharma *et al.,* 2009b). They resist denaturation at extreme of temperature and pH because of their strong and compact multimeric structures (Littlechild, 2015).

NHases and amidase coexist in a bienzymatic pathway in most microorganisms. NHases produces amides during nitrile hydrolysis, whereas the amidase completes the hydrolysis process converting the amide into a carboxylic acid and ammonia (Brady *et al.,* 2004).

As discussed previously, the genes which are associated with nitrile metabolism and/or regulation are known as nitrile gene clusters with other enzymes of this pathway (aldoxime dehydratase, nitrile hydrates, etc) (Bhalla *et al.*, 2018). The nitrile gene clusters associated with the Co-type NHase of *R. rhodochrous* J1 and the Fe-type NHase of *Rhodococcus* sp. N771 are extensively studied and well characterised. The amino acid sequence for the amidase purified and characterised from *R. rhodochrous* J1 was

significantly similar to those of amidases from other *Rhodococcus* sp. Figure 1.9 (Fournand and Arnaud, 2001).

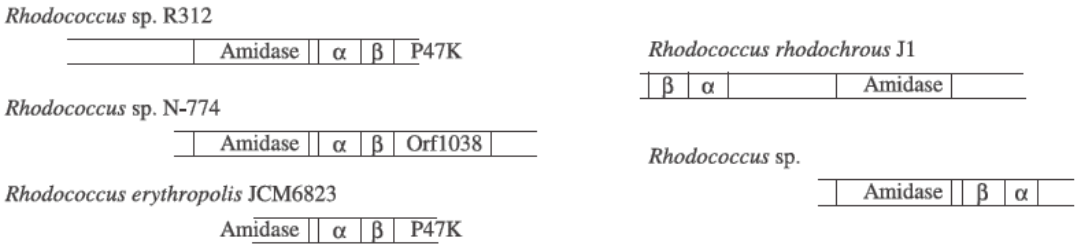

Fig. 1.9 Structural organization of the nitrile hydratase and amidase genes found in *Rhodococcus* sp., *R. rhodochrous* J1 and *R. erythropolis*. The α and β represent the subunits of the nitrile hydrates genes associated with the bienzymatic system. The ORF and P47K represent proteins who's function is still not clear.

1.4 Aldoxime Dehydratases

Aldoxime degrading enzymes have been extensively studied over the past few years as another route for nitrile production from many bacterial and fungal genera (Martínková *et al.*, 2008), Figure 1.10. However, in-depth investigations and industrial applications are restricted to *Bacillus* sp. OxB-1, *Rhodococcus* sp. N-771 and *Pseudomonas chlororaphis* B23, as demonstrated by Bhalla *et al.* (2018). Biotransformation of aldoximes to nitriles is carried out at neutral pH with the expectation of a pure product in contrast to the chemical method of dehydration, which requires harsh conditions. Besides, phenylacetaldoxime (PAOx) degrading and pyridine-3-aldoxime-degrading abilities were found to be widely distributed in bacteria, actinomyces, fungi, and some yeasts. All of the active strains exhibited not only the aldoxime-dehydration activity to form nitrile but also nitrile-hydrolyzing activity (Kato and Asano, 2006).

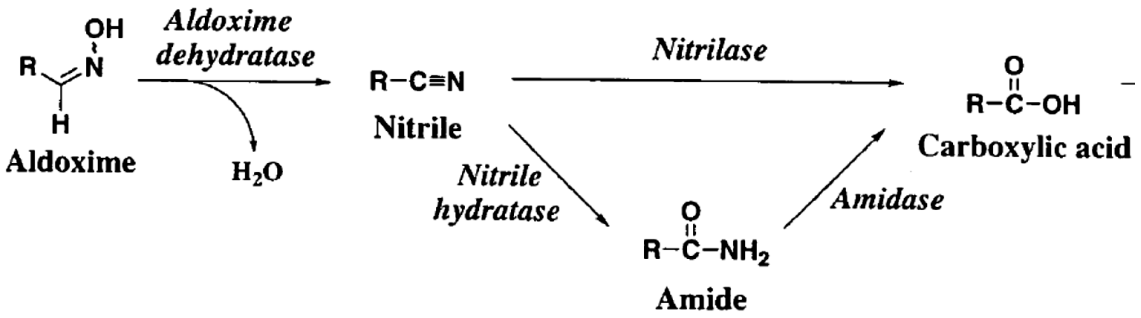

Fig. 1.10 Schematic for the catalytic conversion of an aldoxime to a nitrile via Aldoxime Dehydratase, with successive nitrile-hydrolyzing activity (Kato *et al.,* 2004).

In addition, Kato *et al*. (2000) carried out a study with the use of PAOx and pyridinium oxime (PyOx) as a model compounds of alkylaldoxime and arylaldoxime respectively, to determine the aldoxime degrading ability in bacteria. The aldoxime degrading activity was exhibited by numerous *Rhodococci*. Of the 975 microorganisms evaluated for aldoxime degrading activity, 98 degraded PAOx and 107 degraded PyOx.

It has been shown that the aldoxime and nitrile metabolising genes coexist due to a shared metabolic pathway of aldoxime metabolism. *Bacillus* sp. OxB-1 was one of the first bacteria to exhibit both aldoxime and nitrile degrading abilities. The bacteria has the ability to catalyse the conversion of Z-PAOx to phenylacetonitrile (PAN), and then, activities of nitrilase and amidase acting on PAN and phenylacetamide (PAAm), respectively, to form phenylacetate (PAA) (Asano and Kato, 1998). Also, the purification and characterization of NHases which is involved in aldoxime metabolism from the (E)-pyridine-3-aldoxime degrading bacterium, *Rhodococcus* sp. strain YH3-3 was described by the same authors. The strain showed the dehydration of E-pyridine-3-aldoxime to 3-cyanopyridine, which was then converted to nicotinamide by a NHases. Then, the nicotinamide was successively hydrolysed to nicotinic acid by an amidase.

One interesting study to identify genes coding for enzymes catalyzing the aldoxime biosynthesis in *Pseudomonas* sp. K-9 containing the aldoxime–nitrile pathway, was described by Kato and Asano (2006). The gene cluster was composed of genes coding for regulatory proteins, acyl-CoA ligase, amidase (*ami*), NHases (*nha*), NHase activator and some regulatory unknown proteins, as presented in (Figure 1.11). Comparatively,

the genetic organization of the biosynthetic gene cluster found in this bacterium were highly similar to those strains having Fe-containing NHase (Bhalla *et al.*, 2018).

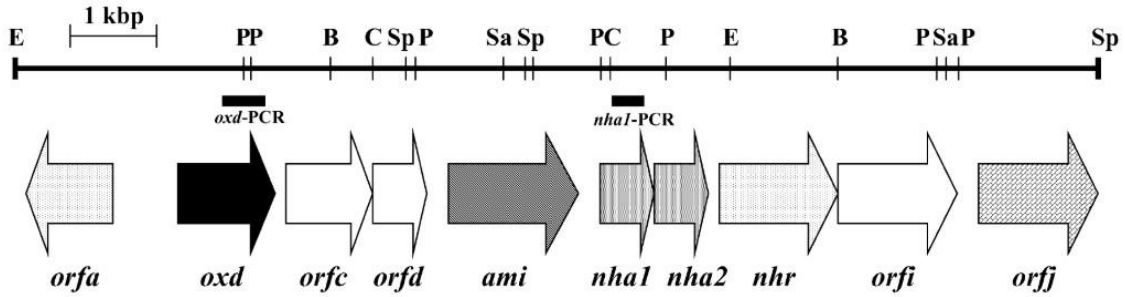

Fig. 1.11 Schematic of the genes present in the 'aldoxime-nitrile' pathway gene cluster in *Pseudomonas* sp. K-9 (Kato and Asano, 2006).

When comparing the cluster of *Pseudomonas* sp. K-9 with those in *R. globerulus* A-4 and *R. erythropolis* N-771, it can be seen that they contain similar genes with the gene arrangement being different. In contrast, the cluster of *Pseudomonas* sp. K-9 and *P. chlororaphis* B23 have a very similar arrangement of genes, and the gene products have remarkable identities with each other, as shown in Figure 1.12.

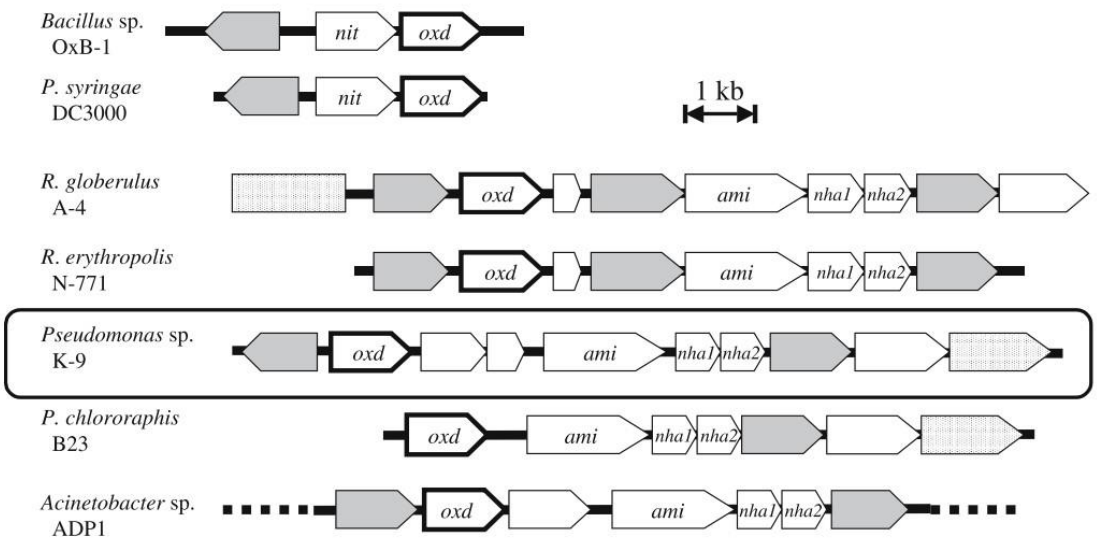

Fig. 1.12 Comparison of the "aldoxime–nitrile pathway" gene cluster in several microorganisms (Kato and Asano, 2006).

The overexpression of the key enzyme in the cluster, OxdK, in *E. coli* was performed by Kato and Asano in 2006. After purificaton, the enzyme was characterized, showing similar characteristics, including, substrate specificity, effects on various compounds and subunit structure, with those known for - aldoxime dehydratase. As a result, the alkylaldoxime-degrading pathway in *Pseudomonas* sp. K-9 was elucidated.

1.5 Screening and selection of new enzymes involved in nitrile metabolism

Microorganisms are the major source of industrial enzymes. The detection of these microbes in natural environments becomes of great importance to verify this biotechnological potential (Sahu *et al.,* 2019). Either screening or selection approaches have been applied for many decades for nitrile-converting microorganisms' isolation, as nitrilase, NHases and amidase can convert their substrates into high-added value compounds (Eman M. M. Abdelraheem *et al.*, 2019).

In general, screening methods are largely readily available and quantitative, but every single colony must be analyzed for enzyme activity. Selection methods are based on

using a selective medium, which allows only the cells with the desired enzyme to grow. Thus, they are more difficult to develop and provide only qualitative results, but have a very high throughput. Selection has been routinely used to obtain nitrile- or amide utilizing microorganisms. A broad spectrum of methods are applicable to assays of their enzymatic activities, but development of the corresponding high-throughput screens is a complex task (Martínková *et al*. 2008).

1.5.1 Conventional screening

Traditionally, microorganisms which have been reported as producers of nitrile-hydrolysing enzymes were screened in a selective medium containing nitriles as sole N source. Consequently, only microbial cells possessing nitrile-metabolizing enzyme activity can grow. As an example, acrylonitrile was used as the sole N source in the isolation of *Arthrobacter nitroguajacolicus* (soil bacteria) as demonstrated by Shen *et al*. (2009), which can perform the convertion of acrylonitrile to yield its the corresponding carboxylic acid - acrylic acid. Another example of conventional screening is the isolation of nitrile-degrading yeasts. Here, phenylacetonitrile was used as the substrate (Oliveira *et al.*, 2013). An *Exophiala oligosperma* strain was isolated, which showed interesting enzyme activity under acidic conditions (Rustler *et al.*, 2008). Other examples include the use of nitrile as a substrate in order to isolate a degrader microorganism of certain nitrilic herbicides, such as *Lysinibacillus boronitolerans* MLH-31 and *Bacillus cereus* MLH-61 bacteria strains, acting as detoxifying agents in the environment (Oliveira *et al.*, 2018). In contrast, a fungal strain (*Fusarium solani*) was isolated by Harper (1977a) from benzonitrile impacted soil-enrichment cultures. In some cases, the carboxylates resulting from nitrile or amide hydrolysis are also used by microorganisms and, therefore, the corresponding substrate can be utilized as sole C and N source (Martínková *et al.,* 2008; Ramteke *et al.*, 2013).

Most nitrile-converting microorganisms were obtained by using a selection technique designed as an enrichment culture, which consists in repeated subcultivations of mixed microbial populations with the target compound. Microorganisms growing faster than other species become dominant in the culture (Asano, 2002). Different microbial strains

are usually selected, when using different nitrile or amide compounds. The strains usually showed high-relative activities for substrates, which were used during the selection procedure (Kaplan *et al*. 2006).

Selection criteria such as temperature or pH can be also varied according to the desired properties of the enzyme. For example, enrichment culture performed at pH 3 with 2-phenylacetonitrile as sole N source afforded an acidotolerant nitrile-hydrolyzing strain of *Exophiala mesophila* (Rustler and Stolz, 2007b). Such microorganisms are promising for the hydrolysis of cyanohydrins, which are unstable at pH above 5 (Martínková, Vejvoda, *et al.,* 2009).

However, the enrichment technique is not always applicable, as some nitriles and amides may inhibit growth. Structurally similar substrates can be sometimes used in that case. For instance, microorganisms catalyzing the hydration of acrylonitrile were isolated by enrichment cultures with acetonitrile or isobutyronitrile (Asano, 2002). This approach was also used for labile substrates such as mandelonitrile that is prone to decomposition into benzaldehyde and HCN, and O-acetylmandelonitrile that is cleaved into mandelonitrile due to the action of ubiquitous bacterial esterases. Therefore, 2-phenylacetonitrile and its substituted derivatives were used as substrates for the selection of bacteria with nitrilase activities towards mandelonitrile (Kaul *et al.,* 2004) and O-acetylmandelonitrile (Layh *et al.,* 1992).

1.5.2 Screening of metagenomic libraries

A set of approximately 200 nitrilases, belonging to at least five major sequence clades, was discovered and published by Vererium Corp. (formerly Diversa Corporation, since acquired by BASF Enzymes) by screening clones expressing the DNA of uncultured organisms from various biotopes. Of these enzymes 137 were characterized in terms of their ability to transform industrially important nitriles (Robertson *et al.,* 2004). For instance, 48 nitrilases were able to hydrolyze mandelonitrile and most of them (44) were (R)-specific. Enzymes suitable for the enantioselective hydrolysis of 3-phenyl propionitrile into (R)-phenyllactic acid or (S)-phenyllactic acid were also identified. Some of the enzymes were able to desymmetrize prochiral 3-hydroxyglutaronitrile, which, by

monohydrolysis, yielded enantioenriched 4-cyano-3- hydroxybutyric acid, an intermediate of the cholesterol-lowering drug Atorvastatin (Lipitor) (Martínková and Kren, 2010). Another example of using functional metagenomics to screen for novel nitrile-hydrolyzing enzymes was described by Bragança *et al.*, (2017). 33 clones expressing nitrile metabolizing activity were confirmed for the ability to use β-hydroxynitriles as substrates.

1.5.3 High throughput screening

Nitrilase activity assays can be based on NH_3 determination, which provide an estimation of the enzyme activity. NH_3 determination from nitriles by nitrilase activity is usually performed by the phenol/hypochlorite method (J.-S. Gong *et al.*, 2011), as well as indophenol blue methods (Scheiner, 1976), the metal ion-based method (Yazbeck *et al.,* 2006), and nesslerization method (Kruse and Mellon, 1953). In addition, a number of screening techniques for nitrile-metabolizing enzymes detection can be used for analysis, like high-performance liquid chromatography (HPLC), gas chromatography, mass spectrometry, capillary electrophoresis and nuclear magnetic resonance (NMR) spectroscopy (Fiet *et al.,* 2006; Zhu *et al.*, 2007). Although effective, these methods are tedious, expensive and time-consuming (Santoshkumar *et al.,* 2010). Consequently, a rapid, simple and efficient screening approach is required (Sahu *et al.,* 2019).

In parallel, high-throughput screening methods employed for determining nitrile-converting enzymes has increased in the past decades (Gong *et al.*, 2017). Methods based on formation of a chromophore and pH indicators enable rapid and specific detection of nitrile-hydrolyzing activity by using whole cells or purified enzymes (Black *et al.*, 2015). Santoshkumar *et al.*, (2010) focused on a novel plate method for detection of aliphatic- nitrile-metabolizing bacteria. This may be an interesting prerequisite method to screen a large number of microbes possessing nitrile-degrading enzyme activity for their ability to form precursors of pharmaceutical interest.

Coady *et al*. (2013) also presents another strategy that facilitates the rapid identification of bacterial isolates demonstrating nitrile hydrolysing activity. The strategy incorporates toxicity, starvation and induction studies along with subsequent colorimetric screening for

activity, further focusing the assessment towards the substrates of interest. This high-throughput strategy uses a 96 well plate system, and has enabled the rapid biocatalytic screening of 256 novel bacterial isolates towards β-hydroxynitriles. Results obtained demonstrated the strategy's potential to rapidly assess a variety of β-hydroxynitriles including aliphatic, aromatic and dinitriles.

As an alternative to the previous method, Black *et al.*, (2015) developed a chromogenic method by using o-phthalaldehyde in high-throughput detection of nitrilase activity of crude cell-free extracts as an alternative to the Nessler method. Another excellent example was demonstrated by Sahu *et al.,* (2019). Soil bacteria were rapidly screened by simple dye-based technique – Bromothymol blue, as an indicator, associated to the nesslerization method in order to detect nitrile hydrolyzing enzymes activity based on a colour change. Over 100 bacterial strains were screened based on their capability to convert acrylonitrile into its corresponding products, which would be a useful, fast and efficient screening technique for nitrile-degrading microorganisms.

1.5.4 Screening for enantioselectivity

Many enantioselective enzymes have been identified among nitrilases, NHases and particularly amidases but this enantioselectivity is often still not sufficient to meet the demands of industrial processes. Broad screening for enantioselectivity is likely to provide desired enzymes (Gong *et al.*, 2012; Abdelraheem *et al.*, 2019). Chiral chromatographic methods employed in most previous works are not suitable for fast screening. However, most of the above colorimetric and fluorimetric methods are also applicable to rapid enantioselectivity assays, provided at least one of the enantiomers is available in an optically pure form (Martínková *et al.,* 2008; Martínková and Kren, 2010).

The above pH-responsive method was used for screening of (*R*, *S*)-mandelonitrile-hydrolyzing activity in about 60 microbial strains (Banerjee *et al.,* 2003). The positive candidates were examined for enantioselectivity. The rates of hydrolysis of racemic mandelonitrile and (*R*)-mandelonitrile were compared, since only this enantiomer was commercially available. The interpretation of the results was difficult, when the reaction of both racemate and *R*-enantiomer caused colour change, as the corresponding

enzymes could be either non-selective or *R*-selective. Therefore, it was proposed to run the screens with pure enantiomers. However, a microbe that was selective for (*S*)-mandelonitrile was identified by this method. Nevertheless, it is to be considered that the ratio of reaction rates for separate enantiomers does not truly reflect the enantiomeric preference of the enzyme acting on a racemate. Moreover, racemization of mandelonitrile must be considered at pH above 5 (Rustler and Stolz, 2007b).

An alternative method is based on the identification of strains, which convert about 50 % of a racemic substrate as monitored by a semi-quantitative TLC method. This approach proved successful in the search of amidases specific for (*S*)-piperidine-2-carboxamide and (*S*)- or (*R*)-piperazine-2-carboxamide (Eichhorn *et al.,* 1997).

1.6 Cloning of nitrile-hydrolysing genes

The production and purification of native molecules produced by wild-type organisms comprises a set of operations including the treatment of raw materials, the preparation of growing medium and the production itself, followed by product separation and purification steps (Kamionka, 2011). However, several problems have been identified such as the difficulty in separation and maintenance of wild-type organisms, lower yields in production by traditional processes, the need for products that are more pure and limitations in obtaining more complex desired molecules that were not synthesized/produced by these organisms.

There are a large variety of genes whose manipulation could be perfomed by genetic-based tools developed by recombinant DNA technology and other molecular techniques. These techniques open a new age for modern molecular biotechnology, a molecular science field of great interest and very promising in improving the acquisition of more varied products of biotechnology (Lee, 2011). Thus, the development and optimization of a high throughput screening strategy and new molecular biotechnology techniques, allowing the generation of recombinant enzymes for the green and sustainable synthesis of compounds for industrial purposes, is a logical workflow to pursue (Truppo, 2017). As a result of such workflows, several nitrile-hydrolysing genes from distinct organisms have

been cloned into suitable hosts and studied/exploited (Gong *et al.*, 2012; Nigam *et al.*, 2017; Liu *et al.*, 2019).

The first nitrilase gene cloned and expressed in *E. coli* encoded a bromoxynil-degrading activity from *Klebsiella pneumoniae* subsp. *ozaenae* (Stalker and McBride, 1987). Several other nitrilase genes from different microorganisms have also been cloned and expressed. The *nit*A gene from *R. rhodochrous* K22 was cloned into plasmid pNK21 (Kobayashi *et al.,* 1992). Komeda *et al*. (1996) reported the presence of a downstream region (1.4 kb) in *R. rhodochrous* J1 that is important for the induction of nitrilase synthesis. Sequence analysis shows the existence of an open reading frame (*nitR*) of 957 bp that codes for a positive transcriptional regulator for *nitA* expression. In a specific example of nitrilase gene expression, the nitrilase AtNIT1 from *Arabidopsis thaliana*, a higher plant, was overexpressed in *E. coli*. The recombinant AtNIT1 has properties similar to those of the native enzyme and the nitrilase from *Brassica napus* (Osswald *et al.,* 2002). Recently, a novel bacterial nitrilase (PaCNit) was isolated from *Pannonibacter carbonis* Q4.6 in a coal mine water and overexpressed in *E. coli*. In this study, the recombinant enzyme shows high activity towards arylaceto, aromatic and aliphatic nitriles, making it a promising biocatalyst for industrial purposes (Liu *et al.*, 2019). In another study, the methylotrophic yeast, *Picha postoris*, was used as a host organism to express a nitrilase gene from *Acidovorax facilis* ZJB09122: the nitrilase enzyme produced in recombinant yeast shows greatly improved activity levels when compared with the *E. coli* nitrilase whole cells (Shen *et al.*, 2020).

In the case of NHase, both the H- and L-NHase genes have been cloned from the industrial strain *R. rhodochrous* J1 (Kobayashi *et al.,* 1991). Both these genes showed significant similarity, in the β subunit's N-terminal sequence, with the NHase gene of *Rhodococcus* sp. N-774 (Ikehata *et al.,* 1989).

Expression of H- and L-NHase genes from *Pseudomonas chlororaphis* B23 under the control of a *lac* promoter in *E. coli* is possible only in medium supplemented with $CoCl_2$, indicating that the addition of cobalt ions to the medium causes the appearance of NHase activity. Although *E. coli* is the best-known cloning vehicle, expression of NHase genes from *Rhodococcus* sp. N-774 and *R. rhodochrous* J1 in *E. coli* resulted in accumulation of an insoluble polypeptide with minimal activity. Thus, NHase and amidase genes of *Rhodococcus* sp. N-774 have been introduced into *R. rhodochrous* ATCC 12674, where

they are expressed in active form (Hashimoto *et al.,* 1992). The close spacing of five genes (amidase, α and β-subunit of NHase, P47 K and *OrfE*) in *P. chlororaphis* B23 suggests that these proteins are translated from a single mRNA species. *E. coli* transformants carrying pPCN4, where this 6.2 kb region is placed under the control of a *lac* promoter, expressed NHase (10 % of total soluble protein) (Nishiyama *et al.,* 1991).

The structural genes encoding the α and β-subunits of the stereoselective cobalt NHase from *P. putida* NRRL 18668 have been cloned and sequenced (Payne *et al.,* 1997). A 6-fold over-production of the enzyme has been obtained by the co-expression of a novel downstream gene encoding protein P14 K that appears to be part of an operon that includes the structural genes for the α and β subunits of NHase. Another NHase gene from *Pseudonocardia thermophila* JCM 3095 has been cloned, and the expressed enzyme shows high thermal stability (Yamaki *et al.,* 1997). Also, the genes encoding an enantioselective NHase from *R. erythropolis* AJ270 have been cloned and an active NHase has been produced in *E. coli* (Song *et al.,* 2008).

Mayaux *et al*. (1990) cloned the amidase and α-subunit of the NHase gene from *Brevibacterium* sp. R312. A D-stereospecific amino acid amidase gene (*daaA*) was cloned from *Ochrobactrum anthropi* SV3 (Komeda and Asano, 2000). A wide-spectrum amidase gene from *B. stearothermophilus* BR388 was cloned and over expressed in *E. coli* (Cheong and Oriel, 2000). All this leads to a better understanding of, and improvements in, enzyme function for biotechnological applications.

1.7 Enzyme improvement in biotechnology

Developments in biocatalysis have been largely fuelled by consumer demands for new products, industrial attempts to improving existing process and minimizing waste, coupled with governmental measures to regulate consumer safety along with scientific advancements. The progress in the field of enzyme or reaction engineering has allowed access to means to achieve these ends, such as directed evolution, *de novo* protein design, use of non-conventional media, using new substrates for old enzymes, active-site imprinting, altering temperature, etc. Utilization of enzyme discovery and improvement tools therefore provides a feasible means to overcome this problem.

Judicious employment of these tools has resulted in significant advancements that have leveraged the research from laboratory to market thus impacting economic growth; however, there are further opportunities that have not yet been explored (Kaul and Asano, 2012).

1.7.1 Factors affecting the production of nitrile-hydrolyzing enzymes

Numerous factors, such as some culture conditions like carbon source, nitrogen source, inducer and conversion conditions like temperature, pH, reaction time, cosolvent etc., do affect the activity and enantioselectivity, and consequently the biomass (Chen *et al.,* 2009).

In fact, sources of carbon and nitrogen are essential nutrients for both cell growth and enzyme production (Mao *et al.*, 2005; Do Nascimento *et al.*, 2019). In some cases, the concentration of these nutrients regulates enzyme yield in a process observed during growth under conditions where the degradative exceeds biosynthetic pathways - catabolite repression, in order to ensure preferencial utilization of carbohydrates, e.g., glucose, lactose and glycerol (Nelson and Cox, 2017). As an example, when glycerol was added into the medium as sole C source as an alternative to glucose, the nitrilase yield of *Pseudomonas putida* MTCC 5110 showed a little lower compared with other sugar (Banerjee *et al.,* 2006). Notably, among the several tested carbon sources, mannitol, starch, sodium acetate and glucose showed the best performance for nitrilase production in *Rhodococcus* sp., *Alcaligenes faecalis*, *P. putida*, *Alcaligenes* sp. and *Paenarthrobacter nitroguajacolicus* (Hu *et al.*, 2007; Shen *et al.*, 2009; Y.-C. He *et al.*, 2010; Nageshwar *et al.*, 2011).

NHases and nitrilase are generally inducible, with very few being constitutive. Namely, the activity could be detected only in the presence of suitable inducers. Substrate, product, or their analogs usually function as inducers, with the exception of some extremely toxic nitriles, such as mandelonitrile, in which case, the growth of the microorganism was completely inhibited. Urea and ε-caprolactam, potential NHases inducers, play important roles in the induction of NHase activity. Moreover, with the addition of different inducers, microorganisms harboring versatile nitrile-converting

enzyme activities exhibited various activities. A distinguished instance was *R. rhodochrous* J-1, a currently utilized organism in commercial synthesis of acrylamide in Japan, which was found to contain two inducible NHases, one of which was specific for aliphatic nitriles induced by urea and the other for aromatic nitriles with cyclohexanecarboxamide and crotonamide as the inducers (Yamada and Kobayashi, 1996).

As far as NHases are concerned, metal ions, predominantly including Fe^{3+} and Co^{2+} are essential in the exhibition of its activity. In case of Fe^{3+} type NHases, only with the addition of Fe^{3+} in the nutrient broth acted as co-factor could NHases activity be observed, as so with the Co^{2+} type NHases (Wieser *et al.,* 1998); however, nitrilases require no metal ions or other cofactors for their activity. Instead, they are evidenced to have catalytically essential cysteine residues (Chen *et al.,* 2009). In contrast, Badoei-Dalfard *et al.,* (2016) demonstrated that the nitrilase and biomass production of *P. aeruginosa* RZ44 were enhanced when Na^{+}, Fe^{2+} and Mg^{2+} were used. On the other hand, addition of the Cu^{2+}, Mn^{2+} and Co^{2+} were shown to prevent cell growth and nitrilase yield.

Other factors could influence the nitrilase bioconversion, such as fluctuations in temperature and/or pH. In fact, nitrilase-catalyzed reactions generally occur under relatively mild conditions, which make them suitable for use as an alternative to traditional reactions (Eman M. M. Abdelraheem *et al.*, 2019). Temperature of 30 to 55 ºC and pH 7.0 to 8.0 were optimum for bioconversion of the nitriles by nitrilase for most bacteria and fungi, respectively (tables 1.7 and 1.8), as demonstrated by Gong *et al.*, (2012). Thus, few nitrilases are capable of resisting the extreme conditions. Yanase *et al*. (1983), however, reported an example of exception to this remark: a novel alkali-tolerant nitrilase from *Pseudomonas* sp. 13 was purified and characterized. With this enzyme the optimum nitrilase activity and stability was recorded at 10 mM 2-mercaptoethanol and pH 9.5.

Another example was relayed by Sonbol *et al*., (2016);the nitrilase gene (*nitras*-ATII) was isolated from the Atlantis II Deep of the Red Sea LCL, overexpressed in *E. coli* and characterized towards dinitriles. As a result, NitraS-ATII showed a high thermostability profile (70 °C) compared to closely related nitrilases from bacteria. Also, the enzyme exhibits tolerance towards high levels of different heavy metals. In addition, a new highly thermostable nitrilase from archaea was isolated (Cabrera and Blamey, 2017); the gene encoding a nitrilase from the hyperthermophile *Pyrococcus* sp. MC-FB was cloned,

expressed in *E. coli* and characterized. The enzyme showed high activity at 90 °C and pH 7.0 towards 2-cyanopyridine (aromatic nitrile), being the first archaeal nitrilase described that has significant aromatic activity so far.

Table 1.7 The reported bacteria with nitrilase activity (Gong *et al*., 2012).

Bacteria	Formation type	Molecular weight (kDa)	Optimum pH / temp(°C)	Stability pH / temp(°C)	Substrate specificity
A. faecalis ATCC 8750	Inducible	460	7.5 / 40-45	6.5-8 / below 50	Arylacetonitriles
A. faecalis JM3	Inducible	275	7.5 / 45	7-8 / 20-50	Arylacetonitriles
A. faecalis ZJUTB10	Inducible	-	7.7–8.5 / 35	- / below 35	-
Acidovorax facilis 72W	Inducible	570	- / 65	5-10 / below 60	Aliphatic dinitriles
Acinetobacter sp. AK226	Inducible	580	8 / 50	5.8-8 / below 60	Aliphatic, heterocyclic nitriles
Acinetobaeter sp. APN	Inducible	-	-	-	α-Aminonitriles
Alcaligenes sp. ECU0401	Constitutive	376	8 / 40	- / below 50	Aliphatic and aromatic nitriles
Bacillus pallidus Dac521	Inducible	600	7.6 / 65	6-9 / below 65	Aromatic nitriles
Bacillus subtilis ZJB-063	Constitutive	-	-	-	Arylacetonitriles
Brevibacterium strain R312	Constitutive	-	7 / 35	- / below 30	-
Geobacillus pallidus RAPc8	Inducible	600	-	-	Aromatic nitriles
Klebsiella ozaense	Constitutive	37	9.2 / 35	-	Bromoxynil
N. globerula NHB-2	Inducible	-	-	-	Aromatic, unsaturated aliphatic nitriles
Nocardia sp. NCIB 11216	Inducible	560	8 / -	-	Aromatic nitrile
P. aeruginosa 10145	Inducible	-	-	-	Aromatic nitriles
P. fluorescens DSM 7155	Inducible	130	9 / 55	-	Arylacetonitriles
P. fluorescens Pf-5	-	138	7 / 45	- / below 65	Dinitriles
P. putida	Inducible	412	7 / 40	6.5-8 / below 50	Arylacetonitriles
Pseudomonas sp. 13	Inducible	1,000	9 / 55	7-11 / below 60	β-Cyano-L-alanine
Pyrococcus abyssi GE5	Inducible	60	7.4 / 80	4.5–8.5 / 60-90	Aliphatic dinitriles
R. erythropolis ZJB-0910	Inducible	-	7.5 / 30	- / below 30	β-Hydroxy aliphatic nitrile
R. rhodochrous J1	Inducible	78	7.6 / 45	- / 20-50	Aliphatic, aromatic nitriles
R. rhodochrous K22	Inducible	650	5.5 / 50	- / below 55	Aliphatic nitriles
R. rhodochrous NCIMB 11216	Inducible	45.8	8 / 30	-	Aromatic nitriles
R. rhodochrous PA-34	Inducible	45	7.5 / 35	- / below 35	Aromatic, aliphatic nitriles
Streptomyces sp. MTCC 7546	Inducible	-	-	-	Aliphatic nitriles

Table 1.8 The reported filamentous fungi with nitrilase activity (Gong *et al.,* 2012).

Filamentous fungi	Formation type	Molecular weight (kDa)	Optimum pH / temp (°C)	Stability pH / temp(°C)	Substrate specificity
A. furmigatus	Inducible	-	-	-	**α-Aminophenylacetonitrile**
F. oxysporum* f sp. *melonis	Inducible	550	- / 40	6-11 / below 40	**Aromatic nitriles**
***A. niger* K10**	Inducible	>650	8 / 45	7.2-9 / below 30	**Aromatic nitriles**
***F. solani* O1**	Inducible	580	8 / 50	7 -9 / 35-50	**Aromatic nitriles**
***F. solani* IMI 196840**	Inducible	550	8 / 45	7.8-9.1 / below 50	**Aromatic nitriles**

Note: "-" indicates no data available.

1.7.2 Modification of medium components and process parameters

After the enzyme has been optimized for operation on the substrate of interest, further enhancement of the process productivity can be brought about by medium engineering and varying process parameters such as agitation and aeration. A remarkable effect of these parameters has been demonstrated for *E. coli* cells expressing the enantioselective arylacetonitrilase from *Pseudomonas putida*, the catalyst exhibiting optimal mandelonitrile-hydrolyzing activity at low agitation and low to intermediate aeration rates (Banerjee *et al.,* 2009). Similarly, as in the production of glycolic acid (Wu *et al.,* 2008), the economic viability of this process was increased by replacing the inducer IPTG with lactose. In the transformation of nitriles as hydrophobic compounds, tolerance to organic cosolvents is an imporant property of the enzymes used. A systematic study of the performance of arylacetonitrilase in the presence of various water-miscible and water-immiscible solvents was assayed with immobilized cells of *A. faecalis*, and a significant correlation between the solvent physicochemical properties and enzyme activity was observed (Kaul and Banerjee, 2008).

In most cases, a fermentation medium that supports the production of the metabolite of interest is first developed based on information available in the literature, the researchers' past experiences, and trial and error. In industrial settings, this medium becomes the

starting point for further optimization. The primary drive here is to increase product titer and to improve the economy of a fermentation process. A secondary drive includes the development of a simplified metabolite-purification process (Walker, 2005).

The basic nutrients for the growth of microorganisms include carbon, nitrogen, and minerals. Depending on the organism and the fermenter conditions used for growth and metabolite production, additional nutrients such as amino acids, vitamins, nucleotides, or even specialty chemicals might be required. There is an array of carbon and nitrogen sources available from which to choose. Some are in relatively pure form, whereas others occur in complex forms such as the byproducts of the food and agricultural industries. Pure or high-quality raw materials, although expensive, are more consistent from batch to batch and vendor to vendor. The byproducts of the food and agricultural industries are often less consistent in quality. Pure or higher-quality carbon and nitrogen sources are generally used for high-value products such as therapeutic proteins, whereas the less expensive raw materials are used for the production of low-cost and high-volume commodity items such as organic acids or bulk chemicals. When developing fermentation media, one must consider cost, availability, and lot consistency. In addition, fermentation media that facilitate downstream processing have an added advantage (Madigan *et al.,* 2017).

Once a fermentation medium is developed, the next phase is to optimize the media composition to achieve the best possible benefit. This task can be tedious and labour-intensive. The traditional method of media optimization is to alter one ingredient at a time until its optimum concentration is identified while the remaining ingredients are held constant. New techniques such as statistical experimental design have become available to assist fermentation scientists (Rao *et al.,* 2004).

Normally, in designing a statistical based experiment, it involves several steps such as (1) Selection of responses (performance characteristics of interest) that will be observed; (2) Identification of the factors (the independent or influencing factors) to be studied; (3) The different treatments (or levels) at which these factors will be set in different individual experiments; (4) Consideration of blocks (the observable noise factors that may influence the experiments as a source of error of variability) (Kumar and Baldi, 2013).

The major drawback of the statistical approach is that there are no precise guidelines for the sequence of experiments to be conducted and the level of combinations of different independent variables for each experiment. The system of laying out the conditions of experiments involving multiple factors was first proposed by Sir R.A. Fisher in 1920s, popularly termed as “fractional design of experiments” (Montgomery, 2017). A full fractional design identifies all the possible combinations for a given set of factors. Since most industrial experiments usually demand a significant number of factors, a full factorial design results in performing a large number of experiments. To reduce the number of experiments to a practical level, only a small set with all the possibilities are selected. The method of selecting a limited number of experiments which generates the most useful information is known as a partial fractional design (Weissman and Anderson, 2015).

The performance of improved strains selected from a screening process using Design of Experiment (DOE) and process optimization must be validated in pilot-scale fermenters. Once validated, the improved strains can be used for commercial production (Politis *et al.,* 2017).

1.8 Biotechnological aplications of nitrile-converting enzymes

Over the past few years, Biocatalysis has become increasingly important as a tool in the synthesis of pharmaceutical intermediates and products (Adams *et al.*, 2019). Various studies on nitrile biocatalysis have been perfomed throughout the past few decades. In the chemical industry, nitrilases have proven valuable in the manufacture of several compounds of high-added value, such as feedstuff additives, pharmaceutical Intermediates and bulk chemicals, by using microbial cells as prerequisite to harbour such enzymes (Egelkamp *et al.*, 2019).

The trend towards switching existing marketed racemic formulations to single isomer drugs rapidly gained pace from the 1990s as a result of thalidomide (Contergan) case. In fact, the difficulties of racemic drug formulation may arise from regulatory, safety and toxicology aspects, as demonstrated in Table 1.9. (Caldwell, 1996). As a consequence, progress in the pharmaceutical industry has led to a paradigm shift in drug development (Danhof *et al.*, 2018).

Table 1.9 If a drug is used in the form of a racemic mixture, then at least six situations are possible due to the two enantiomers (Crabtree, 2009).

Enantiomers have equal pharmacological activity
One enantiomer is biologically active, the other inactive and innocuous
One enantiomer is biologically active, the other toxic
The two enantiomers have unequal degrees of the same activity (i.e. different pharmacokinetics or metabolism rates)
The two enantiomers have different kinds of pharmacological activities (e.g. agonist and antagonist)
The two enantiomers vary in spectra of pharmacological action and tissue specificity

As a result, protein engineering of nitrilase has also been practiced to improve the substrate and product tolerance and specific activity. A high-activity biocatalyst based on an *A. facilis* 72W nitrilase was developed, where protein engineering and optimized protein expression in an *E. coli* transformant host were used to improve microbial nitrilase specific acticity for glycolonitrile by 33-fold compared to the wild-type strain (Wu *et al.,* 2008). Gene site saturation mutagenesis (GSSM) evolution technology was employed to improve enantioselectivity of nitrilase-catalyzed desymmetrization of 3-hydroxyglutaryl nitrile to afford (*R*)-4-cyano-3-hydroxybutyric acid, an intermediate to the cholesterol-lowering drug Lipitor (Grace DeSantis *et al.,* 2003). Changing Ala to His in position 190 provided a 10 % increase in the enantiomeric excess at the commercially relevant 3 M substrate reaction concentration (Chen *et al.,* 2009).

In fact, all the nitrile-metabolizing enzymes hydrolyze a number of structurally diverse nitriles. Several commercially important organic compounds, such as *p*-aminobenzoic acid, benzamide, acrylamide, nicotinic acid, pyrazinoic acid, thiophenamide, etc., have been prepared from the corresponding nitriles using microbial cells (Banerjee *et al.,* 2002).

1.8.1 Acrylamide

Enzymatic synthesis of acrylamide using NHase it is one of the most famed stories in pharmaceutical industry, with 650,000 tons per annum production (Abdelraheem *et al.*, 2019). The first successful commercial biocatalyst process was also the first industrial bulk chemical biotransformation for biocatalysis in Japan and Germany (Crabtree, 2009). The whole cells of *R. rhodochrous* J1, with high activity of NHases are used for conversion to acrylonitrile towards commercial production of acrylamide (Figure 1.13) (Schmid *et al.,* 2001). This biotransformation process was scale up to over 65,000 tons per year by Mitsubishi Rayon. High yield (>99.99 %) and excellent enantioselectivity (>99.99 %) were reached in this setting by biocatalysis, with no unwanted by-products, whereas using traditional methodology resulted in the production of acrylic acid by-products (Jiao *et al.*, 2019). Furthermore, the reactions are typically conducted under mild condition (5 ºC), thus, no inhibitors of polymerization are required (Eman M. M. Abdelraheem *et al.*, 2019).

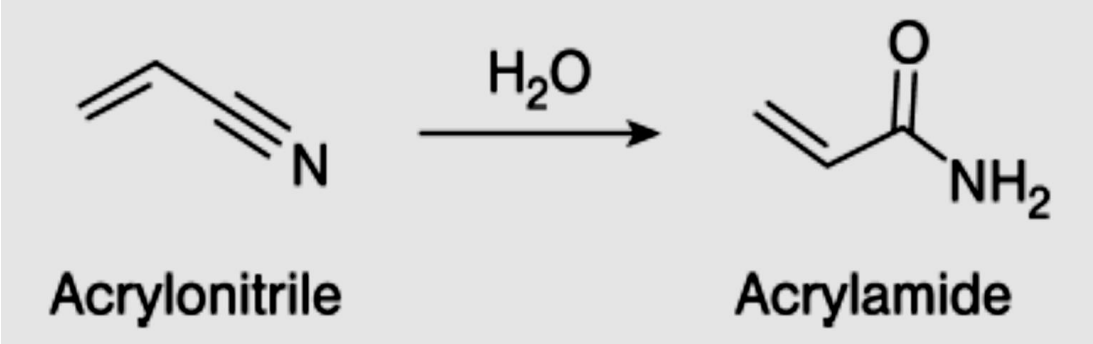

Fig. 1.13 Biotransformation of acrylamide from acrylonitrile by *R. rhodochrous* J1 (Caldwell, 1996).

1.8.2 Nicotinamide and Nicotinic Acid

Nicotinamide and its carboxylic acid (nicotinic acid) are B-complex vitamins used as animal feed supplementation, and also in pharmaceutical industries (Sharma *et al.*, 2006a). The global market for nicotinic acid and its derivatives market is predicted to be

valued at $46.45 billion by 2026 (ICRWorld Research, 2019), with demand predicted to be at least 22,000 tonnes per year (Pai *et al.*, 2014).

Nicotinic acid can be produced from either 3-methylpyridine or 2- methyl-5-ethyl pyridine oxidation but under harshes conditions of high temperature and pressure, and also with the formation of undesired by-products. For that reason, efficiency is always less than 100 percent and require expensive catalysts. On the other hand, enzymes from microrganisms (nitrilase or NHases and amidase) can act as an alternative to chemical processes for the production of nicotinic acid and its derivatives (Gong, Lu, *et al.*, 2012).

Nitrilase, the enzyme that selectively converts the nitrile directly into the acid with no amide intermediate, was used in order to hydrolyse 3-cyanopyridine to nicotinic acid, as shown by Sharma *et al.*, (2006b). The nitrilase of *Nocardia globerula* NHB-2 was induced by propionitrile and consequently, performed the reaction with higher conversion rates of 3.21 mg h^{-1} in comparison to *R. rhodochrous* J1 and *Bacillus pallidus* Dac521, with 2.28 mg h^{-1} and 0.55 mg h^{-1}, respectively.

An alternative to the nicotinic acid production route is the use of NHases, yielding nicotinamide from 3-cyanopyridine, as demonstrated in Figure 1.14. The nicotinamide production reaction was developed industrially by Lonza in Switzerland, Mitsubishi Rayon in Japan and BASF in Germany. Also, the production of acrylamide is performed using the same *R. rhodochrous J1* strain harbouring NHases for nicotinamide synthesis from 3-cyanopyridine (Eyal and Charles, 1990; Crabtree, 2009).

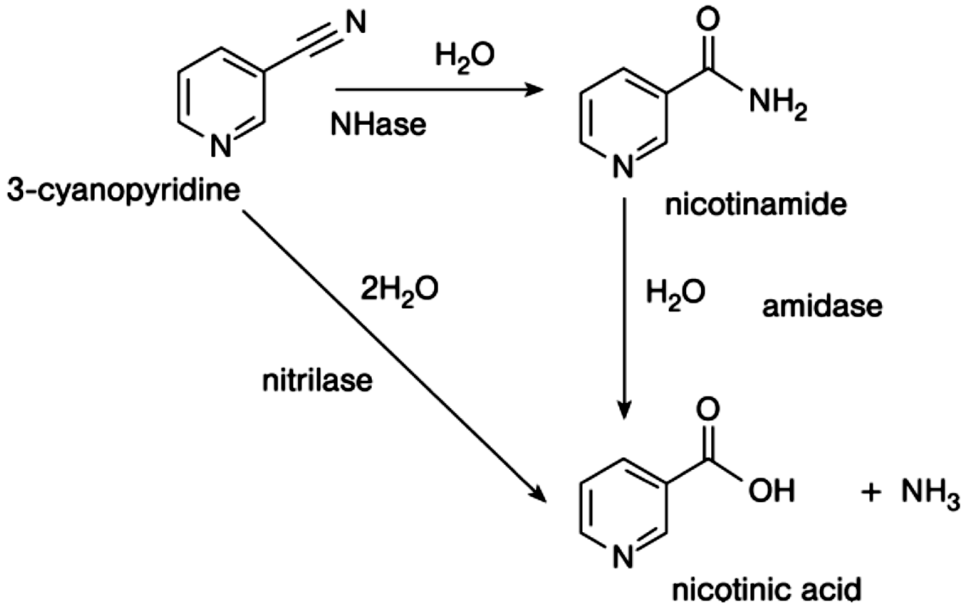

Fig. 1.14 Biotransformation of nicotinic acid from 3-cyanopyridine by NHases (Crabtree, 2009).

1.8.3 Atorvastatin

High cholesterol levels can increase the risk of heart disease. Worldwide, an estimated 2.6 million people are affected by high cholesterol according to the World Health Organization (WHO). In this scenario, statins are considered one of the most successful selling cholesterol-lowering drugs in the world (Hoyos *et al.,* 2019). The worldwide drug market for statins is enormous. For example, the atorvastatin (marketed as Lipitor®) with global sales of $12.9 billion in 2010, is the greatest blockbuster drug in pharmaceutical history. Total sales have exceeded $125 billion since its approval, with analysts predicting sales of US$1.85 billion in 2024 (Chen *et al.*, 2019).

One option for atorvastatin biosynthesis starts from the conversion of 3-hydroxyglutaronitrile into its biocatalytic product (R)-4-cyano-3-hydroxybutyric acid by nitrilases enzymes. This reaction requires nitrilase-catalyzed high regioselectivity and stereoselectivity (Crabtree, 2009).

In the specific example of biocatalyzed synthesis of atorvastatin, over 200 nitrilaes were screened in order to convert 3-hydroxyglutaronitrile into (R)-4-cyano-3-hydroxybutyric acid, yielding >95 % of final product and over 90 % *ee* (DeSantis *et al.*, 2002).

Another outstanding example was reported by Müller (2005). Interestingly, the nitrile 3-hydroxyglutaronitrile (3-HGN) was converted at high substrate concentration by the mutated enzyme at lab-scale reaction. The reaction yielded 96 % (R)-4-cyano-3-hydroxybutyric acid with an excellent *ee* value of 98.5 % when starting from 3 M racemic 3-HGN. Additionally, a *Pseudomonas fluorescens*-based expression system was used for nitrilase production for further studies towards the biocatalysis process. Optically pure (R)-4-cyano-3-hydroxy-butyrate was obtained (Figure 1.15) with a yield of 100 % and ≥99 % *ee* from 3 M racemic substrate concentration at a slightly basic pH (7.5) at 27 °C (Bergeron *et al*., 2006).

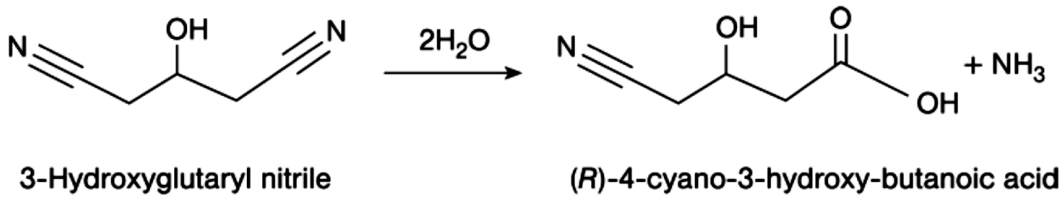

Fig. 1.15 Biotransformation of (R)-4-Cyano-3-hydroxy-butanoic acid from a-Hydroxygluraryl nitrile (Crabtree, 2009).

1.8.4 5-Cyanovaleramide

The conversion of adiponitrile to 5-cyanovaleramide is required for the synthesis of a new chemical used in agriculture (Chen *et al.*, 2009). This reaction can be performed chemically with a stoichiometric mixture of adiponitrile with water and a manganese dioxide (MnO_2) catalyst under harsh operating conditions. As an alternative to this process, a biochemical synthesis *via* microbial biotransformation was developed by DuPont, a chemical company (Figure 1.16) (Zaks, 2001). One such example was reported by Chen *et al.*, (2013). 5-cyanovaleramide, an herbicide intermediate, was used in the synthesis of the optically active weed control herbicide. The process uses whole cells of *Pseudomonas* sp. SY031 under conditions at pH 6.4, 10 mM of adiponitrile at 35 ˚C. Enzymatic reaction required lower temperatures and no by-products were detected, which represents an advantage over the classical chemical process.

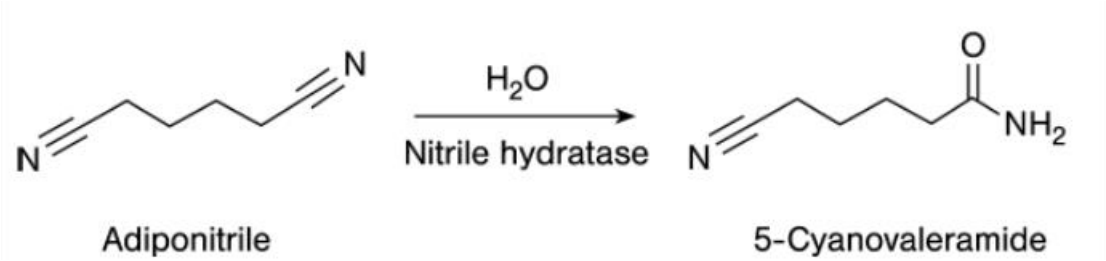

Fig. 1.16 Industrial chemical hydration of adiponitrile to 5-cyanovaleramide using whole cells of *P. chlororaphis* B23 (Zaks, 2001).

1.8.5 (R)-(−)-mandelic acid

(R)-(－)-mandelic acid is used as a chiral building block for the syntheses of several agricultural and pharmaceutical products (He *et al.*, 2010). In addition, the production of (R)-(－)-mandelic acid can be achieved by biotransformation and physicochemical approaches. The latter are technically difficult as reported by He *et al.*, (2008). (R)-(−)-mandelic acid, however, can be successfully produced using mandelonitrile as substrate by biotransformation methods (Chen *et al.*, 2017). The hydrolysis of mandelonitrile for (R)-(－)-mandelic acid production generally exhibits advantages because of reduced

byproduct formation and high enantioselectivity under mild conditions. Notably, the main source of mandelonitrile-specific nitrilases are the gram-negative *Alcaligenes* bacteria (Gong *et al.*, 2017). Additionally, a novel nitrilase was isolated from an opportunistic pathogen, *Burkholderia cenocepacia* J2315, which efficiently converts mandelonitrile to yield its corresponding (R)-(−)-mandelic acid, as shown in Figure 1.17, with 98.4 % of enantiomeric excess (Wang *et al*., 2013).

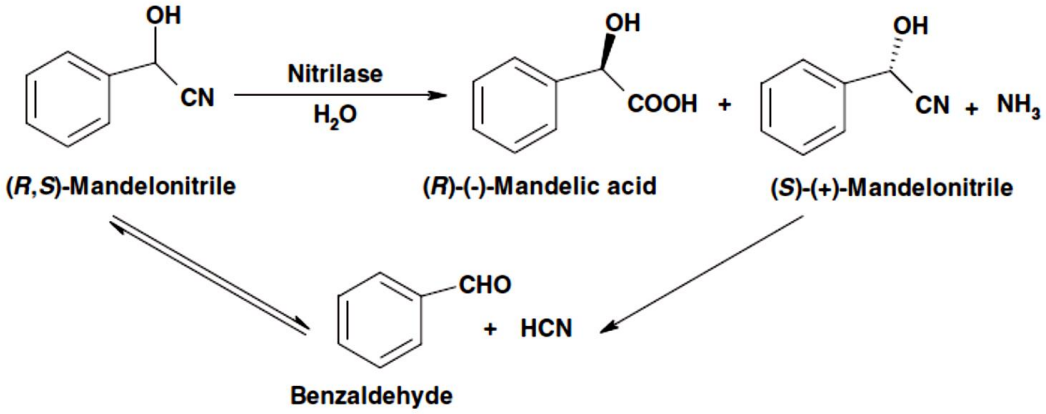

Fig. 1.17 Biosynthesis of (R)-(−)-mandelic acid from (R,S)-mandelonitrile with nitrilase (Xue *et al*., 2011).

1.8.6 Bioremediation and biodegradation potential

Nitrile compounds show widespread environmental occurrence, which may explain the prevalence of nitrilases in microbial cells (Howden and Preston, 2009). However, accidental release of nitriles into the environment can lead to accumulation of these potentially toxic compounds in biota over time (Egelkamp *et al.*, 2019). In this way, nitrile-converting enzymes play a large role in the degradation of highly toxic nitriles in nature, namely bioremediation (Gong, Lu, *et al.*, 2012). Benzonitrile, acetonitrile, acrylonitrile and dichlobenil represent such typical compounds, which are used commonly in agriculture for synthetic rubbers, pesticides, herbicides, plastics and pharmaceuticals (Banerjee *et al*., 2002). Thus, the bioremediation strategy using nitrile-degrading enzymes has been

illustrated as an efficient method for degrading compounds which are toxic in environmental wastes. (Martínková *et al*. 2009; Veselá *et al*. 2010).

Due to the potency and efficiency of biodegradation, similar studies were also conducted in China, in Taiwan, and moreover, some achievements were obtained (Lee and Wang, 2004; Wang, Lee and Chen, 2004; Kao *et al.,* 2006). Nitrile-converting enzymes have also participated in the cyano group-containing herbicide decomposition. A bromoxynil-degrading soil microorganism *Agrobacterium radiobacter* was used for degradation of the herbicide under non-sterile batch and continuous conditions. The efficacy of degradation was enhanced by addition of ferrous, cobaltous or cupric ions (Müller and Gabriel, 1999).

1.9 Basis of Research Work

Given to their various traditional and industrial applications, researchers from the Department of Science, Waterford Institute of Technology, Ireland, showed interest in the development of recombinant enzymes towards the production of pharmaceutical intermediates using biotransformations. In order to establish an effective process of biotransformation, firstly, O'Mahony *et al*. (2005) determined the structure of the NHases gene cluster in *Microbacterium sp*. AJ115, *R. erythropolis* strains AJ270, AJ300 and ITCBP. This study indicated that the NHases in these strains is an Fe-type enzyme and that this enzyme is inducible and is not photosensitive. In addition, the NHases /amidase enzymes of strain AJ270 are inducible with acetonitrile or acetamide. Subsequently in 2008, Song *et al*. reported successfully the expression of the AJ270 NHases using *E. coli* as a host. This enzyme has excellent regioselectivity in hydrolyzing a variety of aliphatic dinitriles and shows excellent enantioselective hydrolysis of racemic trans- and cis-2-arylcyclopropanecarbonitriles. As a result, this study showed that the recombinant enzyme retained the ability to enantioselectively transform 4-benzoyloxyglutaronitrile to the S-(+)-3-benzoyloxy- 4-cyanobutyramide monoamide. Moreover, Coffey *et al*. (2009) performed a study to determine the presence of any nitrilase genes from multiple bacterial strains and to develop a real-time PCR method for detecting any isolated nitrilase gene from soils and bacterial isolates, since the bacterial enzymes capable of nitrile hydrolysis have significant industrial applications. In this study, an identical nitrilase gene (*nit*1) was isolated from the four strains, and the nitrilase from strain AJ270 cloned and expressed

in *E. coli*. Analysis of the recombinant nitrilase has shown it to be functional with activity demonstrated towards phenylacetonitrile.

In 2013, Coady *et al*. reported a high throughput screening strategy for the rapid identification of bacterial isolates possessing nitrile hydrolysing activity towards a variety of β-hydroxynitriles including 3-hydroxybutyronitrile, 3-hydroxy-3- phenylpropionitrile and 3-hydroxyglutaronitrile, which represent an aliphatic, aromatic nitrile and dinitrile respectively. In this study, the application of this strategy was also demonstrated to a successful enantioselectivity screening study, resulting in the identification of an isolate demonstrating >99.9 % ee for 3-hydroxybutyronitrile specifically. In addition, the cost-effective screening strategy developed in this study can be used to screen a large number of microorganisms for their ability to transform aliphatic, aromatic nitriles and dinitriles within a short time period.

Currently, culture enrichment techniques have allowed microorganisms with nitrile-hydrolyzing enzymes to be isolated (Coady *et al.,* 2013). In addition, uncultured environmental samples can be explored by the metagenomic approach (Daniel, 2005). Most of the well-characterized NHases, amidases and nitrilases, which are largely of bacterial origin, have been obtained by selection methods allowing only the positive strains to grow on a minimal medium with a nitrile as the sole nitrogen source (Martínková *et al.,* 2008; Coffey *et al.,* 2009, 2010; Coady *et al.,* 2013). On the other hand, filamentous fungi seem to be a rich but so far little exploited source of nitrile-hydrolysis enzymes.

In this way, our goal was to (1) isolate and screen bacteria with activity towards three pharmaceutically relevant β-hydroxynitriles from environmental samples collected worldwide; (2) bioprospect fungi with potential for nitrile biotransformations; (3) apply functional metagenomics to search for novel nitrile hydrolyzing enzymes from various environmental samples; and (4) to express in *E. coli* a nitrilase gene from *Burkholderia* sp using a statistical design of experiment for culture conditions optimization approach. This will allow the purification of recombinant enzyme, which is the form/stage required by potential industrial partners/customers for screening/scale-up/go-to-market or indeed any collaboration with industry.

CHAPTER 2

THE IDENTIFICATION OF NITRILE-METABOLIZING BACTERIA AND ENZYMES TOWARDS THE PRODUCTION OF PHARMACEUTICAL INTERMEDIATES USING BIOTRANSFORMATIONS

2.1 General introduction

While enzymes are part of the cellular processes of living organisms, or indeed secreted by cells (e.g. filamentous fungi, bacteria, yeasts, algae, plants and animals) it is also possible to exploit them in isolated forms for commercial applications. The use of enzymes in the transformation of organic compounds has been known for over 100 years (Abdelraheem *et al.*, 2019) However, it was only from the second half of the 1990s that the real potential of these biocatalysts in organic synthesis began to be exploited. During this period, a large number of enzyme-catalyzed reactions have been developed and one of the main contributions of such biosynthetic methods has been in obtaining enantiomerically pure intermediates or final products of stereocontrolled synthetic routes (Costa and De Amorim, 1999).

Most drugs have an effect explained based on the theory of receptors. The receptor molecules in the organism are proteins that show high affinity for binding to a certain molecular structure in a manner analogous to the enzyme-substrate connection. The binding of a substrate to a receptor activates a mechanism, which manifests itself as a biological response. This mechanism can be a modification in the activity of an enzyme or transport of ions, and so on. Whatever its physiological function, the receptors have one thing in common: they are chiral molecules and can therefore be enantioselective for its binding with messenger molecules (Aboul-Enein and Wainer 1997).

Currently, the importance and implications of chirality in the development of new drugs are well recognized by both academia and industry. Some years ago, the development of drugs containing only the most active enantiomer was not considered viable (Srinivas *et al*. 2001). However, after decades of pharmacology and therapeutics, a rediscovery of stereochemistry was remarkable in the early 1980s. This rediscovery was prompted by the emergence of new technologies that enabled the preparation of pure enantiomers in quantity, leading to a renewed interest in the role of stereochemistry in the action of drugs (Truppo, 2017; Cheng, Xia and Zhou, 2020).

Most nitriles found in nature are toxic, mutagenic and carcinogenic (Chen *et al.*, 2009; Egelkamp *et al.*, 2019). Despite the high toxicity, they are important intermediates in the chemical industry for the production of amides, carboxylic acids, and various other products (Yu *et al.*, 2021). In recent years, there is a considerable interest in the utilization

of enzymes or whole cell bacterial isolates to afford enantiomerically pure pharmaceutical actives and intermediates in biotechnology and organic chemistry. This is due to the many advantages offered by biotransformations for the conversion of nitriles because these biological systems offer the possibility of using environmentally friendly ("green") reaction conditions (Crabtree, 2009; Fessner, Turner and Wang, 2011; Józwiak, Lough and Irving W. Wainer, 2012; Adrio and Demain, 2014; Kim, 2015; Abdelraheem *et al.*, 2019).

Hydrolysis of nitriles by microbial isolates may proceed via two different enzymatic pathways. In the first route, nitrilases catalyse the direct hydrolysis of nitriles into the corresponding carboxylic acid and ammonia. In an alternative pathway NHases may convert nitriles to the corresponding amide, which is then hydrolysed to the corresponding carboxylic acids and ammonia by an amidase enzyme (Martínková and Kren, 2010; Gong, Lu, *et al.*, 2012; Frederick *et al.*, 2020).

Hann *et al*. (2003) have shown that *β*-Hydroxy nitriles such as 3-hydroxybutyronitrile, 3-hydroxyglutaronitrile and 3-hydroxy-3-phenylpropionitriles can act as sources of *β*-hydroxy carboxylic acids by hydrolysis reactions, but can, however, be prone to elimination reactions under classical acid/base conditions. On the other hand, nitrile biocatalysis can selectively facilitate this hydrolysis without affecting other acid- or alkali-labile functional groups present (DeSantis *et al.,* 2002; O'Reilly and Turner, 2003) whose products can be widely used as chiral precursors for pharmaceutical compounds. For instance, 3-hydroxy-3-phenylpropionic acid and its derivatives, have been used as precursors to chiral drugs such as an important anti-depressants fluoxetine, tomoxetine, nisoxetine and norfluoxetine (Kamal *et al.,* 2002; Hammond *et al.,* 2007). Furthermore, of particular commercial interest, has been the nitrilase catalysed hydrolysis of 3-hydroxyglutaronitrile, the ethyl-ester of which is an intermediate to the cholesterol lowering drug atorvastatin (Lipitor) (DeSantis *et al.,* 2002; Bergeron, Chaplin, *et al.,* 2006). Indeed, several enzymes show potential and actual use in the production of various therapeutic statins (Hoyos *et al*., 2019).

A major consideration in the development of a specific biotransformation is to find the ideal biocatalyst. However, to establish an effective biotransformation process, a detailed analysis of the factors that influence the development and optimization of the integrated biotechnological process is required, which depends on: a) the biocatalyst, b) the

bioconversion and c) the isolation and purification of the product (Oliveira *et al.,* 2015). In this way, with careful selection of biocatalytic systems, it is possible to produce enantiomeric compounds; amides and carboxylic acids with high enantiopurity, making the isolation of microorganisms as “nitrile biocatalysts” a large but potentially rewarding challenge. With this background, the current chapter was focused on identification of nitrile-metabolizing bacteria and enzymes towards the production of pharmaceutical intermediates using biotransformations. This will allow further the cloning, expression and purification of recombinant enzyme(s), which is the form/stage required by potential industrial partners/customers for screening/scale-up/go-to-market or indeed any collaboration with industry.

2.2 Material and methods

All media, solutions, buffers and antibiotics used in this work were made up with distilled deionised water, and sterilized when required. Also, all chemicals used were of analytical grade. Media was made up fresh when required and sterilized by autoclaving before use. All kits were stored and used according to manufacturer’s instructions, unless otherwise stated.

2.2.1 Soil samples, enrichment culture and microbial isolation

Soil samples were previously collected by the research group (Coffey *et al.,* 2010). These soil samples were collected from a range of sites (e.g., suburban, forest, estuaries) from Ireland, England, Wales, France, Poland, South Africa, Estonia, Germany, Austria, Spain, Czech Republic and Australia. The isolation of bacteria from these soils in this study was carried out according to Coffey *et al.,* 2009 with some modifications: 0.5 g sample of each soil was incubated in 50 ml M9 minimal medium supplemented with 10 mM nitrile as the nitrogen source. Each soil was incubated in three shake flasks simultaneously, each with one of three different nitriles - 3HBN, 3HPPN or 3HGN as the sole source of N. These soil cultures were incubated for 28 days at 25 °C and 175 rpm, with samples taken on days 2, 7 and 14. Flasks were allowed to stand for 2 h before sampling to allow larger soil particles to settle. Two 1 ml samples were withdrawn from the top of the culture and dispensed in sample tubes. These 1 ml portions were centrifuged for 1 min at 1,000 x g to pellet remaining large soil particles. An 800 µL portion of the resulting supernatant was

transferred to fresh sample tubes for immediate further analysis, or storage at -70 °C after addition of glycerol to a final concentration of 20 % (v/v). Soil supernatants were serially diluted from 10^{-1} to 10^{-6} and all dilutions were plated onto M9 agar supplemented with the same nitrile as used for enrichment, at the same concentration. Single colonies were picked and serially streaked to fresh M9 agar containing nitrile to obtain pure isolates. After that, isolates were submitted for activity screening in order to determine activity against the nitrile.

2.2.2 Substrates of interest

The nitriles chosen for this study - 3-hydroxybutyronitrile (3HBN), 3-hydroxyglutaronitrile (3HGN) and 3-hydroxy-3-phenylpropionitrile (3HPPN) - are β-hydroxynitriles (Figure 2.1), which can act as sources of β-hydroxycarboxylic acids by biotransformation. These products could be widely used as chiral precursors for pharmaceutical compounds. Racemic 3HBN and 3HGN were purchased from ENAMINE®, and 3HPPN from Sigma®. All other chemicals were of analytical grade and obtained from Macron Fine Chemicals™ and Acros Organics Chemicals.

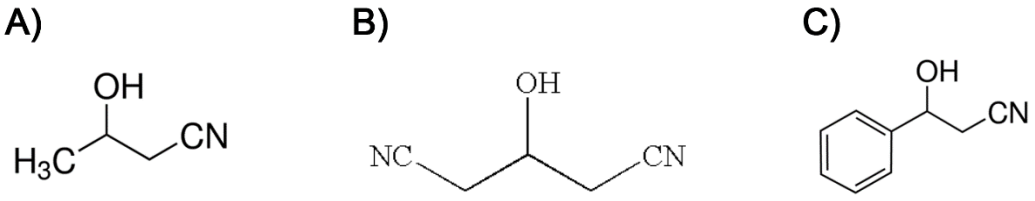

Fig. 2.1 Structure of β-hydroxy nitriles of interest: (A) 3-HBN; (B) 3HGN; and (C) 3HPPN.

2.2.3 Bacteria maintenance and growth medium

The nitrile-metabolizing bacterial strains were isolated from environmental samples collected worldwide. The isolated strains were maintained on YPD plates [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose, 2 % (w/v) nitrogen-free ultrapure agar-agar (Merck)]. For long-term storage, cells were maintained frozen at −70 °C in 20 %

(v/v) glycerol. M9 minimal media [1 X M9 salts, 1 X trace elements solution, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM $FeSO_4$, 0.2 % (w/v) glucose] supplemented with 10 mM nitrile substrate with or without 2 % (w/v) agar was used for bacteria isolation and activity screening. All strains were maintained on YPD agar [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose, 1.5 % (w/v) agar].

2.2.4 Toxicity screening

After isolation, the bacterial isolates were subjected to toxicity studies, each with the other 2 nitriles of interest. By attempting to grow the different isolates in rich medium in the presence of β-hydroxynitriles, the isolates that are sensitive to the nitrile and cannot grow in its presence were determined and excluded from further screening.

Cells were grown in YPD broth containing 10 mM concentrations of the nitriles of interest and checked daily for growth by measuring the optical density A_{600nm} using a NanoDrop® 2000 spectrophotometer (Thermo Scientific). Cells were then subcultured in nutrient agar, containing the key nitrile, using a 96 well stamp format and incubated for up to 5 days. After that, plates were examined visually for the appearance of colonies.

The tolerant isolates then underwent a starvation process in order to ensure that the nitrile would be utilised as the sole N source during the induction stage. The subsequent induction stage involved attempting to grow the isolates in M9 minimal media using 10 mM nitrile as the N source.

2.2.5 Activity screening for nitrile metabolism: Induction of bacteria for activity screening

The tolerant isolates then underwent a starvation process to remove any excess energy from previous culturing in rich media, in order to ensure that the β-hydroxynitrile would be utilised as the sole nitrogen source during the induction stage. 96-well Megablocks® (Sarstedt Ltd, Ireland) containing 600 µL of M9 minimal media supplemented with 10 mM nitrile substrate were inoculated with the isolates and incubated with shaking for 48 h at 25ºC and 250 rpm. For storage of grown cultures, 400 µL of 20 % (v/v) glycerol was added to each culture well and Megablocks were sealed and frozen at −70 °C.

In order to screen the isolates to determine activity levels, a suitable analytical screening method was used. Bacterial growth on key nitriles as the sole N source results in the release of ammonia (Figure 2.2). The amount of ammonia liberated during nitrile degradation was monitored by Nesslerization method as result of the nitrile-degrading enzyme activity.

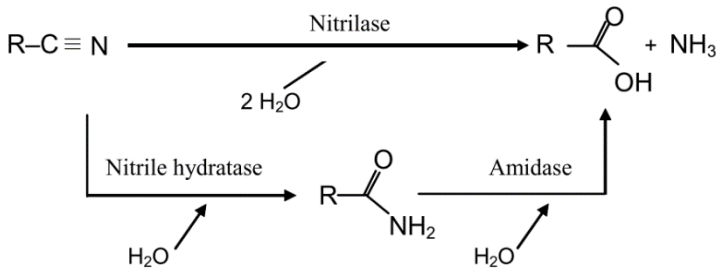

Fig. 2.2 Ammonia is liberated by nitrilase-catalysed hydrolysis (Rao *et al.*, 2010).

2.2.6 Standard curve for Nessler's colorimetric ammonium assay

A stock solution of 100 mM ammonium chloride in 1 L of distilled water was prepared. Standards were prepared via dilutions from the stock solution with the following concentrations of 0.1 mM, 0.5 mM, 1 mM, 1.5 mM and 2 mM ammonium chloride. Aliquots of the standards were reacted with Nessler's ammonium assay and the absorbance was then measured at 425 nm.

2.2.7 Microscale Nessler's ammonium assay

Induced bacterial isolates were screened for activity towards 3HBN, 3HGN and 3HPPN as described by Coady *et al*. (2013) with some modifications. In addition, each isolate was tested to each of three nitriles in order to investigate the existence of enzymatic activity for other nitriles (substrate specificity).

2.2.8 DNA isolation

The procedures used for extraction and purification of bacteria genomic DNA were those described by Sambrook and Russell (2001), with some modifications. Fresh bacterial

cells were grown in 5 mL LB [1 % (w/v) tryptone; 0,5 % (w/v) yeast extract; 1 % (w/v) NaCl] at 37 °C to saturation. The cell mass was collected by centrifugation for 30 sec at room temperature at maximum speed, resuspended in 0.1 mL lysis buffer [2 % Triton X-100; 1 % sodium dodecyl sulfate (SDS); 100 mM NaCl; 10 mM Tris pH 8; 1 mM EDTA], and transferred to 1.5-mL microcentrifuge tube. Next, 0.2 mL of PCI [phenol pH 8-chloroform-isoamyl alcohol (25:24:1)] and 0.3 g of glass beads were added. The cells were broken by using vortex for 1 min, followed by centrifugation at 12,000 rpm for 10 min at 4 ºC. The supernatant was transferred to a new tube, 0.5 mL ethanol 95 % (v/v) was added, and the mixture was kept at −20 °C for at least 20 min. Total DNA was pelleted by centrifugation (14,000 rpm, 10 min), washed with 70 % (v/v) ethanol, and dried at room temperature. The DNA samples were dissolved in 30 µL ultrapure water and kept at −20 °C.

2.2.9 Agarose Gel Electrophoresis

Agarose (Sigma, Cat.# A9539) was added to 100 mL of 1X TAE buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid, pH 8), to a concentration of 0.8 % (w/v) unless otherwise stated. The solution was heated in a microwave oven until all agarose was completely dissolved. The solution was allowed to cool to ~50 °C. Ethidium bromide (10 mg/ml, Sigma, Cat.# E-1510) was then added to the agarose (5 µl/100 mL) and mixed before pouring into the appropriate gel tray. The gel was then placed in the gel tank and 1X TAE buffer added until the gel was sufficiently submerged.

5 µl aliquots of DNA samples were mixed with 1 µl loading dye [0.25 % (w/v) bromophenol blue, 40 % (w/v) sucrose] before being added to the wells of the gel. Electrophoresis was then applied and run at 5 V/cm to obtain maximum resolution of DNA fragments until sufficient migration was achieved. All DNA bands were viewed under UV illumination and photographed using a Syngene Gene Genius Bio-Imaging system.

2.2.10 NHase and amidase genes screening

The following combinations of primers used, as shown in Table 2.1, were designed by Coffey *et al.,* (2010) in order to amplify the *NHase* and *amd* genes. The complete α subunit genes were amplified using the forward primer NHA-F and the reverse primer NHA-R. The complete β subunit genes were amplified using the forward primer NHB-F

and reverse primer NHB-R. The complete αβ genes were amplified using NHA-F and NHB-R. The complete amidase gene was amplified using the forward primer Amd1-F and the reverse primer Amd1-R.

The amplicons were synthesized using the amplification parameters: 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 40 s, followed by 1 cycle of 72 °C for 5 min, with the exception of complete αβ gene amplifications which required an extension time of 1.5 min and of complete amidase gene which required an extension time of 2 min.

Table 2.1 Primers used for PCR amplification of the α and β subunit gene and the complete αβ genes for NHase gene, and for the complete amidase gene (amd) [Coffey et al., (2010)].

Primers	Sequences	Amplicons
NHA-F	5'-ATGTCAGTAACGATCGACCAC	~600 bp
NHA-R	5'-AGGCAGTCCTTGGTGA CGAT	
NHB-F	5'-ATGGATGGAGTACACGATCT	~600 bp
NHB-R	5'-TCAGGCCGCAGG CTCGAGGT	
NHA-F	5'-ATGTCAGTAACGATCGACCAC	~1300 bp
NHB-R	5'-TCAGGCCGCAGG CTCGAGGT	
Amd1-F	5'-ATACGCGTGAATTCGTGGCGACAATCCGACCTGAC	~1782 bp
Amd2-R	5'-GGTGTTGAGTCGGAGTGGATCTTCGAAACTTCCTAG	

2.2.11 Nitrilase genes

All degenerate primers for this screening were previously designed (Dooley-Cullinane *et al.*, 2019) based on phylogenetic analysis of the novel nitrilase subfamily clades (Robertson *et al.,* 2004). The target clades and primers with the expected amplicons are shown in Table 2.2. The gene screening for nitrilase genes of these clades was carried out using touchdown PCR parameters: initial denaturation 5 min at 95 °C, followed by 2 cycles of 1 min at 95 °C, 1 min at 58 °C (annealing) and 1 min at 72 °C (extension),

followed by decreasing the annealing temperature by 1 °C every 2 cycles to 50 °C; followed by 20 cycles of 1 min at 95 °C, 1 min at 50 °C and 40 sec at 72 °C. After that, final extension of the amplification process by 8 min at 72 °C followed by cooling at 4 °C.

Table 2.2 Clade-specific primers and predicted nitrilase clade PCR amplicons [Coffey et al., (2010)].

Clades	Forward Primer	Reverse Primer	Amplicons
1A	5'-ATGRTCTGGGGVCARGGHGA	5'-TCDCCYTGBCCCCAGAYCAT	287 bp
1B	5'-CAYGARCGSATGRTSTGGGG	5'-TCCATCATBCKYTTKCGYTT	440 bp
2A1-20	5'-GSVYTBTGCTGYTGGGARCA	5'-RTARTGVCCRGCVGGRTC	487 bp
2A21-37	5'-GSVYTBTGCTGYTGGGARCA	5'-GARTARTGSCCGRCSGGRTC	512 bp
3A	5'-CAYCGCAARCTSCARCCSAC	5'-TTCATSAKBSCSTCRATCTG	125 bp
4A	5'-CAYCGCAARYTGRWGCCSAC	5'-CATCYWRTKYTCCCAGCA	125 bp
5A	5'-TGCTGGGARMAYYAYATGCC	5'-TCSGSVCGCGMRTARTGSCC	412 bp
5B	5'-TGCTGGGARMAYYAYATGCC	5'-TCRCCDWYRTTRATCCAYTC	250 bp
6A	5'-CAYCGYAAGCTCRTGCCVAC	5'-CATDHRGTTYTCCCAGCAGA	250 bp
Burk	5'-TGCTGGGARMAYYAYATGCC	5' -TCSGSRCGCGCRTARTGGCC	402 bp

2.2.12 Strain identification

Bacterial isolates were identified using molecular approaches. PCR for the amplification of the 16S rRNA gene regions was performed using primers 63f and 1387r (Marchesi *et al.,* 1998). A total reaction volume of 50 µl containing 50 ng of genomic DNA, 10 pmol/µl of each primer, 25 µl 2x GoTaq® Green Master Mix (Promega), and sterile water were used for the PCR reactions under the amplification parameters: 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C, followed by 1 cycle of 8 min at 72 °C.

DNA sequencing of PCR products was performed using the BigDye 3.1 kit (Applied Biosystems) as per the manufacturer's instructions and analysed using an ABI Prism 310

Genetic Analyser (Applied Biosystems, CA, USA). Commercial DNA sequencing was carried out by GATC Biotech, Germany (now part of Eurofins Genomics). After that, nucleotide sequences were analyzed using BLAST software from the GenBank (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine homology with existing species and to allow identification of the isolates (Altschul *et al.,* 1990).

2.2.13 General procedure for enantioselectivity screening

The general procedure for enantioselectivity screening towards racemic nitriles was carried out according to Coady *et al.,* (2013). Chiralcel AD-H and OJ-H columns (Daicel Chemical Industries®) were used for the resolution of carboxylic acids [mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA] and amides and nitriles (same mobile phase conditions with the exception of TFA) respectively. Both, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm. Each isolate was submitted to each of three nitriles in order to investigate the existence of enantioselectivity towards other nitriles (crossed enantioselective screening)

2.2.14 Biotransformation of 3-hydroxybutyronitrile

A final concentration of 10 mM racemic nitrile was added in 6 mL of 100 mM potassium phosphate buffer pH 7.2 containing induced cells (OD_{600nm} = 1), and incubated at 25 °C for 24 h and 250 rpm. The reaction was quenched after 24 h by removal of the cellular biomass by centrifugation at 3000 × *g*. After that, the supernatant was acidified to pH 2 by the addition of 2 M HCl, and then extracted with ethyl acetate. In addition, the extracts were dried over $MgSO_4$ and the solvent removed under vacuum. From this step, the products were submitted to the benzylation procedure: 0.06 mM Silver oxide, 0.24 mM benzylbromide and 2 mL dichloromethane were added and the mixture stirred in the dark for 24 h. 2 mL of acetone was added to the reaction mixture, and then filtered through a 0.45 µm filter and the solvent was removed under vacuum. 1 mL of mobile phase was added before the solution was injected on the Chiral HPLC system. All experiments were performed in triplicate. % enantiomeric excess (% *ee*) was calculated from the ratio of the enantiomer by the difference in peak area divided by the sum of the peak areas for the major and minor enantiomers.

2.2.15 Biotransformation of 3-hydroxy-3-phenylpropionitrile

The biotransformation was carried out in a suspension of phosphate buffer (0.1M, pH 7.0) containing induced cells (OD_{600nm} = 1). The cells were incubated at 25 °C for 30 minutes with mechanical shaking. Racemic nitrile (10 mM) was added in one portion to the flask and the mixture incubated at 25 °C with mechanical shaking (250 rpm) for 24 hours. The reaction was quenched by removing the biomass through centrifugation and the resulting aqueous solution was acidified to pH 2 with aqueous HCl (2M). Extraction with ethyl acetate gave, after drying over anhydrous MgSO4, removal of the solvent under vacuum, the crude products and the remaining nitrile. All experiments were performed in triplicate. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase. *ee* % (enantiomeric excess) was calculated from the ratio of the enantiomer by the difference in peak area divided by the sum of the peak areas for the major and minor enantiomers.

2.3 Results

2.3.1 Bioprospecting for β-hydroxynitriles-degrading isolates from microbial biocatalysis library

A total of 22 environmental soil samples collected worldwide previous to this study were used. Table 2.3 shows the number of bacteria isolated using three different nitriles as the sole nitrogen source by selective enrichment culture techniques.

Table 2.3 Number of bacteria strains isolated with each nitrile substrate.

Nitrile substrate	Strains
3-hydroxybutyronitrile	126
3-hydroxyglutaronitrile	192
3-hydroxy-3-phenylpropionitrile	21
Total	**339**

2.3.2 Activity screening strategy for bacterial nitrile metabolism

All the isolates were submitted to activity screening in order to ensure that the key nitriles were indeed used as the sole N source via enzyme activity. As these isolates using β-hydroxynitriles as a sole N source resulted in the release of ammonia, nitrile-metabolism enzyme activity was monitored using the Nessler assay. Nitrile hydrolyzing activity was calculated by relating the sample absorbance to the standard curve (Figure 2.3a). All experiments were performed in triplicate and the average absorbance used to calculate activity.

Based on ammonia production (Figure 2.3b), the isolates were divided into three subgroups: (1) 0.1-0.29 mM/L = low activity; (2) 0.3-0.7 mM/L = medium activity; and (3) >0.7 mM/L = high activity).

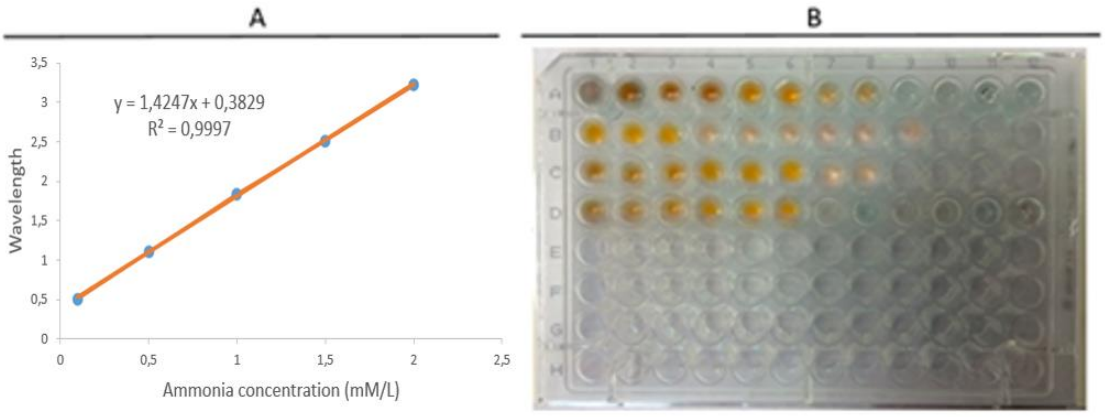

Fig. 2.3 Activity screening for nitrile metabolism. (A) Standard curve for the detection of ammonia using the Nessler's Colorimetric assay. (B) An example of library screening for nitrile hydrolyzing enzyme activity in a 96-well microtest plate.

The results are shown in the following Table 2.4

Table 2.4 Number of isolates demonstrating activity towards each nitrile.

Nitrile substrate	Low activity	Medium activity	High activity
3-hydroxybutyronitrile	117	2	7
3-hydroxyglutaronitrile	185	7	*--
3-hydroxy-3-phenylpropionitrile	18	0	3

*no activity in this range was detected.

After the first round of screening, each bacterial isolate was submitted to substrate specificity screening and the results are represented in Table 2.5.

Table 2.5 Number of bacterial isolates showing crossed enzymatic activity towards each nitrile.

Nitrile substrate	Number of isolates	Isolates from 3HBN screening			Isolates from 3HGN screening			Isolates from 3HPPN screening		
		Low activity	Medium activity	High activity	Low activity	Medium activity	High activity	Low activity	Medium activity	High activity
3HBN	126	-	-	-	183	5	4	18	3	0
3HGN	192	119	7	0	-	-	-	19	2	0
3HPPN	21	*0	0	0	181	9	2	-	-	-

*no growth was detected.

2.3.3 Conventional PCR screening of isolates for NHase, amidase and nitrilase genes

All isolates were screened for the presence of nitrile hydrolyzing genes using conventional PCR with whole cells as template. PCR products were obtained from α and/or β genes of NHase, complete amidase gene and 1B, 5A, 5B, 6A and Burk predicted nitrilase clades genes (Table 2.6) (complete results available in Appendix III).

Table 2.6 Total of positive isolates for nitrile hydrolyzing gene screening using conventional PCR.

Nitriles	α and/or β genes of NHase			Amidase	Predicted nitrilase clades				
	α	β	αβ		1B	5A	5B	6A	Burk
Isolates from 3HBN	53	51	51	51	77	22	16	8	7
Isolates from 3HGN	31	30	30	30	58	43	39	3	3
Isolates from 3HPPN	3	3	3	3	3	3	3	1	1

All PCR products obtained were subjected to agarose gel electrophoresis. One example is given from the library screening for α, β and αβ subunits genes of 4 different bacterial isolates (*Nocardia coeliaca* strain 1A, *Klebsiella oxytoca* strain 2A, *N. coeliaca* strain 3A and *R. erythropolis* strain 29A), which resulted in amplification of a diagnostic DNA fragment of 600-bp; 600-bp and 1,200-bp respectively (Fig. 2.4). *R. erythropolis* AJ270 was used as positive control. No non-specific amplication band was found.

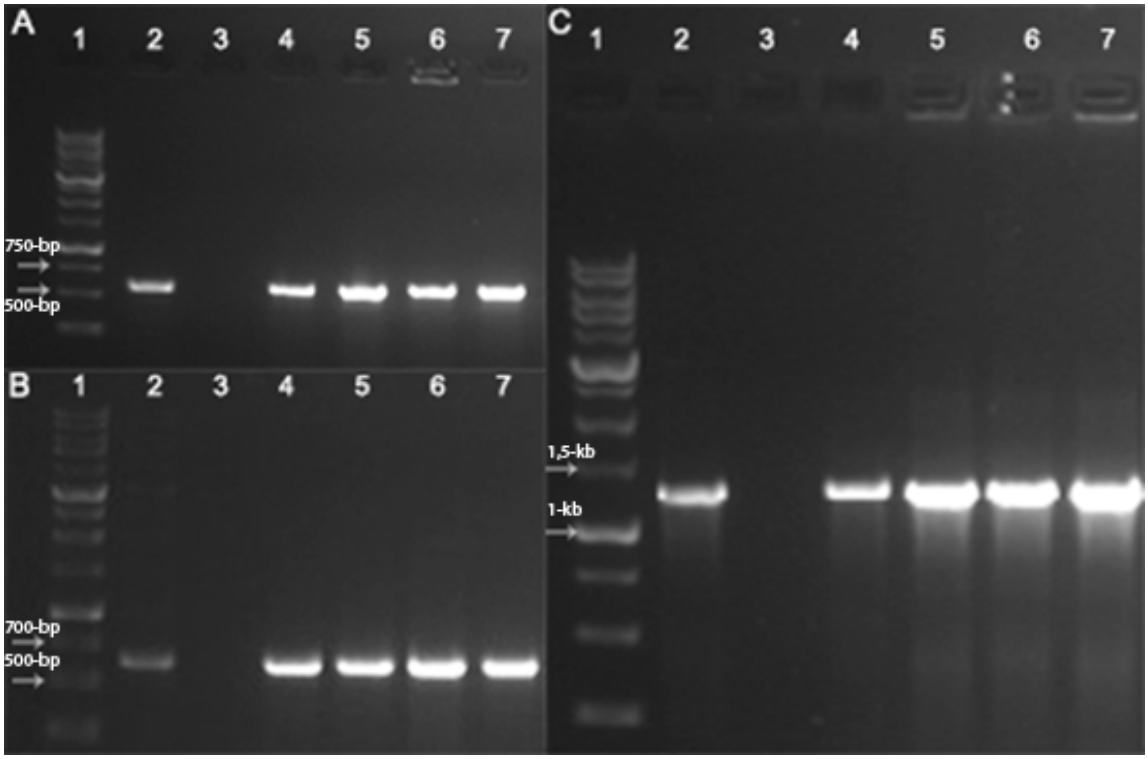

Fig. 2.4 Agarose gel electrophoresis [0.9 % (w/v)] showing NHase gene screening PCR products. (A) α subunit gene of 600-bp. (B) β subunit gene of 600-bp. (C) αβ subunits genes of 1,200-bp. Lane 1, 1-kb CLS-MDNA ladder; lane 2, PCR product from positive control - *R. erythropolis*; lane 3, negative control; lanes 4-7, different bacterial isolates: *N. coeliaca* strain 1A, *K. oxytoca* strain 2A, *N. coeliaca* strain 3A and *R. erythropolis* strain 29A, respectively.

After the gene screening, the results generated were crossed with those from activity screening and 19 bacterial isolates were chosen for enantioselective analysis according to ammonia production from Nessler's colorimetric activity essay and positive bacterial isolates for nitrile metabolism genes (Table 2.7). In addition, the PCR amplification of the 16S rRNA gene was positive for the 19 isolates, Figure 2.5.

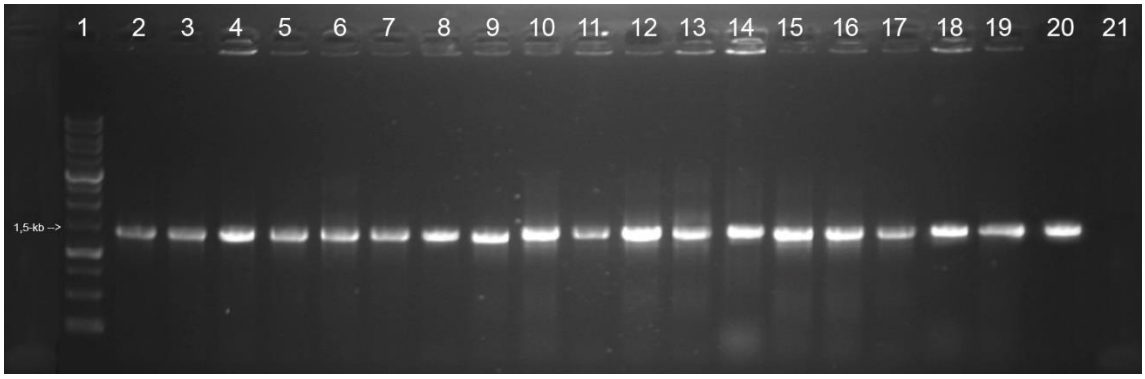

Fig. 2.5 Agarose gel electrophoresis [0.9 % (w/v)] showing the amplification products of the 16s rRNA PCR for the 19 isolates. Promega 1 kB ladder (lane 1); promising bacterial isolates (lanes 2-20); negative control (lane 21).

Table 2.7 Activity and gene screening profile for a selection of bacterial isolates. Isolates were screened for activity and nitrile hydrolyzing enzymes gene. The chosen isolates demonstrated medium or high activity based on ammonia production (0.3-0.7 mM/L = medium activity; and >0.7 mM/L = high activity).

Isolates ID	Nitrile substrate	NH_3 (mM/L) produced	Corresponding encoded gene*
1A	3HBN	1.32	α; β; αβ; Ami; 1B; 5A; 5B; 6A
2A	3HBN	0.83	α; β; αβ; Ami; 1B; 5A; 5B; Burk
3A	3HBN	0.81	α; β; αβ; Ami; 1B; 5A; 5B
29A	3HBN	0.52	α; β; αβ; Ami; 1B; 5A; 6A
31A	3HBN	0.64	α; β; αβ; Ami; 1B; 6A; Burk
35A	3HBN	0.77	α; β; αβ; Ami; 1B; 5A; 6A
39A	3HBN	1.09	α; 5A; 5B
46F7A	3HBN	1.06	α; β; αβ; Ami; 1B; 5A; 5B; 6A
48A	3HBN	1.85	α; β; αβ; Ami; 6A
1B	3HGN	0.32	α; 1B; 5A; 6A
5B	3HGN	0.65	α; β; αβ; Ami; 1B; 5A; 5B
6B	3HGN	0.44	α; β; αβ; Ami; 1B; 5A; 5B
7B	3HGN	0.31	α; β; αβ; Ami; 1B; 5A; 5B
12B	3HGN	0.62	α; β; αβ; Ami; 1B; 5A; 5B; 6A
13B	3HGN	0.66	α; β; αβ; Ami; 1B; 5A; Burk
39B	3HGN	0.49	α; β; αβ; Ami; 1B; 5A; 5B; 6A
1C	3HPPN	2.03	α; β; αβ; Ami; 1B; 5A; 6A
3C	3HPPN	2.17	α; β; αβ; Ami; 1B; 5A
6C	3HPPN	2.04	α; β; αβ; Ami; 1B; 5A; Burk

*α; β; αβ correspond to α and β gene subunits, and the complete αβ genes of NHases, respectively. Ami corresponds to the complete amidase gene. 1B, 5A, 5B, 6A and Burk correspond to predicted nitrilase clades gene.

2.3.4 Screening for enantioselective biotransformations of 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile

Having narrowed down the bacterial isolates from 339 to 19 having medium to high activity towards β-hydroxynitriles, it was decided to focus attention on determining the enantioselectivity of the isolates. Initial enantioselectivity screening involved the biotransformation of racemic 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile (Table 2.8 and Figure. 2.6).

Table 2.8 Enantioselective biotransformations of racemic 3-hydroxybutyronitrile catalysed by novel bacterial isolates. The three promising isolates are underlined.

Isolates ID	Strains	(S)-Acid *ee* (%)[a]	(S)-Nitrile *ee* (%)[a]	Activity subgroups	16s rRNA Accession Number
1A	*Nocardia coeliaca* strain 1A	96.53	91.45	High	NR_104776.1
2A	*Klebsiella oxytoca* strain 2A	>99.99	93.03	High	NR_112010.1
3A	*Nocardia coeliaca* strain 3A	68.42	14.42	High	NR_104776.1
29A	*R. erythropolis* strain 29A	41.42	1.42	Medium	NR_074622.1
31A	*Rhizobium nepotum* strain 31A	23.47	2.47	Medium	NR_117203.1
35A	*Pseudomonas umsongensis* strain 35A	52.64	12.64	High	NR_025227.1
39A	*P. umsongensis* strain 39A	43.72	23.72	High	NR_025227.1
46F7A	*R. erythropolis* strain 46F7A	94.46	95.64	High	NR_074622.1
48A	*K. oxytoca* strain 48A	57.45	16.45	High	NR_102982.1

1B	*Rahnella aquatilis* strain 1B	38.02	2.44	Medium	NR_074921.1
5B	*P. brenneri* strain 5B	41.74	11.55	Medium	NR_025103.1
6B	*P. brenneri* strain 6B	60.65	18.93	Medium	NR_025103.1
7B	*P. brenneri* strain 7B	51.99	11.76	Medium	NR_025103.1
12B	*R. jialingiae* strain 12B	42.08	10.11	Medium	NR_115708.1
13B	*Raoultella terrigena* strain 13B	32.24	18.22	Medium	NR_113703.1
39B	*R. erythropolis* strain 39B	51.02	22.45	Medium	NR_074622.1
1C	*R. erythropolis* strain 1C	7.06	1.18	High	NR_037024.1
3C	*R. erythropolis* strain 3C	10.16	3.04	High	NR_119125.1
6C	*R. erythropolis* strain 6C	11.09	2.31	High	NR_074622.1

[a] Determined by HPLC analysis using a chiral column (see materials and methods in section 2.2.14).

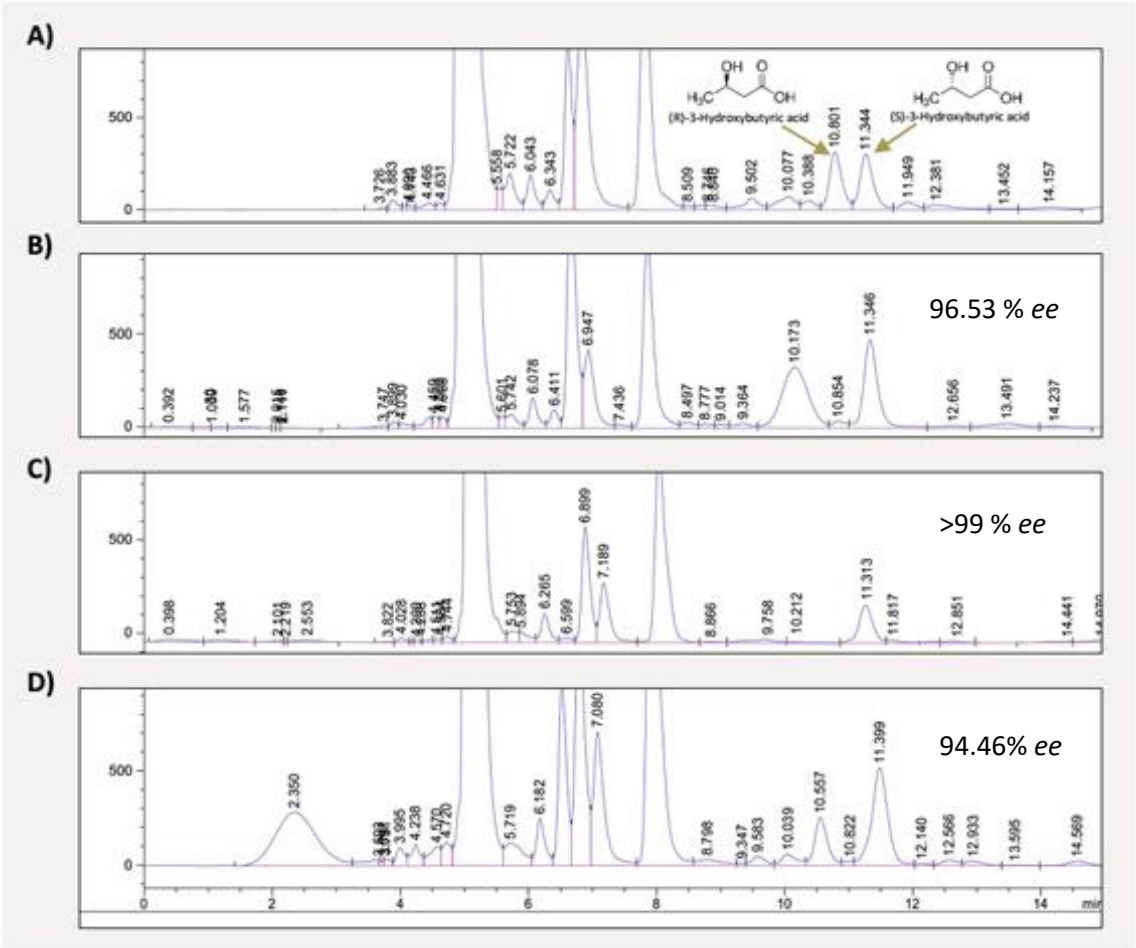

Figure 2.6 Chiral HPLC Chromatograms. (A) Standard of racemic 3-Hydroxybutyric acid. Biotransformation of 3-hydroxybutyronitrile by isolate 1A (B); isolate 2A (C) and isolate 46F7A (D). Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm.

This chiral screening study identified three promising isolates (>90 % *ee*) which demonstrated exceptional enantioselectivity towards 3-hydroxybutyronitrile. On the other hand, chiral screening study towards 3-hydroxy-3-phenylpropionitrile, however, showed poor enantioselectivity, not exceeding 16% *ee* as seen in Figure 2.7. This may be due to steric hindrance by the aromatic ring present on 3-HPPN influencing the enzymatic activity function. Notably, all 3 isolates demonstrated activity results within the high activity range.

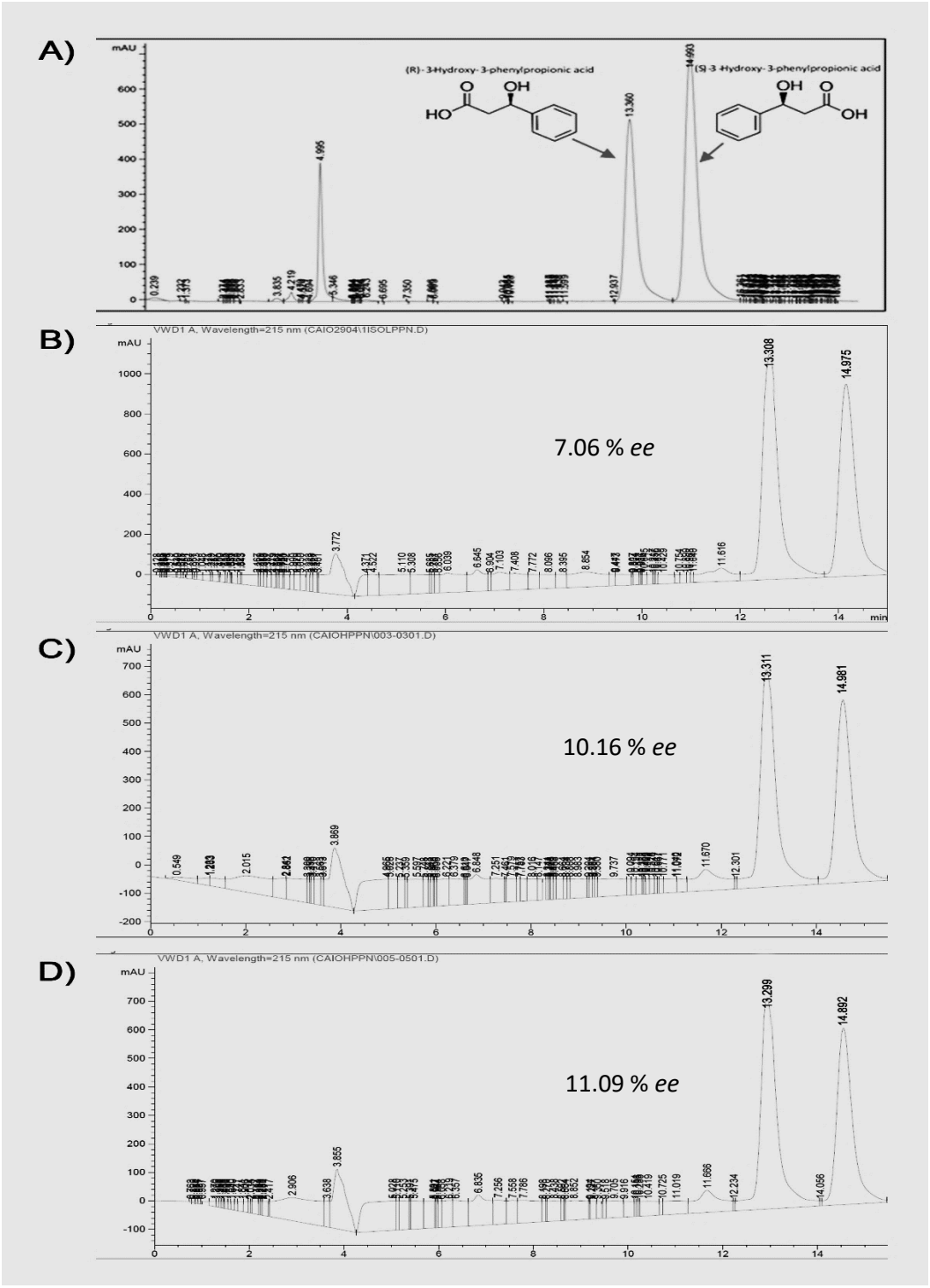

Fig. 2.7 Chiral HPLC Chromatograms. (A) Standard of racemic (R)-3-Hydroxy-3-phenylpropionic acid and (S)-3-Hydroxy-3-phenylpropionic acid. Biotransformation of 3-Hydroxy-3-phenylpropionitrile by isolate 1C (B); isolate 3C (C) and isolate 6C (D). Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm.

After the first round of screening, each bacterial isolate was submitted to crossed enantioselective screening. The results of enantioselective biotransformations were determined on isolates from 3-hydroxybutyronitrile towards 3-hydroxy-3-phenylpropionitrile and from 3-hydroxy-3-phenylpropionitrile towards 3-hydroxybutyronitrile (Chromatograms in appendix I).

2.4 Discussion

The pharmaceutical industry requires amides and acids for use as intermediates in the manufacture of many drugs and chemicals (Schmid *et al.*, 2001; Pollard and Woodley, 2007; Abdelraheem *et al.*, 2019; Janssen and Stucki, 2020). These may be obtained by traditional chemical methods as demonstrated by Mohy El Dine *et al*. (2015). However, more suitable and specific methods for obtaining these are required, with various advantages offered by green enzyme-based technology.

In fact, enzymes govern all the major biochemical events taking place within an organism. Since most of them are highly selective with respect to the chirality of a substrate, each enantiomer of a given compound such as a pharmaceutical intermediates will cause different biological effects (Crossley, 1992; Torre and Albericio, 2020). Consequently, single enantiomers are preferred over racemates.

The prospection for new nitrile-metabolizing enzymes remains relevant to provide efficient biocatalysts for biotechnological applications. Notably, these enzymes are widely spread in the environment and there are many methods to discover new nitrile-metabolizing enzymes. In the present study, this chapter has described the isolation and screening of microbes from environmental samples collected worldwide. These isolates have been shown to be capable of hydrolyzing three pharmaceutically relevant β-hydroxynitriles (3-hydroxybutyronitrile, 3-hydroxyglutaronitrile and 3-hydroxy-3-phenylpropionitrile), which can act as sources of β-hydroxy carboxylic acids via hydrolysis reactions and whose products can be widely used for industrial purposes. For instance, 3-hydroxy-3-phenylpropionic acid and its derivatives have been used as precursors to chiral drugs such as atomoxetine and nisoxetine (Hammond *et al.,* 2007). Additionally, of particular commercial interest, 3HGN is an intermediate in the enantioselective synthesis of cholesterol-lowering drugs (Bergeron *et al.*, 2006; Hoyos *et al.,* 2019). On the other hand, 3HBN is an intermediate used to prepare 2-Methylazetidine, which is a derivative

of Azetidine, a useful building block in the synthesis of polypeptides and other nitrogen containing compounds with potential biological properties (Drouillat *et al.*, 2012). For this reason, the production of 2-Methylazetidine using biotransformation has become an emerging interest to the pharmaceutical industry, including development of treatments for neurological diseases, such as Parkinson's disease, Tourette's syndrome and attention deficit disorder (Degennaro *et al.*, 2017; Behnke *et al.*, 2019). Therefore, with careful selection of biocatalytic systems complemented with microbial screening/selection techniques, it is possible to produce enantiomeric compounds with high purity.

The work undertaken in this project is part of the Molecular Biotechnology and Biopharmaceutical Research group at Waterford Institute of Technology in Ireland. This research group had previously collected soil samples from various locations around the world. These samples had been chosen due to suspected elevated metal content, chemical contamination, or simply as suburban uncontaminated soils with different microbiomes present. All the microbial strains were isolated using the enrichment technique with three different nitriles acting as sole nitrogen source, as shown in Table 2.3. Several bacteria, yeast and filamentous fungi that exhibit nitrilase and/or NHases activities have been isolated through enrichment strategy, as reported by Shen *et al.*, (2021). In fact, these isolates showed higher activities when compared to those isolated from non-enriched cultures, as demonstrated by Gong *et al.*, (2011), Kamal *et al.*, (2011) and Jin *et al.*, (2013), which increases the possibility of identifying ideal strains that harbor target enzymes. For instance, Arfi and Nigam (2020) isolated the bacteria *Staphylococcus* sp. which performed maximum activity for the biotransformation of 3-cyanopyridine to nicotinic acid when the enrichment strategy was used.

The screening of microbes in this study was performed in three stages. In the first stage, the bacterial isolates were submitted to a suitable analytical screening method in order to determine activity. 339 resulting isolates were then quantitatively screened for activity towards these industrially relevant nitriles. This method that was used in the first stage was previously published as a high throughput screening strategy to assess the nitrile metabolising ability; as the bacterial isolates utilizing β-hydroxynitriles as a nitrogen source resulted in the release of ammonia, enzyme activity could be monitored using the technique of Nesslerization (Coady *et al.,* 2013). Therefore, this method allowed for the

rapid identification of isolates demonstrating activity towards β-hydroxynitriles. An advantage of this screening method is to allow for the rapid screening of bacterial isolates potentially containing nitrile-metabolizing enzymes, using 96-well microtest plates.

Based on the ammonia production, the bacteria were divided into three subgroups, as shown in Table 2.4. Only 2.6 % demonstrated medium activity towards 3HBN and 3HGN, whereas 2.9 % were classified as having high activity towards 3HBN and 3HPPN. Additionally, each isolate was submitted to crossed activity screening in order to investigate the activity of each isolate towards the three different nitriles.

As shown in Table 2.5 a total of 4 and 2 bacteria isolated from 3HBN and 3HPPN, respectively, demonstrated high activity towards 3HGN. Interestingly, this nitrile has no reported natural occurrence. Of 339 isolates, 100 % hydrolyze this substrate, exhibiting the ability to hydrolyze both nitrile substituents of 3HGN (Table 2.5). However, the number of isolates with activity towards 3HPPN was poor, with only 3 bacterial isolates demonstrating activity after 24 h, due to its toxicity to microbial cells, which seems common in this class of nitrile (longer aliphatic groups), as investigated by Coady *et al.*, (2013) and Brady *et al.*, (2004).

Several other high-throughput assays have been reported over the past few years in order to identify new nitrile-metabolizing enzymes from microbial cells, including the use of pH indicators, or formation of ammonia and acid generation (Xue *et al.*, 2016; Egelkamp *et al.*, 2020a). Meghavarnam and Janakiraman (2019) developed a simple and efficient screening technique based on the principle of change in culture medium pH. Bromothymol blue was used as pH indicator dye. Its colour change, from blue to dark green-yellow at pH 6-7.4, falls into the pH range of enzyme activity. 108 bacterial strains were screened for enzyme activity, in which 17 strains showed positive for nitrile-metabolising enzymes. However, as this method uses cell suspension it could be only used for qualitative screening due to interference with the absorbance readings (Martínková *et al*., 2008). In another study, after the development of a chromogenic reagent to rapidly verify nitrilase activity as a substitute for Nessler's reagent, 23 recombinant strains expressing nitrilases were used to demonstrate the utility of this high-throughput screening method. In short, nitriles were hydrolyzed and the NH_3 produced was allowed to react with *o*-phthalaldehyde to form a colourless intermediate reagent, which on acidification becomes intensely coloured. Then, fluorescence intensity could be

measured by UV/visible spectroscopy (Black *et al.*, 2015). Other high-throughput screening using advanced methods, including flowcytometry, microfluidic and microarray platform were reported by Shen *et al.*, (2020). However, these methods do not have the advantage of being cost-effective.

Following the preliminary screening in the first stage, the second stage entailed of molecular approaches to screening those microbial isolates for nitrile metabolism genes in order to allow focused screening of a subset (the most promising or lucrative) of the library for enantioselectivity performance, as the complete library could not be tested due to time and logistical restraints. The aim at this stage was to use PCR assays for the rapid detection of any nitrilase genes in the bacterial library, which would allow the isolation of novel nitrilases. Primers were previously designed based on 137 new nitrilase sequences discovered from the screening of >600 biotope-specific environmental DNA libraries described by Robertson *et al*. (2004). The primer sequences were designed based on conserved regions within these clades of nitrilases, including also more recent nitrilase sequence additions to the database. Additionaly, primers were used to amplify α and/or β subunits of NHase genes and the complete gene of amidase, again based on conserved regions of these genes from the database. These results showed that 24 % of the library presented PCR products for the complete α and β subunits of NHase and amidase genes. Also, 84 % of isolates presented a predicted nitrilase gene. In addition, 63 % of bacterial isolates presented each predicted gene encoding these three enzymes.

After that, all the results generated were compared to those from activity screening and from the initial 339 bacterial isolates, a total of 19 bacterial isolates were chosen for the third stage of screening - enantioselective analysis. These 19 strains were chosen based on having medium to high activity towards β-hydroxynitriles and possessing at least one predicted nitrile-metabolism gene.

Next, the third stage of screening was focused on determining the enantioselectivity of the 19 bacterial isolates nitrile-metabolising activity. The strains were screened for enantioselective biotransformations of each nitrile to produce the corresponding carboxylic acids, which are the expected products of nitrile-metabolism enzymes.

The hydrolysis of 3-Hydroxyglutaronitrile by microbial cells has been observed by DeSantis *et al.*, (2002), Robertson *et al.*, (2004), Oliveira *et al.,* (2015), Xue *et al.*, (2016),

Bragança *et al.*, (2017) and Chen *et al.*, (2019). On the other hand, there are few studies reporting the use of 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile as key nitrile substrates targeted for their potential in biocatalytic synthesis of chiral pharmaceutical intermediates (Brady *et al.*, 2004; Coady *et al.*, 2013; Mareya *et al.*, 2020).

The biotransformation of racemic nitrile was initially investigated with the formation of the corresponding acids. These results demonstrated that all 19 isolates screened, showed poor *ee* % towards 3HPPN (<16 %). In agreement with these results, poor enantioselectivity for the hydrolysis of this substrate has been observed by Brady *et al.*, (2014), which was less than 5 %. One explanation is due to sensitivity towards the phenyl ring on the enzymatic reaction, as noticed by Coady *et al.*, (2013).

Generally, the nitrile-metabolizing enzymes in bacterial isolates have shown relatively poor enantioselectivity (Brady *et al.*, 2004). Under optimized reaction conditions, however, the enantioselectivity and activity for nitrile hydrolyzing bacteria could be improved (Shen *et al.*, 2021). Wang *et al.*, (2020) for the first time utilized *R. erythropolis* CCM2595 for the bioconversion of dinitrile to degraded herbicide and chemical raw material by using different temperature and pH conditions. The NHases was further employed to catalytic reactions that could convert adiponitrile into 5-Cyanovaleramide with *ee* value greater than 95 %. Robertson *et al.*, (2004) utilized environmental DNA (eDNA) to create a library of more than 130 unique nitrilase enzymes. Their genes were expressed in *E. coli* and the enzymes were characterized for activity on 3HGN, mandelonitrile and phenylacetaldehyde cyanohydrin. Different conditions (temperature, pressure, pH, salinity) were used in order to determine the range of enzymatic specificity. After several rounds of screening, they reported a few of the nitrilases showing the ability to hydrolyze the key nitriles with enantiomeric excess of >90 % for (S)-4-cyano- 3-hydroxybutyric acid >95 % for (R)-4-cyano-3- hydroxybutyric acid.

In another study, Mareya *et al.*, (2020) employed different pH and temperature conditions to retain enantioselectivity and activity on *R. erythropolis* SET1. It was found >99 % *ee* towards 3HBN. Consistent with the results in this study, 3 strains, *N. coeliaca* strain 1A, *K. oxytoca* strain 2A and *R. erythropolis* strain 46F7A, presented with enantiomeric excess of >90 % towards 3HBN. Of these 3 promising isolates, *K. oxytoca* strain 2A showed exceptional >99.99 *ee* % towards 3HBN, indicating that this bacterial isolate is

highly enantioselective and possibly enantiospecific with 100 % *ee* of (S)-acid. When comparing the acid *ee* % to the nitrile *ee* %, it is assumed that the isolate may contain a nitrilase enzyme, as these types of enzymes hydrolyse (S)-nitrile to produce its corresponding (S)-acids. To the author's knowledge, this is the first study concerning the hydrolysis of 3HBN using the bacteria *K. oxytoca*. As the commercial synthetic process generally requires greater than 98 % enantiomeric excess (Crabtree, 2009; Abdelraheem *et al.*, 2019), with the nitrile-metabolizing gene isolation of this strain for further cloning in a bacterial host for heterologous expression, it would be possible to produce enantiomeric compounds with high enantiopurity.

In summary, the work reported in this chapter describes the identification of isolates with nitrile hydrolysing activity towards *β*-hydroxnitriles. This was achieved via the high-throughput screening strategy. The addition of a toxicity screen towards the various nitriles further focuses this approach. By attempting to grow the different isolates in rich medium in the presence of *β*-hydroxnitriles, the isolates that are sensitive to the nitrile and cannot grow in its presence outside of a community culture were identified and excluded from further screening. In subsequent enantioselectivity screening of a selection of isolates demonstrating the desired activity towards *β*-hydroxnitrile, an isolate demonstrating a highly enantioselective if not enantiospecific activity towards 3-hydroxybutyronitrile in particular, was identified - *Klebsiella oxytoca* strain 2A. Consequently, an efficient method for the potential production of (S)-3-hydroxybutyric acid from racemic 3-hydroxybutyronitrile was established using this bacterium; strain 2A demonstrated high activity towards 3-hydroxybutyronitrile and the highest enantioselectivity. This highlights the importance of carefully selecting the activity specifications of isolates chosen for enantioselectivity screening. By using this strategy, other nitrile-metabolizing bacteria towards the production of pharmaceutical intermediates using biotransformations could also be identified. Further studies to elucidate the full sequence of the responsible genes should be carried out, to allow cloning and expression of recombinant enzyme towards detailed performance studies and realizing commercial potential.

CHAPTER 3

HIGH THROUGHPUT FUNGAL SCREENING STRATEGY DEVELOPMENT TO IDENTIFY FUNGI WITH NITRILE BIOTRANSFORMATION POTENTIAL

3.1 Introduction

Pharmaceutical research and manufacturing is increasingly turning to biotechnology for the more efficient synthesis of complex compounds. Biocatalysis, the core of the biotechnology industry (also known as white biotechnology) is rapidly evolving in organic synthesis, the historic backbone of the pharmaceutical industry, offering advantages in green-technology and stereoselective control (Prado, 2003; Pollard and Woodley, 2007; Crabtree, 2009; Gong *et al.*, 2012; Winkler *et al.,* 2021).

Currently, chemical processes in the pharmaceutical industries have drawn attention to the application of biocatalysis using enzymes or whole cells as catalysts (Torre and Albericio, 2020). The particular properties of enzymes allow them to be used to create new chiral centers via asymmetric reactions, which is an effective strategy for the optical creation of pure molecules for the manufacture of many drugs (Nigam *et al.*, 2017). Thus, the participation of enzymes in chemical synthesis in this context has many advantages, including the fact that they operate under mild conditions of temperature, pH and pressure, with excellent regio- and enantioselectivities in some cases (Kaplan *et al.*, 2013; Hoyos *et al.,*2019). Consequently, the biotransformation studies can be carried out to obtain new products and/or discover new enzymes with potential for industrial and/or biotechnological use (Sudhakaran *et al.,* 2017).

Nitriles are considered crucial intermediaries in synthetic organic chemistry due to the ease they can be transformed into amides and/or carboxylic acids for the industrial synthesis of high-value pharmaceutical intermediates (Banerjee *et al.,* 2002; Oliveira *et al.*, 2018). For this reason, the use of microorganisms in the development of nitrile biotransformations is a viable alternative to chemical hydrolysis due to the ability of some microbial cells, such as fungi, to produce large amounts of biomass, consequently resulting in large amounts of the enzyme of interest in a short period of time (Adrio and Demain, 2014).

Culture enrichment techniques have allowed microorganisms with nitrile-hydrolyzing enzymes to be isolated (Coady *et al.*, 2013; Oliveira *et al.*, 2018). These enzymes are widely distributed in nature, including yeast and filamentous fungi (Rucká *et al.*, 2019). Most of the well-characterized NHases, amidases and nitrilases, which are largely of bacterial origin, have been obtained by selection methods allowing only the positive strains to grow on a minimal medium (MM) with a nitrile as the sole nitrogen source (Martínková *et al.*, 2008; Coffey *et al.*, 2009, 2010; Coady *et al.*, 2013; Sahu *et al.,* 2019;

Xu *et al.*, 2020). On the other hand, fungi seem to be a rich but so far little exploited source of nitrile-hydrolysis enzymes.

The goal of the research described in this chapter was to isolate, screen and identify fungi with activity towards the β-hydroxynitriles from environmental samples collected in Ireland. This work reports a high throughput screening strategy for the rapid identification of fungal isolates possessing nitrile hydrolyzing activity towards a variety β-hydroxynitriles including 3-hydroxybutyronitrile, 3-hydroxy-3-phenylpropionitrile and 3-hydroxyglutaronitrile, which represent an aliphatic, aromatic nitrile and dinitrile respectively. This work also demonstrates the application of this strategy to a successful enantioselectivity screening study, resulting in the identification of an isolate demonstrating highly enantioselectivity using the whole mycelial cells.

3.2 Material and Methods

3.2.1 Fungi cultivation

All fungi were precultured in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose]. MM (minimal medium) (1.5 g/L KH_2PO_4, 0.5 g/L KCL, 0.5 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4$, 0.01 g/L $ZnSO_4$, 10 g/L glucose, 150 µg/mL chloramphenicol, 25 µg/mL rose Bengal, pH adjusted to 6.8 with 1 M NaOH) supplemented with 10 mM nitrile substrate with or without 2 % (w/v) agar was used for fungi isolation and activity screening.

The starting cultures were kept on YPD agar at 4 °C for the growth of the strains and transferred to the fresh medium every 2 months.

3.2.2 Key nitriles as substrates

The nitriles used for this study were 3-hydroxybutyronitrile (3HBN), 3-hydroxyglutaronitrile (3HGN) and 3-hydroxy-3-phenylpropionitrile (3HPPN). Racemic 3HBN and 3HGN were purchased from ENAMINE®, and 3HPPN from Sigma®. All other chemicals were of analytical grade and obtained from Macron Fine Chemicals™ and Acros Organics Chemicals.

3.2.3 Isolation of yeasts and filamentous fungi

The yeasts and filamentous fungi were isolated from environmental samples collected in Ireland based on the premise that these samples would have been exposed to industrial waste from an old tannery industry or metal content. 10 g of soil sample was suspended in 100 mL of sterile saline solution followed by serial dilution from 10^{-2} to 10^{-4} and then all dilutions were plated onto MM agar supplemented with nitrile as the sole nitrogen source as described previously. The plates were incubated for 8 days at 25 °C. Fungal conidia and/or mycelia and yeasts cells were picked and serially streaked to fresh MM agar containing nitrile to obtain pure isolates. Then isolates were submitted for activity screening in order to determine activity towards nitriles.

3.2.4 Maintenance of yeasts and filamentous fungi

The isolates were maintained on YPD plates [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose, 2 % (w/v) agar] supplemented with 150 µg/mL chloramphenicol. For long-term storage, yeasts were maintained frozen at −70 °C in 20 % (v/v) glycerol, and also, the filamentous fungal spore suspensions were maintained frozen at −70 °C in 50 % (w/v) glycerol in water supplemented with 150 µg/mL chloramphenicol.

3.2.5 Inoculum preparation of filamentous fungi and estimation of mycelial growth

Fungal spores were extracted from MM agar plates using a spatula and suspended in 10 mL of sterile distilled water. The spore suspensions were homogenized and underwent vortex agitation for 10 seconds, and then filtered through a funnel containing sterile gauze. The number of spores per mL was determined in a Neubauer chamber.

In order to measure the fungal dry weight, each spore suspension was added to 50 mL of MM liquid media and incubated for 8 days at 25 °C and 160 rpm and then filtered through a 0.22 µm filter. The filter was kept along with mycelium fungi and then subjected to drying at 105 °C for 24 h, followed by dry weight measurement.

The fungal mycelia were transferred to plates containing MM supplemented with 10 mM nitriles, and then incubated for 8 days at 25 °C. The diameter of mycelia was measured daily and biological duplicates were performed.

3.2.6 Yeast cell growth and the relation between OD reading (A_{600nm}) and dry cell mass concentration (g L^{-1})

To determine the cell biomass dry weight, yeast culture samples from the exponential growth phase (10 mL) in MM supplemented with 10 mM nitriles were centrifuged at 3000 g for 15 min at 4 ºC, and then the cell pellet was resuspended in 6 mL of sterile distilled water. A volume of 3 mL was distributed into three crucibles of 1 mL each and dried at 105 °C for 24 h to determine the dry mass. A linear regression of the absorbance (A600nm) versus dry mass (g L-1) allowed the determination of the dry biomass corresponding to 1 unit of absorbance at 600 nm.

3.2.7 Induction of yeasts and fungal extracts for activity screening

96-well Megablocks® (Sarstedt Ltd, Ireland) containing 600 µL of M9 minimal media supplemented with 10 mM nitrile substrate were inoculated with the isolates and incubated with shaking for 48 h at 25 °C and 250 rpm. For storage of grown cultures, 400 µL of 20 % (v/v) glycerol was added to each culture well and Megablocks were sealed and frozen at −70 °C. For filamentous fungi, the mycelium was filtered, washed 2 times with Tris/HCl (50mM, pH 8) and resuspended in 5 mL MM media supplemented with 10 mM nitrile substrate (approximately 5 mL of MM media per 1 g of wet mycelium) and then incubated for 48 h at 25 °C and 160 rpm.

3.2.8 Standard curve for Nessler's colorimetric ammonium assay

A stock solution of 100 mM ammonium chloride in 1 L of distilled water was prepared. Standards were prepared via dilutions from the stock solution with the following concentrations of 0.1 mM, 0.5 mM, 1 mM, 1.5 mM and 2 mM ammonium chloride. The absorbance was then measured at 425 nm.

3.2.9 Microscale Nessler's ammonium assay

Induced yeast isolates were screened for activity towards 3HBN, 3HGN and 3HPPN as described by Coady *et al*. (2013) In addition, each isolate was tested against each of the three nitriles in order to investigate the existence of enzymatic activity for the nitriles other than that used to isolate (crossactivity). The high throughput screening strategy was modified for use with filamentous fungi. The reaction mixture (total volume of 0.2 mL) consisting of 100 mM potassium phosphate buffer pH 8, 10 mM nitrile and the mycelial

mass (approximately 50 mg) was shaken at 850 rpm and 25 °C in Thermomixer Compact Eppendorf for 24 h. After incubation, the reaction was quenched by adding 50 µL of 250 mM HCl and were centrifuged at 500 g for 10 min. 20 µL of the supernatant was transferred to a 96-well microtest plates containing 181 µL of Nesslers mastermix [155 µL distilled water, 1 µL 10M NaOH and 25 µL Nesslers reagent (Merck®)]. The reaction was allowed to stand for 10 min and then the absorbance read at 425 nm. Cell blanks contained approximately 50 mg of cell extracts in phosphate buffer. Nitrile blanks contained 200 µL of 10 mM nitrile in phosphate buffer.

3.2.10 Yeast and filamentous fungi DNA isolation

The procedures used for extraction and purification of fungal genomic DNA were those described by Sanchez *et al*. (1993), with some modifications. Isolates were grown in 5 mL YPD at 25 °C to saturation. The cell mass was collected by centrifugation, resuspended in 0.2 mL lysis buffer [2 % (w/v) Triton X-100, 1 % (w/v) sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA], and transferred to 2-mL screw-cap tubes. Next, 0.2 mL of PCI [phenol pH 6.7-chloroform-isoamyl alcohol (25:24:1)] and 0.3 g of glass beads were added. The cells were broken by using a FastPrep homogenizer followed by centrifugation at 10,000 x g for 10 min. The supernatant was transferred to a new tube, 0.5 mL ethanol was added, and the mixture was kept at -20 °C for at least 20 min. Total DNA was pelleted by centrifugation (14,000 x g, 10 min), washed with 70 % (v/v) ethanol, and dried at room temperature. The DNA samples were dissolved in 20 µL nuclease-free water and kept at -20 °C. An aliquot of 1 µL was used as template in PCR reaction.

3.2.11 Gene screening by conventional PCR

NHase and amidase genes: the following combinations of primers, as shown in Table 2.1, were used designed by Coffey *et al.,* (2010) in order to amplify the *nhase* and *amd* genes. *Rhodococcus erythropolis* AJ270 was used as a positive control. The complete α subunit genes were amplified using the forward primer NHA-F and the reverse primer NHA-R. The complete β subunit genes were amplified using the forward primer NHB-F and reverse primer NHB-R. The complete αβ genes were amplified using NHA-F and NHB-R. The complete amidase gene was amplified using the forward primer Amd1-F and the reverse primer Amd1-R. The amplicons were synthesized using the amplification parameters: 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72

°C for 40 s, followed by 1 cycle of 72 °C for 5 min, with the exception of complete αβ gene amplifications which required an extension time of 1.5 min and of complete amidase gene which required an extension time of 2 min.

Nitrilase genes: all degenerate primers and the PCR parameters for this screening were previously designed (Dooley-Cullinane *et al*., 2019) as per 2.2.11.

3.2.12 General procedure for enantioselectivity screening

The general procedure for enantioselectivity screening towards racemic nitriles was carried out according to Coady *et al.,* (2013). Chiralcel AD-H and OJ-H columns (Daicel Chemical Industries®) were used for the resolution of carboxylic acids [mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA] and amides and nitriles (same mobile phase conditions with the exception of TFA) respectively. Both, with a flow rate of 0.8 mL/min and a detection wavelength of 215nm. Each isolate was submitted to crossed enantioselective screening. In addition, there were some modifications to adjust for filamentous fungi, which are described below.

3.2.13 Yeast biotransformation of 3-hydroxybutyronitrile

10 mM racemic nitrile was added in 6 mL of 100 mM potassium phosphate buffer pH 7.2 containing induced cells (OD_{600nm} =1), and incubated at 25 °C for 24 h and 250 rpm. The reaction was quenched after 24 h by removal of the cellular biomass by centrifugation at 3000 × g. After that, the supernatant was acidified to pH 2 by the addition of 2 M HCl, and then extracted with ethyl acetate. In addition, the extracts were dried over $MgSO_4$ and the solvent removed under vacuum. From this step, the products were submitted to the benzylation procedure: 0.06 mM Silver oxide, 0.24 mM benzylbromide and 2 mL dichloromethane were added and the mixture stirred in the dark for 24 h. 2 mL of acetone was added to the reaction mixture, and then filtered through a 0.45 µm filter and the solvent was removed under vacuum. 1 mL of mobile phase was added before the solution was injected on the Chiral NP-HPLC system. All experiments were performed in triplicate. % enantiomeric excess (%*ee*) was calculated from the ratio of the enantiomer by the difference in peak area divided by the sum of the peak areas for the major and minor enantiomers.

3.2.14 Filamentous fungi biotransformation of 3-hydroxybutyronitrile

10 mM racemic nitrile was added in 5 mL of 50 mM potassium phosphate buffer pH 8 containing approximately 1 g of wet mycelium and activated at 25 °C for 24 h and 160 rpm. The reaction was quenched after 24 h by removal of the cell extracts by centrifugation at 3000 × g. After that, the supernatant was acidified to pH 2 by the addition of 2 M HCl, and then extracted with ethyl acetate. In addition, the extracts were dried over $MgSO_4$ and the solvent removed under vacuum. From this step, the products were submitted to the benzylation procedure: 0.06 mM Silver oxide, 0.24 mM benzylbromide and 2 mL dichloromethane were added and the mixture stirred in the dark for 24 h. 2 mL of acetone was added to the reaction mixture, and then filtered through a 0.45 µm filter and the solvent was removed under vacuum. 1 mL of mobile phase was added before the solution was injected on the Chiral HPLC system. All experiments were performed in triplicate as described above.

3.2.15 Biotransformation of 3-hydroxy-3-phenylpropionitrile

All procedures were the same as described above with the exception of the benzylation step. After the extracts were dried over $MgSO_4$ and the solvent removed under vacuum, 1 mL of isopropyl alcohol (IPA) was added before the solution was injected on HPLC system.

3.2.16 Isolate identification

Fungal isolates were identified using molecular approaches. PCR for the amplification of the ITS regions was performed using fungus-specific universal primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS2 (5′-GCATCG ATGAAGAACGCAGC) were used to amplify the ITS1 region, while ITS3 (5'-GCATCGATGAAGAACGCAGC) and ITS4 (5'-GCATATCAATAAGCGGAGGA) were used to amplify the ITS2 region, as described by Leaw *et al*. (2006). A total reaction volume of 50 µl containing 50 ng/ml of genomic DNA, 10 pmol/µl of each primer, 25 µl GoTaq® Green Master Mix (Promega), and sterile water were used for the PCR reactions under the amplification parameters: 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C, followed by 1 cycle of 5 min at 72 °C.

DNA sequencing of PCR products was performed in W.I.T. using the BigDye 3.1 kit (Applied Biosystems) as per the manufacturer's instructions and analysed using an ABI

Prism 310 Genetic Analyser (Applied Biosystems, CA, USA). Commercial DNA sequencing was carried out by GATC Biotech, Germany. Molecular identification of fungi was carried out by sequencing the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions of the rRNA gene operon. After that, nucleotide sequences were analyzed using BLAST from the GenBank (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the homology with the existing species (Altschul *et al.,* 1990).

3.3 Results

3.3.1 Bioprospecting for nitrile-hydrolyzing fungi

Initially, the bioprospecting was conducted on MM (minimal medium) agar supplemented with nitrile as nitrogen sole source. A total of 5 environmental samples collected in Ireland for this study were used. Table 3.1 shows the number of fungi isolated by selective enrichment culture techniques.

Table 3.1 Number of fungal strains isolated with each nitrile substrate.

Nitrile substrate	Strains
3-hydroxybutyronitrile	3
3-hydroxyglutaronitrile	0
3-hydroxy-3-phenylpropionitrile	4
Total	**7**

The growth of the fungi was observed on the surface of agar containing 10 mM 3-hydroxybutyronitrile (3HBN) or 10 mM 3-hydroxy-3-phenylpropionitrile (3HPPN) at 25 °C for 8 days. No colony of fungi growing, however, was observed on 10 mM 3-hydroxyglutaronitrile (3HGN) agar plates after incubation. Table 3.2 summarizes the results of the performance testing of 7 fungi on MM agar in the presence of nitriles and subsequent strain identity.

Table 3.2 Nitrile metabolizing environmental fungi isolated with a corresponding nitrogen source.

Nitrile substrate	Fungal isolates	Samples location	Fungus type	ID
3HPPN	*Candida* sp. CRSB1	River Clodagh, River bed, under bridge, Portlaw, Waterford/IE	Yeast	Lev1
3HBN	*Saccharomyces* sp. CRSB2	River Clodagh, River Bank, Portlaw, Waterford/IE	Yeast	Lev2
3HPPN	*Fusarium solani* CRSB3	River Clodagh, River bed, under bridge, Portlaw, Waterford/IE	Filamentous fungus	F1
3HBN	*Fusarium solani* CRSB4	River Suir, Mount Congreve, under the bridge, Waterford/IE	Filamentous fungus	F2
3HPPN	*Fusarium solani* CRSB DT1	River Clodagh, River bed, Curraghmore Estate, Portlaw, Waterford/IE	Filamentous fungus	F3
3HPPN	*Fusarium oxysporum* CRSB DT2	River Clodagh, River Bank, Portlaw, Waterford/IE	Filamentous fungus	F4
3HBN	*Barnettozyma californica* var. *dimennae* CRSB5	The Comeragh Mountains, Waterford/IE	Filamentous fungus	F5

After the isolation, fungal conidia and/or mycelia and yeasts cells were picked and serially streaked to fresh MM agar containing 10 mM nitrile up to obtain pure isolates for further analysis and molecular identification. Negative control was conducted on the 7 isolates by inoculating on MM agar without addition of nitrile as nitrogen sole source (Figure 3.1). In these experiments, the fungi did not grow on the agar. In fact, this result was important to the selection of the fungi with a potential for biotransformation of β-hydroxynitriles.

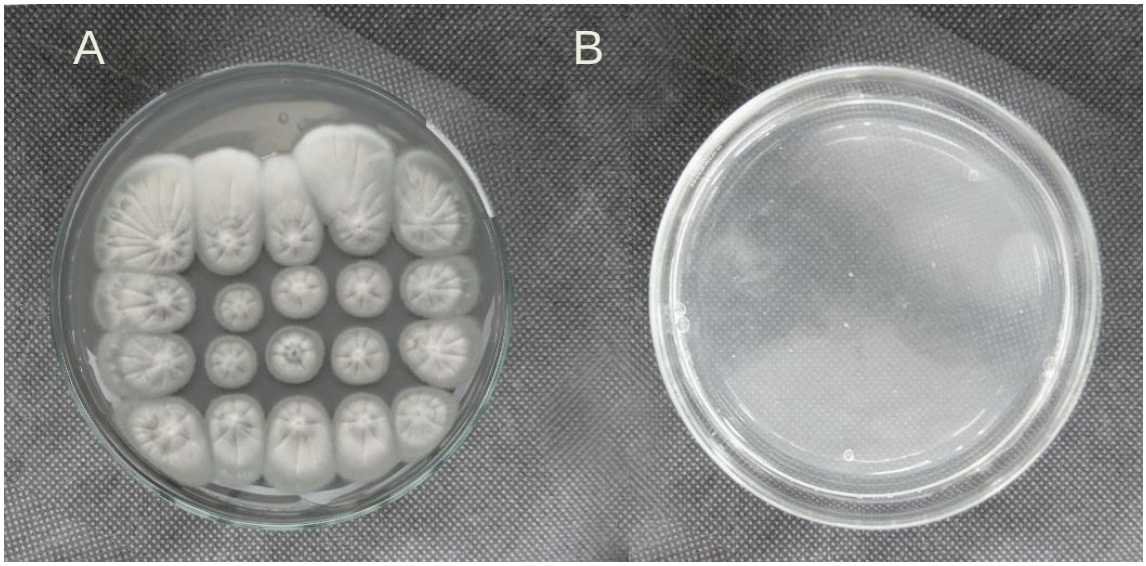

Fig 3.1 Growth of the fungus *Fusarium solani* CRSB DT1 on MM agar supplemented with 10 mM 3-hydroxy-3-phenylpropionitrile (A) and without 3-hydroxy-3-phenylpropionitrile (B).

3.3.2 Analysis of yeast cell and mycelial growth on MM supplemented with 10 mM nitriles

The quantification of growth of filamentous fungal and yeast isolates was performed differently; for yeast, liquid medium was used while for filamentous fungi, MM agar was used. As before, minimal medium containing 10 mM of nitriles as the sole nitrogen source was used. In MM with 10 mM of 3-hydroxybutyronitrile, one A_{600nm} unit was found to be equivalent to 0.337 g L^{-1} and 0.586 g L^{-1} of the dry biomass for *Candida* sp. CRSB1 and *Saccharomyces* sp. CRSB2 respectively. On the other hand, when the yeast cells were cultivated in MM with 10 mM of 3-hydroxy-3-phenylpropionitrile, one A_{600nm} unit was found to be equivalent to 0.523 g L^{-1} and 0.231 g L^{-1} for *Candida sp.* CRSB1 and *Saccharomyces* sp. CRSB2 respectively. In addition, the estimation of fungal mycelial growth was performed on plates (Table 3.3).

Table 3.3 Growth of filamentous fungi on a MM agar with 10 mM of nitrile after 7 days at 25 °C.

Fungal isolates	**Colony diameter (*cm*)**	
	10 mM 3HBN	**10 mM 3HPPN**
Fusarium solani CRSB3	0.8	1.5
Fusarium solani strain CRSB4	1.5	0.5
Fusarium solani strain CRSB DT1	4.7	6.3
Fusarium oxysporum strain CRSB DT2	0.6	1.8
Barnettozyma californica var. *dimennae* strain CRSB5	2.1	0.5

3.3.3 Nitrile-hydrolyzing activity screening

Fungal isolates were prepared as described in Material and Methods, and then, submitted to Nessler's colorimetric assay as per Coady *et al*. (2013), which was calculated by relating the sample absorbance to the standard curve (Figure 2.3a). Each sample was performed in triplicate and the average absorbance used to calculate activity. After the first round of screening, each fungal isolate was submitted to crossed activity screening and all the results are shown in the following Table 3.4.

Table 3.4 Activity screening for fungal nitrile metabolism.

ID fungi	Nitrile substrate	NH_3 (mM/L) produced	Crossed enzymatic activity*	
			Activity towards 3HBN (NH_3 mM/L)	Activity towards 3HPPN (NH_3 mM/L)
Lev1	3HPPN	0.62	0.21	_
Lev2	3HBN	0.91	_	0.26
F1	3HPPN	0.56	0.29	_
F2	3HBN	0.75	_	0.21
F3	3HPPN	1.28	1.01	_
F4	3HPPN	0.66	0.17	_
F5	3HBN	1.09	_	0.32

* No activity was observed on 3HGN after incubation.

3.3.4 Conventional PCR screening of fungi for nitrile-metabolising genes

To screen for the genes involved in nitrile metabolism, all the fungal isolates were submitted to conventional PCR. PCR primers used were those shown on Table 2.1. PCR products using primers NHA-F and NHA-R for α subunit of NHase were obtained from 6 of the 7 fungal isolates and subjected to agarose gel electrophoresis (Table 3.5 and Figure 3.2a). PCR amplification of predicted nitrilase genes were obtained from only 1 of the 7 isolates for the 2A1-20 and 2A21-37 gene clades, while PCR amplification using 4A primers were obtained from 2 of the 7 fungal isolates (Table 3.5 and Figure 3.2b).

Table 3.5 PCR screening of the 7 fungi for nitrile-metabolising gene detection.

Isolates	ID fungi	Nitrile substrate	Corresponding encoded gene
Candida sp. CRSB1	Lev1	3HPPN	α; 4A
Saccharomyces sp. CRSB2	Lev2	3HBN	α
Fusarium solani CRSB3	F1	3HPPN	α
Fusarium solani CRSB4	F2	3HBN	α
Fusarium solani CRSB DT1	F3	3HPPN	α; 2A1-20; 2A21-37; 4A
Fusarium oxysporum CRSB DT2	F4	3HPPN	α
Barnettozyma californica var. dimennae CRSB5	F5	3HBN	–*

*no PCR products were detected.

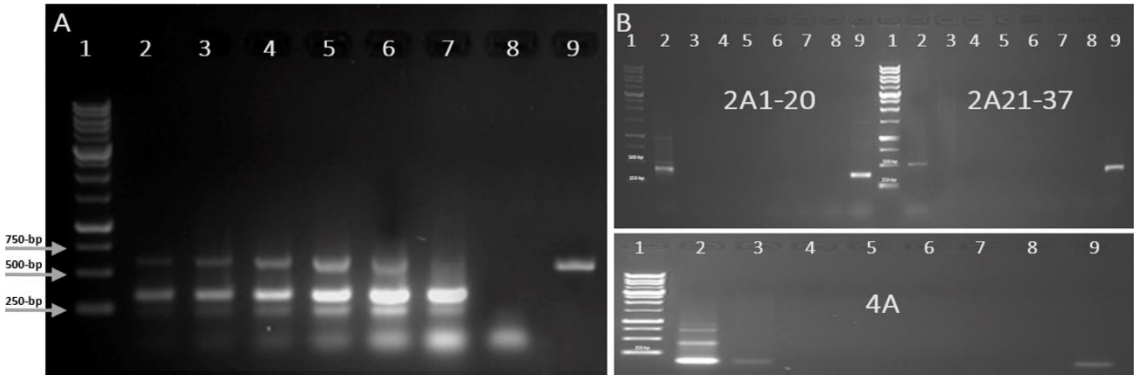

Fig. 3.2 Agarose gel electrophoresis [0.9 % (w/v)] of the complete α subunit gene detected using primers NHA-F and NHA-R to amplify a 600-bp diagnostic fragment (A). 1-kb CLS-MDNA ladder (lane 1). Lanes 2-8: Lev1, Lev2, F1, F2, F3, F4 and F5, respectively; and PCR product from positive control (lane 9). 1-kb CLS-MDNA ladder (lane 1); PCR amplification of predicted nitrilase genes (B) using primers for 2A1-20 clade to amplify a 487-bp; 512-bp (2A21-37 clade) and 125-bp (4A clade) diagnostic fragments) 1-kb CLS-MDNA ladder (lane 1). 7 fungal isolates (lanes 3-9) and PCR product from positive control (lane 2).

3.3.5 Screening biotransformations of 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile by fungi

Initial enantioselectivity screening involved the biotransformation of racemic 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile for both filamentous fungi and yeast cells. The results indicated one promising isolate, *Fusarium solani* CRSB DT1, which demonstrated exceptional enantioselectivity in hydrolysis of 3-hydroxy-3-phenylpropionitrile of >99.99 % *ee* and enantioselectivity towards 3-hydroxybutyronitrile of 98.03 % *ee*. On the other hand, the other fungi, *Fusarium solani* CRSB4, showed low-middle enantioselectivity for both nitriles used (Table 3.6 and Figure 3.3). All fungi were submitted to crossed enantioselective screening and the results are presented in Table 3.6.

Table 3.6. Enantioselectivity screening and crossed screening involved the biotransformation of racemic 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile. The promising isolate is underlined. The result displays (S)-enantiomer product.

Isolates	**Nitrile substrate**	**Activity screening NH_3 (mM/L) produced[a]**	**Crossed enantioselective screening in 3HBN**		**Crossed enantioselective screening in 3HPPN**	
			Acid *ee* (%)[b]	**Nitrile *ee* (%)[b]**	**Acid *ee* (%)[b]**	**Nitrile *ee* (%)[b]**
Candida sp. CRSB1	3HPPN	0.62	1.32	5.41	2.01	9.72
Saccharomyces sp. CRSB2	3HBN	0.91	23.77	26.12	7.31	2.86
Fusarium solani CRSB3	3HPPN	0.56	24.44	2.34	94.44	61.25
Fusarium solani CRSB4	3HBN	0.75	92.94	57.93	25.51	3.58
Fusarium solani CRSB DT1	3HPPN	1.28	98.03	92.84	>99.99	ND
Fusarium oxysporum CRSB DT2	3HPPN	0.66	4.25	1.33	18.74	5.51
Barnettozyma californica var. *dimennae* CRSB5	3HBN	1.09	46.11	13.98	2.25	11.48

[a] Activity subgroups determined using Nessler's colorimetric activity assay based on ammonia production.

[b] Determined by HPLC analysis using a chiral column (see material and methods).

ND: none detected.

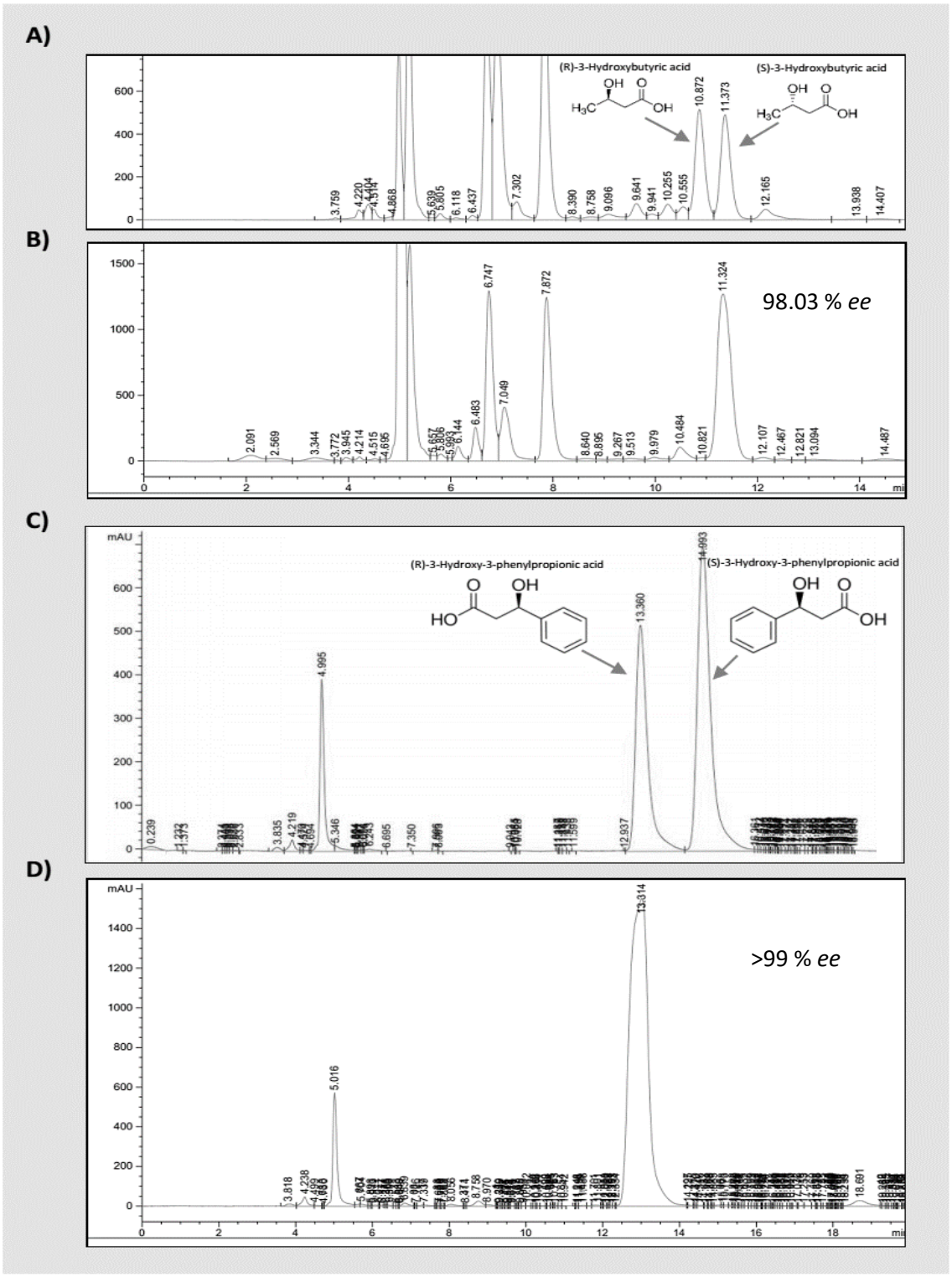

Fig. 3.3 Chiral HPLC Chromatograms. (A) Standard of racemic 3-Hydroxybutyric acid. (B) Biotransformation of 3-hydroxybutyronitrile by F3 isolate. (C) Standard of racemic 3-hydroxy-3-phenylpropionic acid. (D) Biotransformation of 3-hydroxy-3-phenylpropionic acid by F3 isolate. For conditions of HPLC analysis see materials and methods.

3.4 Discussion

Biotransformation of nitriles mediated by microorganisms has attracted considerable attention in the academic and industrial sector to be a sustainable alternative that is considered as green technology. Therefore, the search for microorganisms which contain the enzymes responsible for these biotransformations, for instance, nitrilase, NHases and amidase, becomes very important. Besides their synthetic value, these enzymes play a key role in environmental protection as they have been employed successfully in biodegradation and bioremediation of contaminated areas with organonitriles of anthropogenic sources Martínková (2019).

Bacterial nitrilases and/or NHases and amidases, acknowledged as useful biocatalysts for the biopharmaceutical industry, have been thoroughly described (Komeda *et al.*, 1996; Cramp, Gilmour and Cowan, 1997; Brandão *et al.,* 2003; Kiziak *et al.*, 2005; Banerjee *et al.,* 2006; Naik *et al.*, 2008; Coffey *et al.*, 2009; Sosedov *et al.*, 2010; Coady *et al.*, 2013; Suhaila *et al.*, 2019; Mareya *et al.*, 2020; Guo *et al.*, 2020; Lankathilaka *et al.,* 2020; Yu *et al.*, 2021). In contrast, there are few publications describing these enzymes from filamentous fungi and yeast cells. To date, over 30 nitrilases have been isolated and characterized. Only 4 out of 30 were isolated from fungi (Shen *et al.*, 2021). NHases also occur in filamentous fungi and yeast, as reported by Martínková (2019). However, in agreement with those findings with regards to nitrilases, only a few NHases were also isolated and characterized from native fungi. Therefore, the goal of this work was to bioprospect fungi with potential for nitrile biotransformations from environmental samples collected in Ireland.

Initially, the bioprospecting was performed on agar plates with 10 mM nitriles as N source. A total of 7 fungi were isolated on MM agar with 10 mM of 3-hydroxybutyronitrile (3HBN) and/or 3-hydroxy-3-phenylpropionitrile (3HPPN). The biocatalytic potential of these fungi has been evaluated by comparing the fungal growth on MM agar plates supplemented with 10 mM nitriles, with those grown on MM agar plates in the absence of the N source (control). This result was significant for selecting of fungi with potential for biotransformation of key nitriles since the fungal isolates used the nitriles effectively as a nitrogen source. In fact, the screening of microoragnisms using an appropriate enrichment strategy has proven to be a viable approach for discovering new enzymes with desired properties (Winkler *et al.,* 2021). Interestingly, Jin *et al.*, (2013) reported a

new fungus, *Fusarium proliferatum* ZJB09150, showing higher activities when isolated from soil using 3-cyanopyridine as sole nitrogen source. In another study carried out by Gong *et al.*, (2011), *F. oxysporum* H3 was isolated when glycolonitrile was used as an inducer from an enriched soil sample. For this reason, the enrichment of environmental samples or cultures increases the possibility of identifying ideal strains that harbour target enzymes.

The first studies on nitrile-hydrolyzing enzymes were published in 1964 by Mahadevan and Thimann. This work reported the ability of a number of fungi (mainly *Fusarium* sp) capable of using nitriles as a sole nitrogen source. In agreement with these results *Fusarium* spp strains were also isolated from different environmental samples, as demonstrated in Table 3.2.

Fusarium solani CRSB3, *F. solani* CRSB4, *F. solani* CRSB DT1, *F. oxysporum* CRSB DT2 and *Barnettozyma californica* var. *dimennae* CRSB5 developed in the culture medium, whose colony diameter varied from 0.8 to 4.7 cm towards 10mM 3HBN, and 0.5 to 6.3 cm towards 10 mM 3HPPN (Table 3.3). However, it is important to point out that the growth in the presence of key nitriles was discreet compared to the growth of these fungi in YPD agar plates. In general, after 8 days of incubation on YPD medium, fungal growth was evident on the entire surface of the plate (results not shown). Therefore, it was found that the key nitrile, used as the sole nitrogen source, induced the growth of fungi under these conditions (fig. 3.1).

Previously, benzonitrile was used to induce nitrile-metabolizing enzymes in fungi, as reported by Harper (1977). In another study, several nitriles such as acetonitrile, propionitrile, benzonitrile and isobutyronitrile were used as sole nitrogen sources to isolate different fungi (Goldlust and Bohak 1989). In all the cases, the benzonitrile was the superior substrate acting as an inducer for fungal isolation. Other nitriles have been used in order to evaluate the enzymatic potential of several fungi. Phenylacetonitrile was selected, as described in Oliveira *et al.,* (2015), and the growth of marine fungi on a solid medium was measured. Colony diameter varied from 0.5 to 3.5 cm towards phenylacetonitrile as sole nitrogen source, however, all the fungi were inhibited at 0.17 mM of nitrile per plate.

Additionally, the estimation of mycelial and yeast cell growth using the same culture conditions, with the exception for yeasts cells (which required MM liquid media) was evaluated, in order to investigate if the the amount of nitrile added to the medium does not cause inhibition of fungal growth. Our results showed that after 8 days of cultivation, the fungi demonstrated good growth by using 10 mM nitrile. 3HPPN was the superior substrate for four strains. On the other hand, 3HGN was not accepted by any of these isolates, apparently because of its toxicity. In fact, there is no record in the literature of this nitrile being used by filamentous fungi or yeast cells. In constrast, Chen *et al.*, (2019) and DeSantis *et al.*, (2003) reported the bacteria that could hydrolyse 3HGN to its corresponding acid.

The activities of all yeasts tested were lower towards 3HBN and 3HPPN, aliphatic and aromatic nitriles, respectively. A similar observation has been also made for *Candida fabianii* (Brewis *et al.,* 1995), *C. famata* (Rezende *et al.*, 1999) and *Kluyveromyces thermotolerans* MGBY 37 (Prasad, Sharma and Bhalla, 2005) towards aromatic and aliphatic nitriles. This could indicate that the nitriles impose more pronounced sterical hindrances on the yeast's enzymes.

A significant consideration in the development of specific biotransformation is identifying an appropriate biocatalyst or strain from culture collections by using screening methods (Martínková, 2019). Selective enrichment strategy for the isolation and quantification of nitrile-metabolism microbial cells, where nitrile substrates are used as a nitrogen source, requires efficient methods to screen libraries of isolated strains to identify those possessing the desired reactivity and selectivity. Several high-throughput screening methods have been developed for nitrile hydrolysing enzymes, such as described by Banerjee *et al.*, (2003), Coady *et al.*, (2013), Black *et al.*, (2015), Xue *et al.*, (2016), Nigam *et al.*, (2017) and Sahu *et al.,* (2019) and have been reviewed by Shen *et al.*, (2021). The focus of these screening methods was on bacterial enzyme activity, while few studies focused exclusively on fungal enzyme activity. Ogunyemi *et al.*, (2020) identified the nitrilase activities from several fungi strains using the traditional method described by Almatawah *et al.,* in 1999, by monitoring the ammonia production using a UV-visible spectrophotometer at 630 nm. The same method was performed by Ondřej *et al.*, (2006) to screen fungi from culture collection towards 3-cyanopyridine as the sole source of nitrogen. Traditional screening methods, using small flasks, beaker, or test tube for

NHases and amidase activity, are often very laborious and time-consuming. In this way, a colourimetric method for the determination of ammonia using reagents such as Nessler's provides a promising strategy for rapid screening and, in particular, offers the potential for the evaluation of nitrile hydrolysing enzymes in 96-well plate systems (Coady *et al.*, 2013).

For this reason, the next step of our study was to adapt the method for activity screening described by Coady *et al*. (2013) to use in filamentous fungi. Differents conditions were tested to standardize the parameters for using in filamentous fungi. The adapted screening method is described in materials and methods (section 2.25). Nevertheless, a combination of this strategy screening using filamentous fungi and yeast cells, allowed the development of a novel rapid identification screening for fungi potentially containing nitrile-hydrolysing enzymes. A key advantage of this screening, is the incorporation of the 96-well microtest plate, which enables rapid detection of screening activity. The utilisation of a high-throughput fungal screening method using 96-well plates was only used to characterise the growth of fungi in different carbon sources (Tamminen *et al.*, 2020; Weinberger *et al.*, 2020), biofilm formation and quantification (Pierce *et al.*, 2008), and for small molecules detection (Krysan and Didone, 2008; Garnier *et al.*, 2018; Stocker *et al.*, 2020).

After initial evaluation, fungal isolates were submitted to PCR screening to identify potential nitrile metabolism genes. Surprisingly, our results demonstrated that primers designed for bacterial nitrilase gene amplification, could also be used in fungi. This may be explained due to the similarity of those nitrilase genes in prokaryotic cells. On the other hand, conventional PCR with those primers for α subunit of NHase gene gave a number of products. Curiously, some fungi harbour genes encoding two or three nitrilases, as discussed by Vejvoda, *et al.*, (2009). Moreover, genes coding for both nitrilase and NHases could be found in some strains of *F. solani* (Shen *et al.*, 2021).

The NHase gene was first isolated from *F. solani* by Harper in 1977, but the sequence of the amino acids was never determined. In addition, Torruella *et al.*, (2009) suggested that, after a broader search for several genes in eukaryotes cells, the fungal NHase gene might have originated from a prokaryotic source by a lateral gene transfer event.

Subsequently, all the fungi were enantioselectively screened via biotransformations of each nitrile to produce the corresponding carboxylic acids, which are the expected products of nitrile-metabolism. The chromatographic methods used to determine the enantiomeric excess (*ee* %) of nitrile is described in Materials and Methods. The biotransformation of racemic nitrile was initially investigated with the formation of the corresponding acids. The results demonstrated that *F. solani* CRSB DT1 exhibited exceptional enantioselectivity towards 3-hydroxy-3-phenylpropionitrile of >99.99 % *ee* and enantioselectivity towards 3-hydroxybutyronitrile of 98.03 %. It is promising, because this isolate also showed the best result of the activity screening. *F. solani* CRSB3 also presented good enantioselectivity of >92 % towards 3HBN. Prior to this study, the hydrolysis of these key nitriles was only tested on bacterial cells. To this author's knowledge, this is the first study concerning the isolation of a fungi with the potential to perform the biotransformation of 3HBN and 3HPPN. In agreement with these results, the biocatalytic potential of *Fusarium* sp for aliphatic and aromatic nitrile biotransformation, has been observed by Oliveira *et al.*, (2013). In that study, *F. solani* was used for the conversion of phenylacetonitrile to 2-hydroxyphenylacetic acid, with ee > 99 %. Also, in another study, the *F. solani* strain could convert 2,6-pyridinedicarbonitrile into corresponding carboxylic acids, with ee > 90 % (Vejvoda *et al.*, 2007).

In contrast, the results from this study showed that the yeast cells, *Candida* sp. CRSB1 and *Saccharomyces* sp. CRSB2, exhibited poor enantioselectivity towards both nitriles. As the commercial synthetic process generally requires greater than 98 % enantiomeric excess (Crabtree, 2009), proving the viability of attempting to isolate the genes that encode those nitrile-hydrolyzing enzymes and express them in a suitable host represents a big challenge.

CHAPTER 4

APPLYING FUNCTIONAL METAGENOMICS TO SEARCH FOR NOVEL NITRILE-HYDROLYZING ENZYMES FROM ENVIRONMENTAL SAMPLES

4.1 Introduction

The pharmaceutical industry has several motivations to probe the enormous resource that is uncultivated microbial diversity. Presently, there is a global political pressure to encourage industrial/white biotechnology to substantially impact such industrial production. Thus, the development of novel enzymes as the ideal biocatalyst is very desirable, if not required (Sheldon *et al*, 2020).

The *Prokarya* domain is the most diverse and abundant group of microorganisms on Earth. Archaea and bacterial cells can live in almost all environments. However, environmental microbiologists estimate that about 99 % of bacteria cannot be cultivated in the laboratory (Bodor *et al.*, 2020).

There are two main different approaches in the isolation of microbial genes/enzymes from the environment; culture-dependent and culture-independent methods. Both allow the characterization of and access to some of the diverse microbiome within a sample and therefore are considered complementary (Nakamura *et al.*, 2016). It has been estimated that < 1 % of the microorganisms are detected on plates with culture medium due to selective conditions, depending on the composition of the culture media. On the other hand, the culture-independent methods indicate a predominance of many uncultivated species (Prem *et al.*, 2021). Therefore, microbial enzymes obtained from pure cultures which are currently being used for nitrile metabolism are not an apt indication of the full potential for biocatalysis possessed by microorganisms. Taking this into consideration, in recent years, some culture-independent-based methods have been established to survey the different biodiversities in varied environments (Prayogo *et al.*, 2020).

One example of a culture-independent molecular method is metagenomics, which consists of direct extraction of nucleic acids from the environmental sample(s), followed by cloning into a suitable vector, transformation into host cells and sequencing or screening for the genes and/or functions of interest (Handelsman, 2004), without going through prior DNA amplification steps.

Currently, uncultured environmental samples can be explored by the metagenomic approach. Metagenomics not only provides the diversity profiling of microbial communities but also allows the search for identifying novel genes and proteins of

industrial interest (like nitrile-degrading enzymes) overcoming the uncultivable nature of the microbes (Prem *et al.*, 2021).

Metagenomics has two important goals: (1) to determine the taxonomic composition of the entire microbiome (structural) and (2) to elucidate the total genomes and associated genes of the microbiome (functional). Structural metagenomics identifies the major species, genera, or domains of organisms inhabiting a particular ecological niche to study and understand their roles in evolutionary aspects, biogeochemical cycles, and environmental interactions. In contrast, functional metagenomics is about studying genomic diversity in an environmental sample where metagenomic libraries are established, and clones screened with enzyme substrates (Simon and Daniel, 2011).

A functional metagenomics approach has shown some positive results over the years, as in the study of the determination of complex biosynthetic pathways (Libis *et al.*, 2019), for the discovery of new hydrolytic enzymes, such as amylases, lipases and proteases (Popovic *et al.*, 2015), or to identify nitrilase-encoding genes from plasmid libraries containing metagenomic DNA from different sources (Robertson *et al.*, 2004; Bayer *et al*., 2011; Bragança *et al.*, 2017; Egelkamp *et al.*, 2020b).

The functional strategy selects the clones by the activity where changes caused by enzymatic reactions are detected. It is considered a simple, cheap, and fast technique used in the medical, agricultural, and industrial areas, as it allows faster obtaining of clones (Ngara and Zhang, 2018). This strategy can be done in 3 ways: (a) each clone is individually tested by visual detection in the desired function (growth inhibition, antibiotic resistance or complementary biochemical pathways), allowing to find a limited number of clones; (b) selection of high quantity of clones, using a fast and automated method to separate desired candidates from other clones by fluorescent cell sorting, a technique named by Uchiyama *et al.*, (2005), from SIGEX (Substrate-Induced Gene Expression Selection); and (c) by function selection, where only a rare clone grows among hundreds of thousands of clones analyzed, mainly due to the lack of gene expression efficiency in the host strains (Handelsman, 2004). However, this strategy has the potential to identify new classes of coding genes for both known and new functions (Lam *et al.*, 2015).

The need for the evaluation of a large number of clones for a specific function in functional screening is the main disadvantage. On the other hand, it is possible to use large insert

vectors like fosmids as carriers during metagenomic library construction with higher efficiency and a lower number of clones. As a consequence, the library could be screened not only for industrially relevant genes encoding for new enzymes but also for whole operons (Gonçalves *et al.*, 2015).

For this reason, this current study focused on applying functional metagenomics constructed in fosmid vector to search for novel nitrile hydrolyzing enzymes from environmental samples. This will allow the cloning, expression and purification of recombinant enzymes, which is the form/stage required by potential industrial partners/customers for screening/scale-up/go-to-market or indeed any industrial exploitation. The nitriles chosen for this study were 3-hydroxybutyronitrile (3HBN), 3-hydroxyglutaronitrile (3HGN) and 3-hydroxy-3-phenylpropionitrile (3HPPN), which are β-hydroxynitriles, which can act as sources of β-hydroxycarboxylic acids by biotransformation - these products could be widely used as chiral precursors for pharmaceutical compounds.

4.2 Material and Methods

4.2.1 Substrates of interest

Racemic 3HBN and 3HGN were purchased from ENAMINE®, and 3HPPN from Sigma®. All other chemicals were of analytical grade and obtained from Macron Fine Chemicals™ and Acros Organics Chemicals.

4.2.2 Total DNA extraction and metagenomic fosmid library construction

10 soil samples used in this work as sources of metagenomic DNA were obtained from environmental soils collected from terrestrial and aquatic microenvironments in Co. Waterford, Ireland. They were selected based on potential nitrile contamination from industrial or anthropological practices at the site of collection. Total DNA was extracted according to the previously described protocol (Stevenson and Weimer, 2007). Briefly, the cells were lysed using beads and 20 % (x/v) SDS, DNA was then isolated by purification using phenol/chloroform extraction followed by alcohol precipitation. The

pellet of DNA was resuspended in TE (10 mM Tris/HCl, 1 mM EDTA, pH 8), treated with RNAse at 37 °C for two hours and stored at -20 °C.

Cloning of metagenomic DNA into the vector pSMART® FOS Vector (Lucigen®) and packaging recombinant lambda phage by Gigapack III XL packaging extract (Stratagene®) were performed as per the manufacturer's instructions. Briefly, metagenomic DNA samples were subjected to an end-repair reaction to create blunt ends with 5´ phosphate groups for ligation into the blunt, dephosphorylated vector. Fosmids containing the inserts were then packaged with Gigapack III XL and used to infect the Replicator™ FOS strain. Infected cells were spread on YT+CXIS plates (8 g/L bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl and 15 g/L agar) supplemented with 12.5 µg/mL chloramphenicol, 40 µg/mL X-Gal, 0.4 mM IPTG and 5 % (w/v) sucrose, and incubated at 37 °C overnight for selecting transformants. The titer of the packaged fosmid phage particles was first determined to calculate plating requirements. Clones were transferred to 96-well Megablocks® containing LB broth supplemented with 12.5 µg/mL chloramphenicol and stored after growth at -70 °C in the presence of 20 % (w/v) glycerol.

4.2.3 Fosmid clone selection by functional screening

Individual clones from the metagenomic fosmid library were pre-cultured in 96-well Megablocks® containing 600 µL of TB broth [11.8 g/L bacto-tryptone, 23.6 g/L yeast extract, 9.4 g/L dipotassium hydrogen phosphate (K_2HPO_4; anhydrous), 2.2 g/L potassium dihydrogen phosphate (KH_2PO_4; anhydrous), 0.4 % (w/v) glycerol] supplemented with 8 mL filter-sterilized 50 % (w/v) glycerol and 12.5 µg/mL chloramphenicol, and then incubated at 25 °C and 250 rpm for 16 h. For induction, fosmid clones were grown overnight in TB medium plus 12.5 µg/mL chloramphenicol in the presence of 1X Copycontrol™ fosmid auto-induction solution (Epicenter, Cat No. AIS10F7) at 37 °C and 225 rpm. After growth, the clones were plated on M9 agar supplemented with 10 mM nitrile substrates and incubated for 6 days at 25 °C for nitrile functional screening. An initial screening stage involved the streaking of clones followed by a "bulk screening" strategy which involved transfecting the replicator FOS cells with the remaining phage particles and subsequent auto-induction in TB broth with 12.5 µL/mL chloramphenicol and auto-induction solution. The auto-induced "bulk-clone" library was spread over the surface of an agar plate with functional clones identified over background

growth. All functional screening in solid medium was carried out in triplicate. For long-term storage, cells were maintained frozen at -70 °C in 20 % (v/v) glycerol.

4.2.4 Gene screening by conventional PCR

NHase, amidase and nitrilase gene screening were performed by direct colony PCR of functional clones as described in section 2.2.10. Nitrilase genes were amplifield by using degenerate primers as per section 2.2.11. On the other hand, NHase and amidase were amplified using primers as shown in Table 2.1.

4.3 Results

4.3.1 Metagenomic library screening for clones expressing nitrile metabolizing activity

To identify novel genes coding for nitrile hydrolyzing enzymes from new sources, a fosmid metagenomic library was constructed with total DNA isolated from environmental samples collected in Ireland from terrestrial and aquatic microenvironments. A total of 12,000 clones were obtained from the metagenomic library.

Due to time and material limitations, 10 % of these clones were individually screened for nitrile activity on solid medium by functional screening, indicated by growth using the selected nitrile as the sole source of nitrogen. Of the 1,200 clones screened, 16, 12 and 5 clones showed activity via cell growth and nitrile utilization towards 3-hydroxyglutaronitrile, 3-hydroxybutyronitrile, and 3-hydroxy-3-phenylpropionitrile respectively (Figure 4.1). These clones were re-streaked to ensure growth was due to enzyme activity.

Fig. 4.1 Sample image of restreaked clones on M9 minimal media containing 10 mM 3HGN. Replicator™ FOS strain was used as negative control.

The remaining 90% of the clones from the library were submitted to bulk library screening. Of the 10,800 clones screened, one and four clones demonstrated activity via cell growth and nitrile utilization towards 3-phenylpropionitrile and 3-hydroxybutyronitrile respectively. No clones were detected with activity towards 10 mM of 3-hydroxyglutaronitrile.

4.3.2 PCR screening

A total of 38 functional clones (capable of nitrile utilization) were submitted to gene screening using conventional PCR. An example of PCR amplification with the amidase primers is shown in Figure 4.2. All the results of the DNA fragments amplifications are shown in Table 4.1.

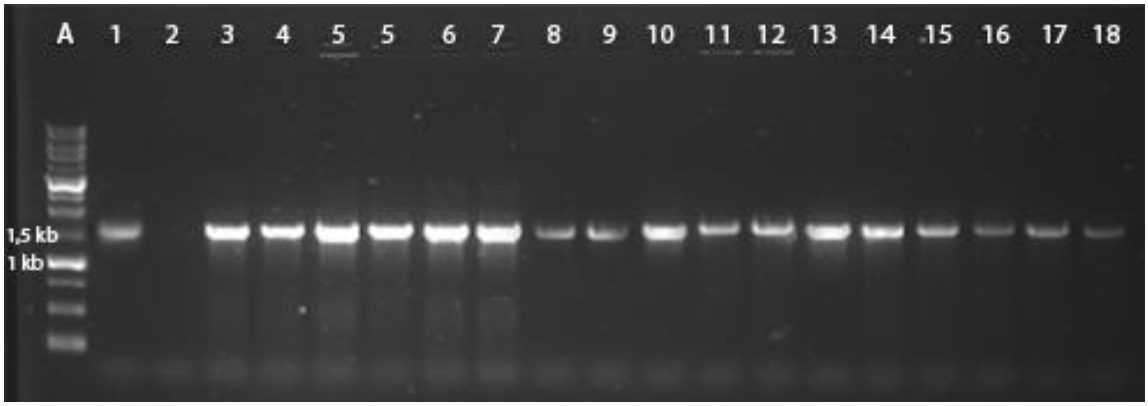

Fig. 4.2 Agarose gel electrophoresis 1.2 % (w/v)] of amidase gene detected using primers to amplify a ~1,5-kb diagnostic fragment. 1-kb CLS-MDNA ladder (lane A). *R. erythropolis* AJ270 positive control (lane 1). Negative control (lane 2). 7 clones capable of growth on 3HPPN (lanes 3-8), 10 clones capable of growth on 3HBN (lanes 9-18).

Table 4.1 Numbers of clones yielding PCR products of the expected size of nitrile hydrolyzing genes.

Functional screening	Amidase	Nitrilase	NHase
Clones from 3HPPN	6	6	-
Clones from 3HBN	10	16	-
Clones from 3HGN	–	16	-

4.4 Discussion

To construct a metagenomic library to search for new nitrile-metabolizing enzymes, this study used DNA fragments representing a mixed microbial soil population obtained from various regions around the south east of Ireland. These environmental samples were selected based on the premise that these samples would have been exposed to industrial waste from an old tannery industry and harbor metal content. A total of 10 of these environmental samples collected in Ireland from terrestrial and aquatic microenvironments were processed into metagenomic DNA libraries containing fragments approximately 38 to 40 kb in size.

This study chose a function-based metagenomic approach, which allows the identification of novel nitrile metabolizing enzymes, making it possible to focus gene screening and therefore detect only enzymes that are functional (Sleator *et al*., 2008). It is a common practice for enhancing the desired functions in a microbial community to induce the growth of specific microorganisms by applying selective enrichment methods to the sample to produce an increased screening hit rate (Michael *et al.*, 2003; Uchiyama and Miyazaki, 2009; Simon and Daniel, 2011; Wang *et al.*, 2012; Rabausch *et al.*, 2013; Lam *et al.*, 2015).

A total of 1.2 x 10^4 clones were obtained from the metagenomic library. The 38 positive clones demonstrating growth on the chosen nitriles were subjected to conventional PCR screening. Our results demonstrated that 42 % of the positive clones contain the complete amidase gene. In contrast, no clones have presented NHase genes of the sequence type targeted by the chosen primers (Fe-type NHase genes with similar homology to those often seen in *Rhodococci*, such as *R. erythropolis* strain AJ270 (accession number AJ490527) or strain N771 (accession number AJ716152). It is important to point out that these clones may have a different NHase that the primers do not amplify due to sequence divergence at the primer binding sites. Also, the NHases primers used in this study are specific to Fe-type NHases genes. So, it is possible that the functional clones contained a Co-type NHases or a novel gene that could not be detected by using these primers. On the other hand, most of them yielded potential PCR products for a nitrilase gene. In contrast, Gong, *et al.*, (2012) reported more than 60 nitrile-metabolizing cells, including species of *Hanseniaspora*, *Geotrichum*, *Exophiala*, and *Rhodotorula* contained the NHases-amidase system, rather than the nitrilase system.

In one metagenomic study, Liebeton and Eck (2004) reported 12 novel NHases. In another study, only one amidase-positive clone was detected by Voget *et al.*, (2003) when a general screening of a soil metagenomic library for biocatalysts was used.

Nitrilase genes are relatively rare in microbial genomes and less than 20 were reported in the literature prior to the application of metagenomics for their detection within an environmental sample (Podar *et al*., 2005). However, two studies using environmental samples collected worldwide from terrestrial and aquatic microenvironments were used to construct a metagenomics library, allowing identification of more than 337 novel

nitrilases, which has dramatically increased the amount of information about these enzymes (Grace DeSantis *et al.*, 2002; Robertson *et al.*, 2004).

In summary, this study consisted of applying functional metagenomics to search for novel nitrile hydrolyzing enzymes using environmental samples collected in Ireland. Gene screening of the positive clones demonstrating the potential presence of nitrilase and amidase genes. It is proposed that this research would be carried out in future to identify complete gene sequences for cloning and expression towards realizing commercial potential. It is believed that by using this molecular approach, one may build an extraordinary bank of clones producing novel nitrile-metabolizing enzymes. Fosmid extracts from these clones would ideally be sequenced. This would give an indication of source species but also allow us to elucidate the genes responsible for the nitrile hydrolysis, and the newly discovered enzymes can be applied for the enantioselective production of carboxylic acid derivatives.

CHAPTER 5

NIT2 CHARACTERISATION AND OPTIMIZATION OF THE FERMENTATION PROCESS FOR RECOMBINANT NITRILASE EXPRESSION IN *ESCHERICHIA COLI* USING STATISTICAL DESIGN OF EXPERIMENTS

5.1 General introduction

Nitrilases (EC 3.5.5.1) are enzymes catalyzing the conversion of nitriles into carboxylic acids and ammonia. These enzymes have potential as commercial biocatalysts for manufacture of many drug intermediates and chemicals (Pace and Brenner, 2001; Coady *et al.,* 2013). Chiral carboxylic acid products of nitrilase reaction e.g., 3-hydroxy-3-phenylpropionic acid or 3-hydroxyglutaronitrile are precursors for synthesis of pharmaceutical compounds (DeSantis *et al.,* 2002; Kamal, Khanna and Ramu, 2002; Bergeron, Chaplin, *et al.,* 2006; Hammond *et al.,* 2007). Several nitrile metabolizing enzymes are reported that show high enantioselectivity toward compounds of industrial interest (Naik *et al.,* 2008; Coady *et al.,* 2013).

Enzymes are typically produced in lower yield in the native organisms. Consequently, the use of microorganisms as 'cell factories' has received increasing interest in industry to provide suitable yields of recombinant enzymes (Bergquist, Morgan and Saul, 2014). Once the enzyme is characterized for desired properties, large scale production in heterologous hosts is essential. Among many systems available for recombinant protein expression, the gram-negative bacterium *Escherichia coli* remains one of the most attractive hosts (Hewitt and McDonnell, 2004; Demain and Vaishnav, 2009), due to its capacity to grow rapidly and at high density on economical medium, simple scale-up process, its well-characterized genetics and the availability of an increasingly large number of cloning vectors and optimized host strains (Jana and Deb, 2005; Terpe, 2006; Gopal and Kumar, 2013). BL21 *E. coli* strain is routinely used for larger scale protein production (Terpe, 2006; Song *et al.*, 2008; Kaplan *et al.*, 2011; Gong, Lu, *et al.*, 2012; Veselá *et al.*, 2012). These strains express T7 RNA polymerase in a regulated manner, thus facilitating regulated expression of the gene of interest (Studier, 1991; Zerbs, Frank and Collart, 2009). However, despite the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently and high-level production of functional proteins in *E. coli* may not be a routine matter and sometimes it is quite challenging (Jana and Deb, 2005; Gräslund *et al.,* 2008).

Many factors contribute to this challenge, including low expression, and the misfolding of the recombinant protein into inclusion bodies (Malhotra, 2009; Papaneophytou and Kontopidis, 2014) which are a major bottleneck for structural biology researches. Leibly *et al*. (2012) showed that more than 30 % of recombinant proteins expressed in *E. coli*

appear to be insoluble. In addition, the production of soluble and active proteins is influenced by several factors as such as expression host, fusion tag, induction temperature and time. In this way, statistical design in microbiology for optimization of culture conditions could be an alternative method for production of recombinant protein due to providing information on variable interactions that escape the “one-factor-at-a-time” method (Walker, 2005).

Increasing productivity and improving quality are important goals in any business. Here, we report the most important factors affecting the production of recombinant nitrilase in a soluble form. To date no studies on medium engineering for nitrile-hydrolyzing enzyme activity within the recombinant *E. coli* whole cell have been reported. Thus, this study formulates an appropriate production medium using statistical optimization that can substantially increase nitrilase production.

5.2 Material and methods

5.2.1 Chemicals

Racemic 3-hydroxybutyronitrile (3HBN), 3-hydroxy-3-phenylpropionitrile (3HPPN) and 4-hydroxyphenylacetonitrile (4HPAN) were purchased from Sigma-Aldrich®. 3-hydroxyglutaronitrile (3HGN) and 2-phenylpropionitrile (2PPN) were purchased from TCI AMERICA®.

All other chemicals used in this study were analytical grade unless otherwise stated. All solvents were HPLC grade. Antibiotics were purchased from Sigma-Aldrich®. Water (MQ) was purified with a Milli-Q-system

5.2.2 Bacterial strains, plasmids and nucleotide sequence analysis

A strain of *Escherichia coli* NovaBlue GigaSingles (NovaBlue is a K-12 strain derivative that offers high transformation efficiency, facilitates plasmid stability, and allows blue/white screening with appropriate plasmids), was used to initially clone the gene of interest. The DE3 lysogen of NovaBlue is potentially useful as a stringent host due to the presence of the *lacIq* repressor encoded by the F’ episome. The genotype of this strain

is as follows; *endA1 hsdR17*($r_{K12}^{-}m_{K12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac [*F′p*roA*$^{+}$*B*$^{+}$ *lacI*^{q}*ZΔM15*::Tn*10*(Tc)]. NovaBlue cells are tetracycline resistant (Tetr), endonuclease deficient (*endA*) and are recombination deficient (*recA*). The *hsdR* mutation prevents the cleavage of cloned DNA. The *lacI* q*Z_M15* gene on the F´ episome allows blue/white colour screening. Also, these recombinant strains are used for routine molecular cloning applications.

E. coli BL21(DE3)pLysS – the genotype of this strain is as follows; F-, *omp*T, *hsd*S_B (r_B, m_B-), *dcm*, *gal*, λ(DE3), pLysS, Cm^r. These cells allow high-efficiency protein expression of any gene that is under the control of a T7 promoter and has a ribosome binding site. BL21(DE3)pLysS is lysogenic for λ-DE3, which contains the T7 bacteriophage gene I, encoding T7 RNA polymerase under the control of the *lac* UV5 promoter. In addition, BL21(DE3)pLysS also contains a plasmid, pLysS, which carries the gene encoding T7 lysozyme. T7 lysozyme lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. Furthermore, this recombinant strain provides tighter control of protein expression for the expression of toxic proteins and is resistant to chloramphenicol.

E. coli BL21(DE3)pLysS was used as the host strain for the nitrilase (*nit*2) gene expression. The nucleotide sequence of the *nit*2 gene from *Burkholderia* sp. was identified by Coffey *et al*. (2009) and has been submitted to GenBank under the NCBI accession number GQ254726. The *nit*2 gene was previously cloned into the expression vector pRSF-2 Ek/LIC and expressed in *E.coli* BL21(De3) and preserved in our laboratory. This strain had previously been shown to produce only insoluble Nit2 protein. Stocked culture was activated at 37 ºC in LB media.

The pGEM®-T Easy Vector (Promega) (Figure 5.1) was used as convenient system to clone PCR products of the *nit2* gene. The pGEM®-T Easy is a pre-linearized Vector containing 3´-T overhangs at the insertion site to provide a compatible overhang for PCR products.

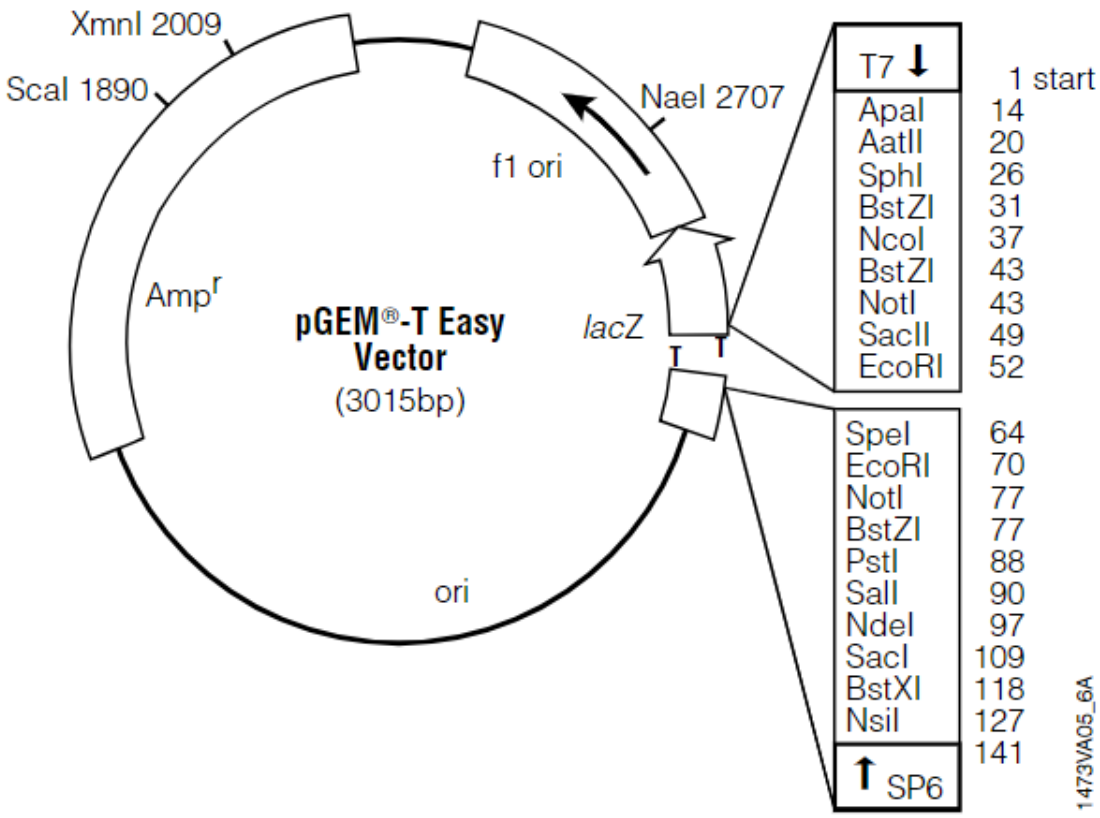

Fig. 5.1 pGEM®-T Easy Vector Map and Sequence Reference Points (Promega™).

The pEXP5-CT/TOPO® expression vector (Figure 5.2) provides a highly efficient, five-minute, one- step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for T7-based and a high-level expression of recombinant protein for inducible expression in *E. coli*.

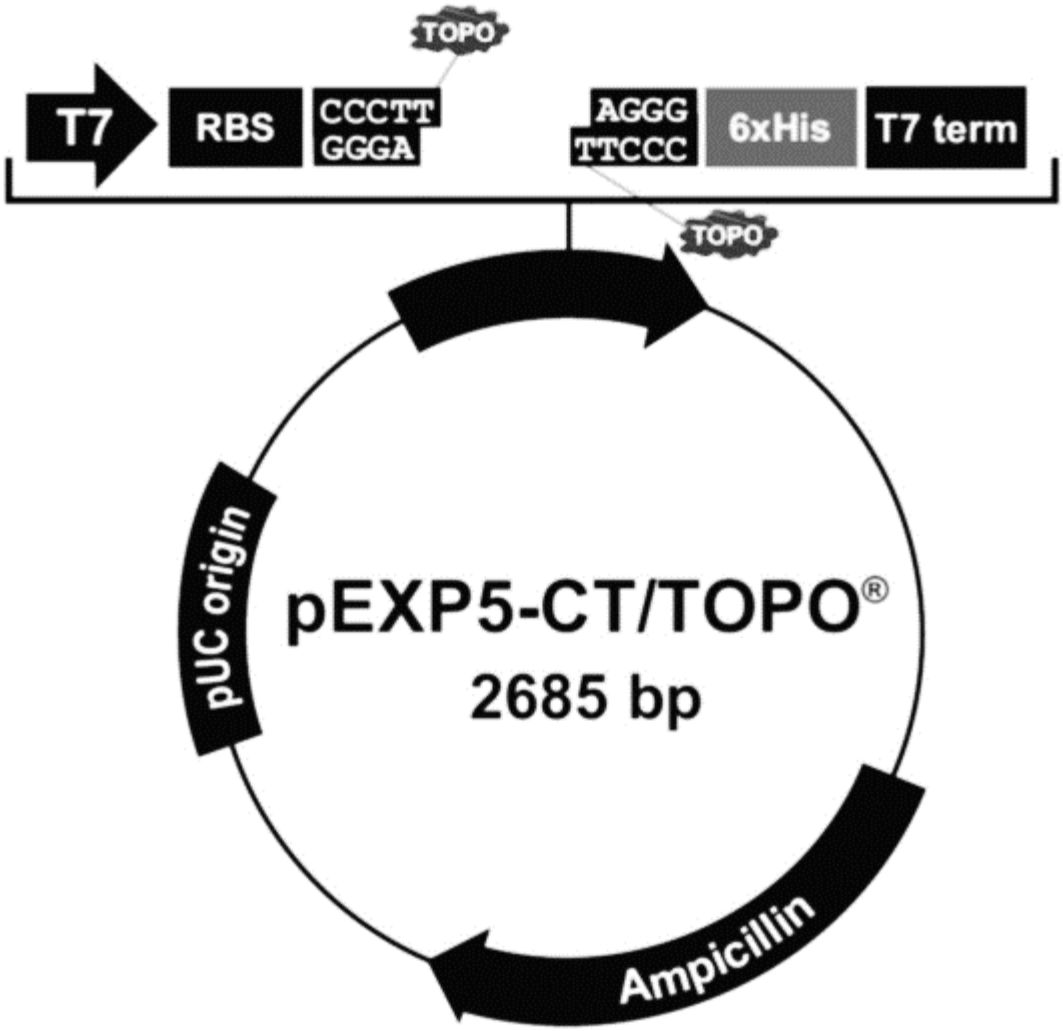

Fig. 5.2 pEXP5-CT/TOPO Map (Thermofisher^{TM}).

5.2.3 Maintenance of *E.coli* transformant strains

E. coli transformants (*E. coli* BL21(DE3) pLysS, Invitrogen or NovaBlue GigaSingles, Novagen) were stored at 4 °C on LB agar (Sigma-Aldrich) containing the appropriate antibiotic (plasmid vector dependant). Plates or broth were inoculated from glycerol stocks when required. Glycerol stocks were prepared as follows; Overnight LB broth (with the appropriate antibiotic) cell cultures were centrifuged in 1 ml aliquots at 12,000 g for 5

mins, before being resuspended and stored in 80 % (w/v) glycerol and 80 % (w/v) LB broth containing 50 µg/mL ampicillin at -70 °C.

For transformants harbouring pGEM®-T Easy and pEXP5-CT/TOPO® vectors, 50 µg/ml of ampicillin (Sambrook and Russell, 2001) were added to broth, agar and glycerol stocks.

5.2.4 Preparation of chemically competent *E. coli* cells

The process of calcium chloride ($CaCl_2$) competent cells preparation, encourages bacterial cells to uptake DNA from the surrounding environment during subsequent transformation through heat-shocking. Competent cell preparation was carried out as described in Chang *et al*. (2017) with some modifications.

10 mL of LB broth was inoculated with a loopful of *E. coli* NovaBlue cells from a fresh LB agar plate (containing 50 µg/ml of Tetracycline) and incubated overnight at 37 °C at 250 rpm, in LB broth containing 50 ug/ml Tetracycline. 1 mL of this culture was then used to inoculate 99 mL of fresh LB (1:100 diluition). The bacteria were grown until an $O.D._{600}$ = 0.4 was reached. The cell suspension was divided into 50 ml aliquots and pelleted by centrifugation at 4ºC at 4000 rpm for 10 mins. Cells were then washed in 5 mL of ice-cold 10mM NaCl and recentrifuged for 10 mins. The supernatant was discarded and each pellet was resuspended in 20mL of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. Centrifugation was repeated for 10 mins. The supernant was discarded and the pellets were combined by resuspending in 5 mL ice cold 0.1 M $CaCl_2$ with 15 % (vv) glycerol. Cells were resuspended in 0.1 mL aliquots and those not to be used immediately were stored at -70 °C.

5.2.5 Subcloning of the *nit*2 gene and construction of pEXP5-CT/TOPO-NIT2

The *nit*2 gene was ligated into the expression vector pEXP5-CT/TOPO® (Invitrogen). Firstly, the *nit2* gene was obtained by polymerase chain reaction (PCR) conducted in a 50 µL reaction mixture, each containing 1 µL culture of *E. coli* pRSF-2 Ek/LIC-NIT2 as template, 1 unit of Q5® High-Fidelity DNA Polymerase (NEB), 10 µL of 5X Q5 Reaction Buffer, 2.5 mM of each dNTP, and 10 pmol of the upstream primer nitfull F2 (5’-

atgcctctcgttcatccgga-3') and downstream primer, nitfull r (5'-catctcttcccggtgcgcc-3'). Then, the recombinant vector of pEXP5-CT/TOPO-NIT2 was transferred into *E. coli* BL21(DE3)pLysS.

5.2.6 Heat-shock transformation of recombinant vector

Plasmid transformations (heat-shock method) were carried out as described in Chang *et al*. (2017) with some modifications:

For transformations ~100 ng of plasmid DNA was added to 100 µL of *E. coli* competent cells, as soon as the cells were thawed on ice. After 30 min of incubation on ice, cells were submitted to heat shocked by placing in a 42 °C water bath for exactly 45 seconds. 1 mL of pre-warmed LB was added before incubation at 37 °C, 200 rpm for 60 min. 100 µL aliquots of cells were then plated out onto warm LB plates containing 50 µg/mL ampicillin. Transformants were analysed after 12-16 h at 37 ºC.

For screening and selection of recombinants, 0.1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) and 100 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were also added to the LB agar plates when required for blue/white colony screening.

5.2.7 Colony screening

Colonies were screened for inserts and correct orientation, without plasmid preparation, by direct colony PCR using a combination of vector-specific primers – T7 Forward primer (Promega, Cat.# Q5011), T7 Reverse primer (Promega, Cat.# Q5021) – with a primer that hybridizes within the insert (Figure 5.3).

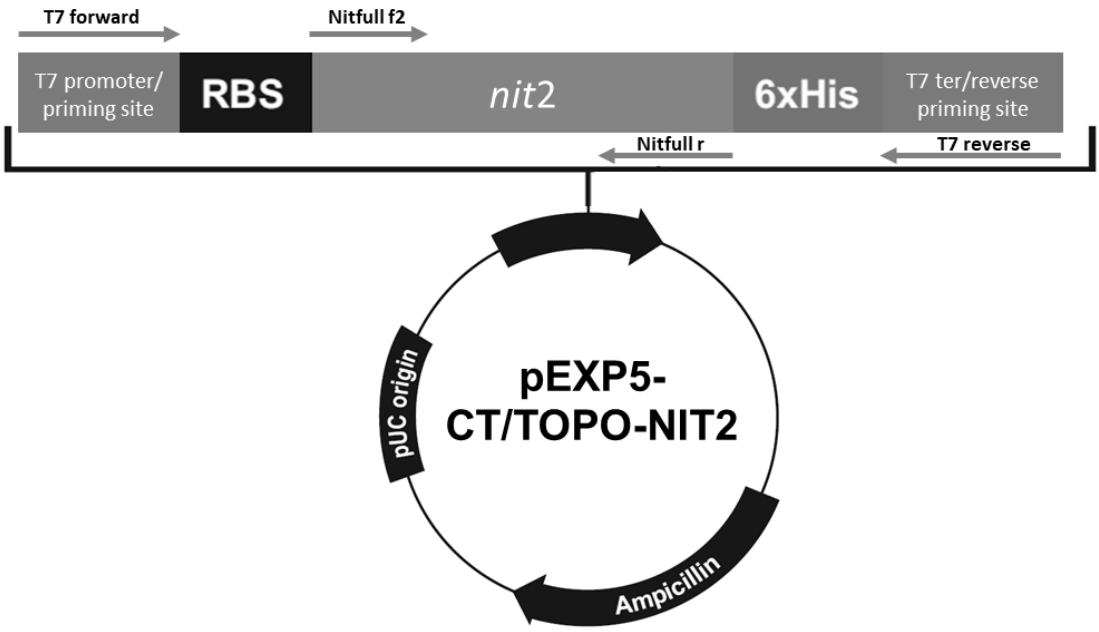

Fig. 5.3 pEXP5-CT/TOPO-NIT2 recombinant construction.

The experiment was carried out as described in Sambrook & Russell (2001), adapted: a colony from LB agar plate was picked by using 10 µl pipet tip and transferred to a 50 µl reaction mixture containing 1X GoTaq®Green Master Mix (2X) and 1.0 µM each primer. The reaction was then, submitted to PCR (section 5.2.10).

5.2.8 Plasmid DNA isolation from *E. coli* clones

Plasmid DNA was isolated from small scale (2 mL) bacterial cultures by treatment with alkali and SDS as described in Sambrook & Russell (2001) with some modifications:

A single colony of transformed bacteria was inoculated into 2 mL of LB broth containing the appropriate antibiotic and incubated overnight at 37 ºC, 250 rpm. To ensure that the culture was adequately aerated, the volume of the culture flasks was ten times greater than the volume of the bacterial culture and the flasks were loosely capped. Bacterial pellets of 1.5 mL of culture were collected after centrifugation at 12,000 rpm for 2 min, 4 ºC. Pellets were resuspended in 100 µL of ice-cold alkaline lysis solution I [50 mM

glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)] by vigorous vortexing. Then 200 µL of freshly prepared Alkaline lysis solution II [0.2 M NaOH, 1 % (w/v) SDS] was added to each bacterial suspension and mixed by inverting the tubes rapidly five times. The bacterial suspension tubes were then stored on ice for 2 min. 150 µL of ice-cold alkaline lysis solution III [5 M potassium acetate, 11.5 % (v/v) glacial acetic acid] was added and mixed by inverting the tubes several times and the tubes were stored on ice for 3 to 5 minutes. The bacterial lysates were centrifuged at 12,000 rpm for 5 min at 4ºC and the supernatants were transferred to a fresh tube. Two volumes of ethanol at room temperature were used to precipitate the nucleic acids. The precipitated nucleic acid was collected by centrifugation at 12,000 rpm for 5 min at 4 ºC. Then the supernatant was removed gently and 1 mL of 70 % (v/v) ethanol was added to the pellet. Next, all the ethanol was removed by evaporation by storing the open tube at room temperature (10 min). Finally, the nucleic DNA was dissolved in 50 µL of 55 ºC sterile pre-warm ultrapure water. The samples were analysed by electrophoresis.

5.2.9 Agarose Gel Electrophoresis

Qualitative analysis of plasmid DNA and PCR products was conducted by agarose gel electrophoresis.

Agarose (Sigma) was added to 100 mL of 1X TAE buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid, pH 8), to a concentration of 0.8 % (w/v) unless otherwise stated. The solution was heated in a microwave oven on medium power for 1 to 2 min until the solution was clear and the agarose was completely dissolved. The solution was allowed to cool into a water bath at 55 °C. Ethidium bromide (Sigma) was then added to the agarose to a final concentration of 0.5 µg/mL and mixed before pouring into the appropriate gel tray. The gel was then placed in the gel tank and 1X TAE buffer added until the gel was sufficiently submerged. 5 µl aliquots of DNA samples were mixed with 1 µL loading dye (0.25 % (w/v) bromophenol blue, 40 % (w/v) sucrose) before being added to the wells of the gel. Electrophoresis was then applied at 5 V/cm until sufficient migration was achieved. All DNA bands were viewed under UV illumination and photographed using a Syngene Gene Genius Bio-Imaging system.

5.2.10 PCR amplification

PCR primers used in this study are shown in Table 5.1. Each 50 µl reaction mixture contained 50 to 100 ng DNA, 1.25 units Taq polymerase (Promega), 1.25 mM $MgCl_2$, 0.2 mM each dNTP and 1 µM of each primer. For PCR products intended for expression, NEB Q5® Hot Start High-Fidelity DNA Polymerase was used.

Table 5.1 PCR primer names and sequences.

Primers	Sequence	Region amplified
T7 forward*	5′- TAATACGACTCACTATAGGG-3′	Flanks the inserts in the pGEM
T7 reverse*	5´- TAGTTATTGCTCAGCGGTGG-3´	Flanks the inserts in the pGEM
Nitfull f2**	5'-ATGCCTCTCGTTCATCCGGA-3'	*nit2* gene
Nitfull r**	5'-CATCTCTTCCCGGTGCGCC-3'	*nit2* gene

*(Sambrook *et al*., 1987); **(Coffey *et al*., 2009).

The PCR products were amplified using the following amplification parameters; 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 57 °C, and 1 min per kb at 72 °C, unless stated otherwise in Results section.

5.2.11 DNA sequence analysis

Softwares used for analysis of DNA sequence data were Sequence Scanner Software 2 (Applied Biosystems) and DNASTAR Lasergene Pro. Database searches were performed using NCBI BLAST tools (Altschul *et al.,* 1990). Phylogenetic trees were constructed using MEGA X (Kumar *et al.,* 2018) with a clustalW alignment of the sequenced nitrilase region.

5.2.12 Identification of significant variables by experimental design

The optimization of the soluble expression of recombinant *nit*2 in *E. coli* BL21(DE3)pLysS was done by design of experiment based statistical optimization by employing Plackett-Burman design (PBD), which is based on the first order model:

$$Y = \beta_0 + \sum \beta_1 X_i$$

where Y is the response (activity determined using Nessler's colorimetric assay), β_0 is the model intercept and β_1 is the linear coefficient, and X_i is the level of the independent variable. This model is used to screen and evaluate the significant factors that influence the response and does not describe interaction among factors.

Five variables were carefully selected and evaluated with eighteen experiments. These variables were: induction temperature at 16 and 30 ºC, IPTG (inducer) concentration at 0,05 and 1 mM, yeast extract concentration at 0,1 and 0,8 % (w/v), ethanol at 1 and 3 % (w/v) and sorbitol concentration at 0,5 and 1 % (w/v). The experiments were designed and analyzed by the screening of factors design with the software package "JMP® 10.0.0" (Copyright© 2012 SAS Institute Inc). Table 5.2 illustrates the factors under investigation and their levels employed in the experimental design. The practical levels were obtained from previous work in the laboratory. The response was the activity towards 4-hydroxyphenylacetonitrile determined using Nessler's colorimetric activity assay, which was conducted in triplicate to gain an average.

5.2.13 Medium engineering

After the identification of significant variables for product yield, the next step in medium engineering was to determine the optimum level of each key independent variable as identified by the screening of factors design. The Box-Behnken design as described by Box and Behnken (1960), which is a fraction of the full factorial, was used to evaluate the quadratic effects and two-way interactions among the screened variables. The optimum levels of the variables were obtained by graphical and numerical analysis using JMP® program. The final optimum experimental parameters were calculated using the Response Surface Design function, which allows for identifying the best combination of each component.

5.2.14 Protein production in shake flasks

1 ml of seed culture was inoculated in 25 mL Luria-Bertani (LB) media [1 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract and 1 % (w/v) NaCl, pH 7.5] in 250 mL shake flask containing 100 µg mL^{-1} of ampicillin. For the purpose of medium engineering, the production medium was prepared in different formula as illustrated in Table 5.2. In all cases, incubation was at 37 ºC, 250 rpm for 24 h. The recombinant nitrilase expression was induced by the addition of IPTG (0,05 or 1 mM) when optical density at 600_{nm} reached ~ 0.8 wherever applicable. Samples taken after overnight induction were analyzed for Optical Density (OD_{600nm}), Nessler's colorimetric ammonium assay and by SDS-PAGE. Control experiments were performed in parallel on the same media without any induction.

5.2.15 Determination of nitrilase activity

Induced recombinant cells were screened for activity towards 3HBN, 3HGN, 3HPPN, 4HPAN and 2PPN as described by Coady *et al*. (2013). Cell blanks contained cells at OD_{600nm} = 0.1 in phosphate buffer. Nitrile blanks contained 200 µL of 10 mM nitrile in phosphate buffer.

5.2.16 Polyacrylamide gel electrophoresis (PAGE)

Gel electrophoresis was performed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a Tris-glycine buffer system as described by (Laemmli, 1970). The gel was stained with Commassie brilliant blue (R-250) and destained by a mixture of methanol, acetic acid and deionized water (1:0,7:8,3, v/v/v).

15 mL of 12% separating gel was used per two gels (4.9 mL distilled water; 6 mL 30 % acrylamide mix; 3.8 mL 1.5 M Tris (pH 8.8); 0.15 mL 10 % (w/v) SDS (pH. 8.8); 0.15 mL 10 % (w/v) ammonium persulfate; 0.006 mL TEMED). 10 mL of stacking gel was prepared per two gels (6.8 mL distilled water; 1.7 mL 30 % acrylamide mix; 1.25 mL 1.0 M Tris (pH 6.8); 0.1 mL 10 % (w/v) SDS (pH. 8.8); 0.1 mL 10 % (w/v) ammonium persulfate and 0.01

mL TEMED). 500 mL of electrode buffer (6 g Tris base, 26.8 g glycine and 1 g SDS/L) was used per gel tank.

5 µl of size standard (SeeBlue™ Plus2 – Thermofisher) was applied per well, and 10 µl of samples were applied. Electrophoresis was performed at 200 Volts, 150 mA, until the blue band of the sample buffer reached the end of the gel.

After electrophoresis, the gels were incubated in a staining tray with staining solution (0.2 g Coomassie Brilliant Blue R250 dye in 50 % (v/v) methanol, 10 % (v/v) glacial acetic acid) with gentle agitation. After 30 minutes staining the gels were subjected to destaining overnight in destain solution (10 % (v/v) methanol, 7 % (v/v) glacial acetic acid) with several changes of destain solution.

Non-denaturing (native) PAGE was performed using the procedure used for SDS-PAGE with the following alterations: the samples were prepared using the NativePAGE™ Sample Buffer (4X) (ThermoFisher Scientific, Cat. No. BN2003) and the NativePAGE™ 5 % G-250 Sample Additive (ThermoFisher Scientific Cat. No. BN2004) without boiling. The samples were electrophoresed through premade 4-16 % Native PAGE Bis-Tris gels, following the manufacturer's specifications (ThermoFisher Scientific, Cat. No. BN1002BOX). In addition, the NativeMark™ Unstained Protein Standard (ThermoFisher Scientific Cat. No. LC0725) was used as a protein ladder.

5.2.17 Nit2 isolation

The *E. coli* clone containing the His-tagged recombinant Nit2 protein were harvested by centrifugation (3200 x g, 20 min, 4 ºC). B-PER Bacterial Protein Extraction (ThermoFisher Scientific, Cat. No. 89821) was used for extraction of soluble protein extract from recombinant *E. coli*, as per the manufacturer's instructions. After that, nickel affinity His-tag purification columns (HisPur Ni-NTA Purification Kit - Thermofisher Scientific, Cat. No. K95001) were used to purify Nit2 according to the manufacturers' protocol. The purified protein was then desalted with the Zeba™ Spin Desalting Columns, 30K MWCO (ThermoFisher Scientific, Cat. No. 87766), following the manufacturer's specifications. Finally, the purified desalted Nit2 was concentrated by ultrafiltration with the Corning®

Spin-X® UF concentrators (Sigma-Aldrich, Cat. No. CLS431485). In addition, concentration of the Nit2 protein was determined by UV measurement at 280 nm using the Nanodrop® ND-1000 spectrophotometer. All purification procedures were carried out at 4 °C.

5.2.18 Biotransformations with the Nit2 purified His-tagged recombinant enzyme

The standard nitrilase assay was conducted in a 200 µl reaction mix in microtiter plates containing sodium phosphate buffer (100 mM, pH 7.0), 10 mM nitrile (4-HPAN), final concentration of purified Nit2 enzyme at 0.015 mg per 200 ul, 30 °C for 2 hours. 50 µL of 250 mM HCl was added to quench the reaction and samples were centrifuged at 16,000 x g for 20 minutes to pellet the denatured protein. The production of the corresponding carboxylic acid and ammonia was determined using the Nessler's Ammonia Assay as in section 5.2.15.

5.2.18.1 Optimum Temperature and pH Determination

To characterise the substrate range of the enzyme, the optimum temperature and pH were first determined using 4HPAN as substrate. Biotransformations were carried out as per section 5.2.18.

To determine the optimum temperature for the recombinant Nit2 recombinant enzyme the biotransformation reaction was carried out as described in section 5.2.18 in the temperature range of 10 °C to 70 °C with 10 mM 4HPAN as the substrate.

A pH range of 4.0 to 9.0 was assessed at the following - 0.1 M sodium acetate buffer (4.0, 4.6, 5.0 and, 6.0), 0.1 M potassium phosphate buffer (6.0, 6.4, 7.0, 7.5 and, 8.0) and 0.1 M tris-HCl buffer (8.0, 8.5 and 9.0) with enzyme assays performed in triplicate. For the pH study, purified enzyme was not elution buffer exchanged into sterile distilled water, but subjected to buffer exchange into the appropriate test buffers listed above, using desalting columns (Zeba Spin Desalting Columns, 7k MWCO) as before.

5.3 Results and discussion

5.3.1 Sequence analysis of *nit2* gene

Bacterial nitrilases have great potential for the production of numerous industrially useful products, with advantages over traditional chemical methods (O'Reilly and Turner, 2003). This potential has resulted in the isolation of nitrilase genes from a range of microorganisms, such as *Burkholderia* sp. LC3, *R. erythropolis* AJ270 and AJ300 (Coffey, 2007), *Serratia* sp. SS10 and *Staphylococcus* sp. (Dooley-Cullinane *et al.,* 2018).

Coffey *et al.,* (2009) developed a real-time PCR TaqMan® assay that allowed nitrilase gene detection directly from soil enrichment cultures without DNA extraction. As a result, the *nit2* gene from a number of *Burkholderia* sp. was isolated by using this assay.

A study published by Robertson *et al*. (2004) identified 137 unique nitrilase sequences from uncultured organisms. These nitrilases were identified and grouped into clades based on their DNA sequence homologies and substrate analysis. In addition, 37 of 137 nitrilases are members of the 2A clade.

The phylogenetic trees constructed from the *nit*2 gene sequences displayed high sequence homology to 7 bacterial strains from the database (Figure 5.4). The *nit2* gene sequence similarities between the seven type strains were 72.55-99.72 % (mean 94,61 %). *R. erythropolis* AJ270 among the other strains showed more than 98 % gene sequence similarity to *nit*2 from *Burkholderia* sp. LC3, indicating that these two nitrilases are closely related. On the other hand, the uncultured organism clone 2A25 nitrilase gene (Robertson *et al.*, 2004) has shown the lowest sequence similarity, 72.55 %. This sequence was used to root the phylogenetic tree, and the nitrilase sequence similarity score of 72.55 % is typical of the nitrilase gene sequences within the 2A clade.

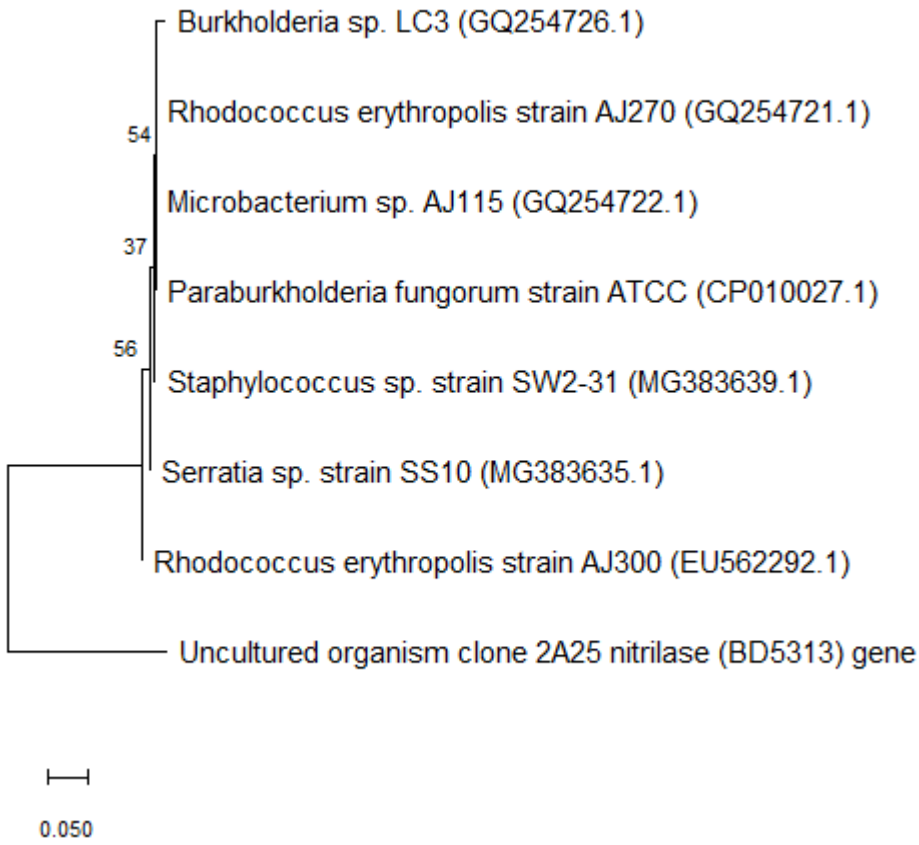

Fig 5.4 Phylogenetic tree of 7 bacterial strains based on *nit*2 gene sequences. The trees were constructed with the neighbour-joining method. Genetic distances were computed by Kimura's two-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The uncultured organism clone 2A25 nitrilase (BD5313) gene was use to root the phylogenetic tree. There were a total of 2579 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Bars, 0.05 substitutions per nucleotide position. Accession numbers of the gene sequences are listed.

The *nit*2 translated amino acid sequence similarity values were 61.46-100 % (mean 85.78 %) from the 7 bacterial strains from the database (Figure 5.4). In addition, the complete *nit*2 gene encodes a protein of 339 amino acids and showed homology of 100 % to predicted nitrilases from *Paraburkholderia fungorum* ATCC BAA-463 (CP010027.1), as demonstrated in Figure 5.5.

Score	Expect	Method	Identities	Positives	Gaps	Frame
700 bits(1807)	0.0	Compositional matrix adjust.	339/339(100%)	339/339(100%)	0/339(0%)	+3

```
Query  1        MPLVHPEYKVAVVQAAPVWLDLDAGIDKTIELINEAAGNGARLISFPETWLPGYPWHIWM  60
                MPLVHPEYKVAVVQAAPVWLDLDAGIDKTIELINEAAGNGARLISFPETWLPGYPWHIWM
Sbjct  2339898  MPLVHPEYKVAVVQAAPVWLDLDAGIDKTIELINEAAGNGARLISFPETWLPGYPWHIWM  2340077

Query  61       GAHAWQVSRGFVQRYFDNSLDYDSPEALRLSTAVQQAGITAVVGVSERSGSSLYMGQWII  120
                GAHAWQVSRGFVQRYFDNSLDYDSPEALRLSTAVQQAGITAVVGVSERSGSSLYMGQWII
Sbjct  2340078  GAHAWQVSRGFVQRYFDNSLDYDSPEALRLSTAVQQAGITAVVGVSERSGSSLYMGQWII  2340257

Query  121      GADGRTIAARRKLRPTHVERAVFGEGDGSDLAVHQVEGLGRIGALCCWEHLQPLSKYAMY  180
                GADGRTIAARRKLRPTHVERAVFGEGDGSDLAVHQVEGLGRIGALCCWEHLQPLSKYAMY
Sbjct  2340258  GADGRTIAARRKLRPTHVERAVFGEGDGSDLAVHQVEGLGRIGALCCWEHLQPLSKYAMY  2340437

Query  181      AQNEQVHVAAWPSFSNYEPFAPALGSEVNNAASRIYAVEGSCFVLAPCAVVSKAMIDELC  240
                AQNEQVHVAAWPSFSNYEPFAPALGSEVNNAASRIYAVEGSCFVLAPCAVVSKAMIDELC
Sbjct  2340438  AQNEQVHVAAWPSFSNYEPFAPALGSEVNNAASRIYAVEGSCFVLAPCAVVSKAMIDELC  2340617

Query  241      DTPEKRELNHVGGGHAVIYGPDGSALVPKLPEDSEGILYADIDLATIVMAKNAADPAGHY  300
                DTPEKRELNHVGGGHAVIYGPDGSALVPKLPEDSEGILYADIDLATIVMAKNAADPAGHY
Sbjct  2340618  DTPEKRELNHVGGGHAVIYGPDGSALVPKLPEDSEGILYADIDLATIVMAKNAADPAGHY  2340797

Query  301      SRPDVTRLFINKTRMQRVEAFALPIDRSENDDLAHREEM  339
                SRPDVTRLFINKTRMQRVEAFALPIDRSENDDLAHREEM
Sbjct  2340798  SRPDVTRLFINKTRMQRVEAFALPIDRSENDDLAHREEM  2340914
```

Fig. 5.5 Alignment of regions of nitrilases from *Burkholderia sp. LC3* (query) (GQ254726.1) and *P. fungorum* ATCC BAA-463 (sbjct) (CP010027.1) (Program version: TBLASTN 2.9.0+).

5.3.2 The construction of recombinant vector

The gene encoding Nit2 (*nit*2) from *Burkholderia* sp (Coffey *et al.,* 2009) was inserted into the pEXP5-CT/TOPO expression vector, which allowed expression of recombinant protein fused to a C-terminal peptide containing a polyhistidine (6xHis) tag. As shown in Figure 5.6, NITFULL PCR product (~1,020 bp) and DNA sequencing indicated that the gene of *nit*2 was successfully obtained by PCR and transferred into a new vector of pEXP5-CT/TOPO-NIT2.

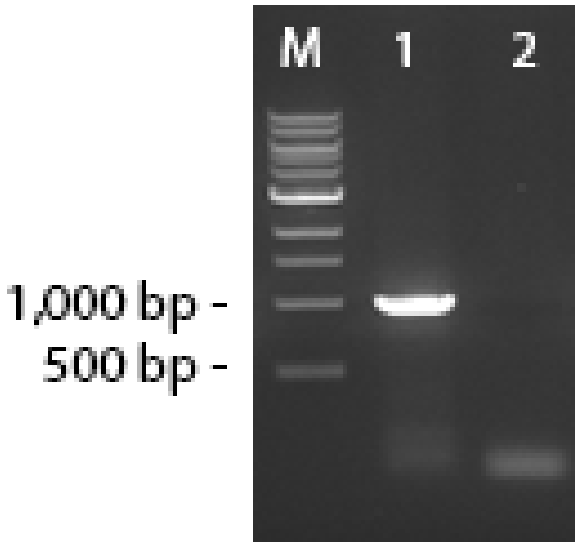

Fig. 5.6 Agarose gel electrophoresis [0.9 % (w/v)] of the PCR product from recombinant vector pEXP5-CT/TOPO-NIT2. M: CSL-MDNA-1KB marker. Lane 1: *nit2* was detected using primers to amplify a ~1,020-bp amplicon. Lane 2: negative control.

5.3.3 Screening of important media components for nitrilase activity by recombinant *E. coli*

The importance of the five variables, namely, induction temperature, IPTG (inducer), yeast extract, ethanol (EtOH) and sorbitol concentrations for nitrilase activity within the whole cell was investigated by Plackett-Burman design. PBD is widely used as a powerful tool for identifying factors with significant influence on protein's production (Souza, Flôres and Ayub, 2006; Ghasemi *et al.,* 2011; Pareek, Singh and Ghosh, 2011; Kumar and Baldi, 2013; Papaneophytou and Kontopidis, 2014). Table 5.2 represents the Plackett-Burman experimental design for 18 trials with two levels of concentrations for each variable and shows the effects of these components on the response and significant levels. Activity assay towards 4-hydroxyphenylacetonitrile was determined via the Nessler's colorimetric assay as detailed in Section 5.2.15, after 24 h of induction, following the Plackett–Burman design. The biotransformations were carried out in 200 µL format containing potassium phosphate buffer pH 7, a final cell OD_{600nm} of 0.1 and a final substrate concentration of 10 mM. The biotransformation was carried out over a 24 h and expressed in terms of concentration (mmol/L) of NH_3 produced by using whole cells, as shown in Table 5.2.

Activities varied according to the medium conditions, from 0.891 to 5.28 mmol/L. The comparisons of nitrilase activity in different media, shows that the medium in trial 6 gave the highest enzyme activity, followed by medium in trial 17 and 18. The best result was achieved with LB medium supplemented with yeast extract at a concentration of 0.8 % (w/v), ethanol concentration of 3 % (w/v), sorbitol concentration of 1 M, IPTG concentration of 0.05 mM at temperature of 16 ºC. The variation of these results highlights the importance of medium engineering in order to obtain higher efficiency.

Table 5.2 The Placket-Burman experimental design matrix.

Run	Pattern	Yeast extract (%)	EtOH (%)	Sorbitol concentration (M)	IPTG concentration (mM)	Temperature (°C)	Activity (mmol/L)
1	0−−++−	0.1	1	1	1	16	3.44
2	0−+−+−	0.1	3	0.5	1	16	2.27
3	0++−+−	0.8	3	0.5	1	16	4.33
4	−−++−−	0.1	3	1	0.05	16	4.01
5	−+−++−	0.8	1	1	1	16	3.06
6	−+++−−	0.8	3	1	0.05	16	5.28
7	+−−−−−	0.1	1	0.5	0.05	16	0.987
8	+−−−−−	0.1	1	0.5	0.05	16	0.891
9	++−−−−	0.8	1	0.5	0.05	16	3.785
10	0−−+−+	0.1	1	1	0.05	30	1.2
11	0+−+−+	0.8	1	1	0.05	30	3.03
12	0++−−+	0.8	3	0.5	0.05	30	3.44
13	−−−−++	0.1	1	0.5	1	30	1.01
14	−−+−−+	0.1	3	0.5	0.05	30	4.11
15	−+−−++	0.8	1	0.5	1	30	2.78
16	+−++++	0.1	3	1	1	30	3.75
17	++++++	0.8	3	1	1	30	4.89
18	++++++	0.8	3	1	1	30	4.92

Increasing the cellular concentration of osmolytes or chaperones, which are essential for proper folding or for protein stability, can prevent the formation of inclusion bodies and/or soluble aggregates as demonstrated by Arsène *et al*. (2000) and Lund (2001). In this study, the strategy for improving the level of recombinant protein expression was by growing and inducing the strains in the presence of sorbitol and ethanol. Blackwell and Horgan (1991) have shown that cells growing during induction period in the presence of sorbitol produced >400-fold higher levels of recombinant protein when compared to the control cultures. Sorbitol supplementation increases the osmotic pressure and consequently, leads to the accumulation of osmoprotectants (e.g., glycine betaine, threholose) in the cell, which stabilize the native recombinant protein structure (Georgiou and Valax, 1996). Additionality, it was revealed in this study that adding ethanol to the medium has the most influential effect on nitrilase activity. This effect may be due to enhanced solubility of recombinant proteins in *E. coli* by inducing the expression of heatshock proteins, as reported by other researchers Chhetri *et al*. (2019). Exposing the cells to a heat shock stress will lead them to produce chaperones and consequently will prevent aggregation and increase the refolding of proteins as reported by Papaneophytou and Kontopidis (2014). On the other hand, acetate is known as an inhibitor of biomass and recombinant protein production (Kleman and Strohl, 1994; De Mey *et al.,* 2007). Therefore, supplementing the medium with yeast extract as a supplement of essential micronutrients, growth factors and amino acids can similarly reduce acetate formation and enhance recombinant protein production by *E. coli* (Leone *et al.,* 2015; Xu *et al.,* 2015). For this reason, in this work, we chose different concentrations of yeast extract in order to limit acetate accumulation.

Based on the statistical analysis, R^2 was found to be 0.938, which means that model could explain 93.8 % of the total variations in the system. The main effects of the screened factors on the nitrilase activity were calculated and presented in the Pareto chart (Figure 5.4). All variables had positive effect on nitrilase activity except temperature of induction which contributed negatively. Thus, increasing the positive variables concentration and decreasing temperature of induction should result in a higher activity of Nit2.

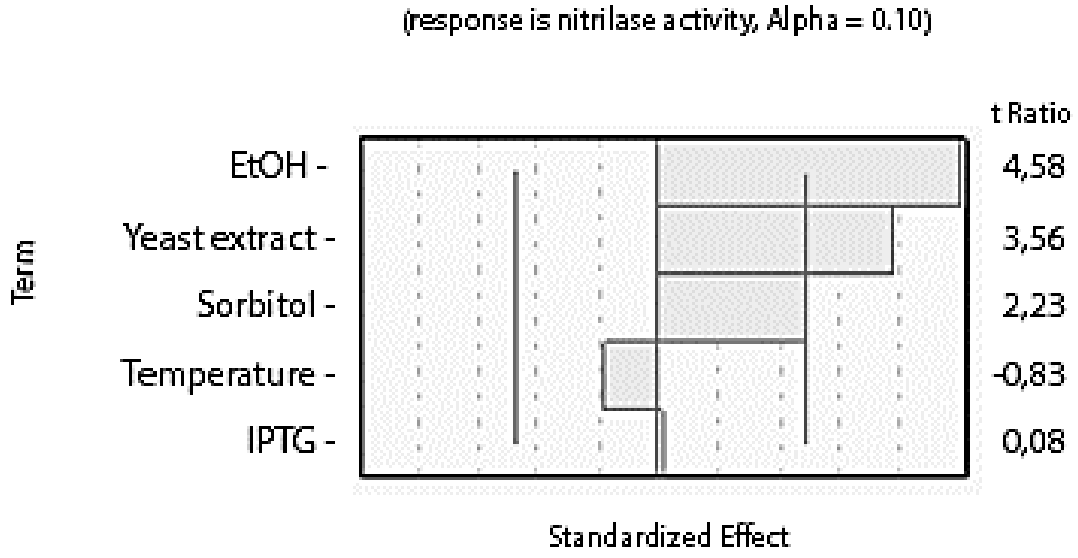

Fig. 5.7 Pareto chart rationalizing the effect of each variable on the nitrilase activity (mmol/L) produced by recombinant *E. coli* BL21(DE3)pLysS.

5.3.4 Medium optimization of screened components for nitrilase activity

The data shown in Table 5.2 was analyzed using JMP® software. The *t*-test and *P*-values were used to identify the effect of each factor on nitrilase activity as shown in Table 5.3. A *P*-value of less than 0.05 indicates that the model terms are significant. The regression analyses data of Plackett-Burman design (PBD) revealed that yeast extract concentration, ethanol concentration and sorbitol concentration were the critical factors that could stimulate the expression of soluble recombinant nitrilase, which affected positively the nitrilase activity within the recombinant whole cells ($P < 0.05$).

Table 5.3 Statistical analysis of Plackett–Burman design for each variable on nitrilase activity.

Variables	Effect	Coefficients	*t*-value	*P*-value
Yeast extract (%)	1.294	0.647	3.56	0.0039*
EtOH (%)	1.664	0.832	4.58	0.0006*
Sorbitol concentration (M)	0.81	0.405	2.23	0.0457*
IPTG concentration (mM)	0.026	0.013	0.08	0.9404
Temperature (°C)	-0.302	-0.151	-0.83	0.4221

*P-value of less than 0.05.

Thus, further experiments with these three variables showing *P*-value below 0.05 in the PBD were investigated using Box-Behnken design (Table 5.4). The response surface plots and their corresponding contour plots were obtained when the data of nitrilase activity were incorporated and analyzed by JMP® software – Response Surface Design (Fig. 5.8). Figure 5.8 (A-C) was based on the final model of response surfaces, which represents the effect of two variables constant at their studied concentration range and at fixed concentration of the third variable.

Table 5.4 Experimental values of nitrilase activity recorded in Box-Behnken design.

Std ID	Pattern	EtOH (%)	Yeast extract (%)	Sorbitol concentration (M)	Activity[a] (mmol/L)
1	000	2	0.45	0.75	3.66
2	00-	3	0.8	1	4.71
3	0−−	2	0.1	0.5	3.11
4	−0−	1	0.45	0.5	2.01
5	+0+	3	0.45	1	3.11
6	−0+	1	0.45	1	1.47
7	+−0	3	0.1	0.75	2.12
8	0+−	2	0.8	0.5	3.88
9	0++	2	0.8	1	2.94
10	0−+	2	0.1	1	3.28
11	−+0	1	0.8	0.75	2.3
12	+0−	3	0.45	0.5	4.27
13	0-0	2	0.8	0.75	3.35
14	−−0	1	0.1	0.75	2.02
15	++0	3	0.8	0.75	5.16

[a]Activity assay towards 4-hydroxyphenylacetonitrile determined using Nessler's colorimetric activity (24 h) assay expressed in terms of concentration (mmol/L) of NH3 produced by using whole cells.

As we can see in the Figure 5.8 (C), the maximum response for nitrilase activity >5.39 <6 mml/L) occurred when ethanol was used at a concentration of 3 % (w/v) and yeast extract and sorbitol were used at a concentration of 0.8 % (x/v) and 0.5 M, respectively. On the other hand, on either side of the optima, the nitrilase activity decreased considerably when less ethanol is added. This suggested that the ethanol concentration in the medium had a very significant effect on the nitrilase activity.

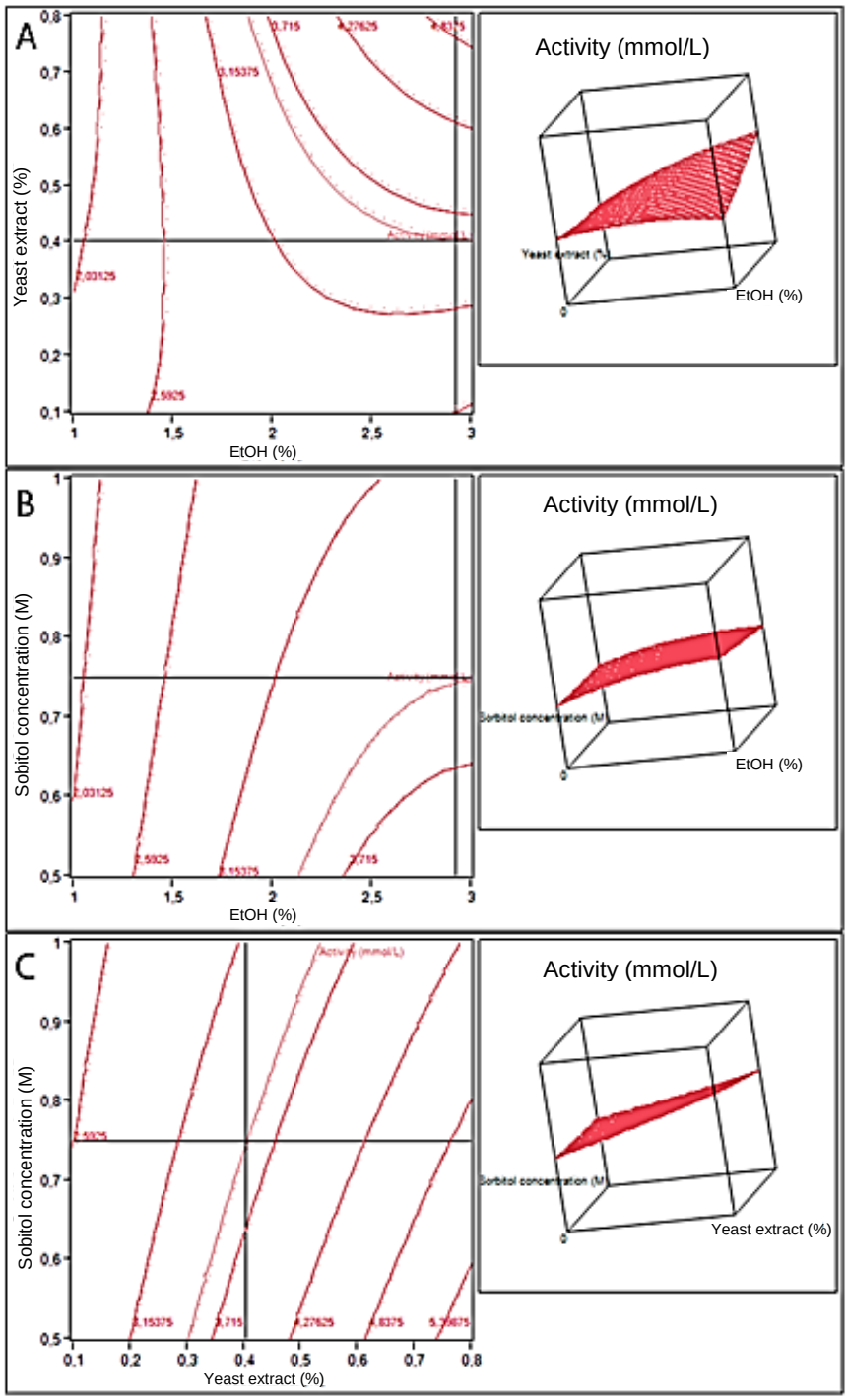

Fig. 5.8 Response surface (3D) and contour (2D) profiler of the combined effects of three selected variables. (A) shows the effect of yeast extract and ethanol concentrations on nitrilase activity (sorbitol concentration: 0,5 M). (B) shows the effect of sorbitol and ethanol concentrations on nitrilase activity [yeast extract concentration: 0,8 % (w/v)]. (C) shows the effect of sorbitol and yeast extract concentrations on nitrilase activity [ethanol concentration: 3 % (w/v)].

As clear from Figure 5.9, which shows the interaction profiles between the positive variables for the nitrilase activity response, the highest activity was encountered when highest concentration of ethanol and yeast extract were used against lowest concentration of sorbitol.

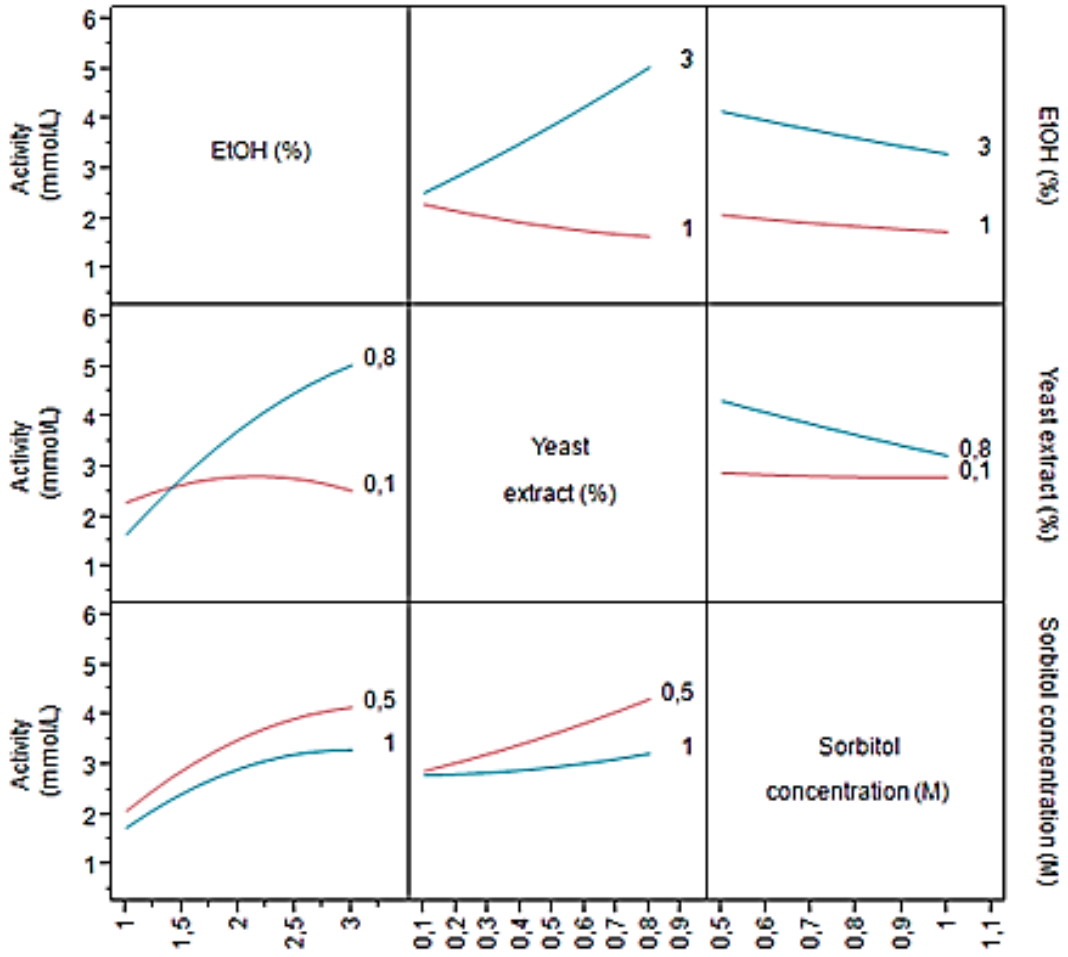

Fig. 5.9 Interaction profiles for the nitrilase activity response.

Thus, the software used have predicted the activity of nitrilase within the studied range of all three variable components (Table 5.5) as shown for each factor on figure 5.9, with 93.4 % confidence interval on the mean response.

Table 5.5 Predicted vs. experimental values for maximum nitrilase activity.

Variables	Values	Experimental activity[a] (mmol/L)	Predicted activity[a] (mmol/L)
Yeast extract (%)	0.8		
EtOH (%)	3		
Sorbitol concentration (M)	0.5	5.541	5.757
IPTG concentration (mM)	0.05		
Temperature (°C)	17		

[a]Activity assay towards 4-hydroxyphenylacetonitrile determined using Nessler's colorimetric activity (24 h) assay expressed in terms of concentration (mmol/L) of NH_3 produced by using whole cells.

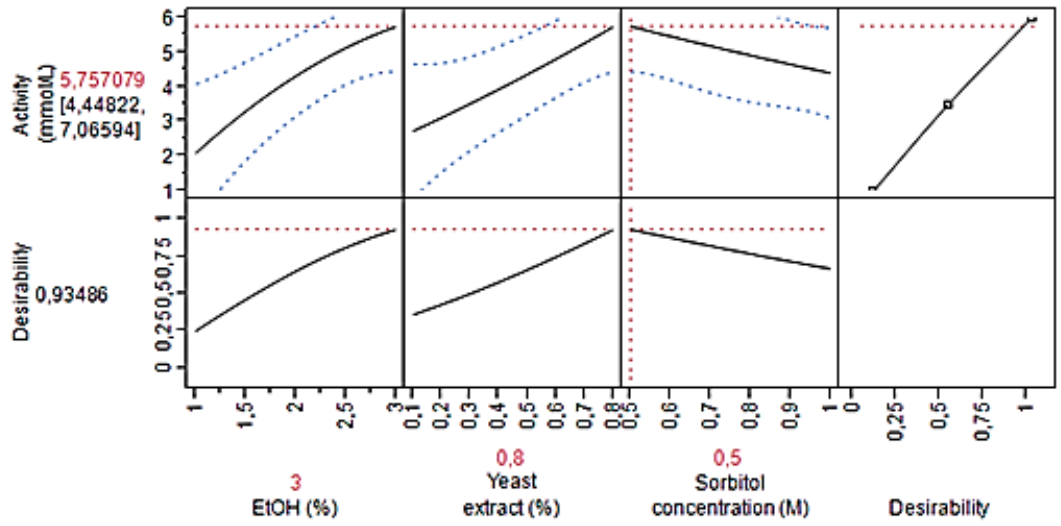

Fig. 5.10 Correlation between the actual and the predicted values (black lines) for the nitrilase activity experiment. The vertical red lines correspond to the current value of the factors. The current value of each factor is also shown in red below the horizontal axis. The red value on the vertical axis is the predicted response based on the current values of the factors. A 95 % confidence interval for the mean effect is plotted with a blue line about the predicted values.

A maximum nitrilase activity yield (5.75 mmol/L) was predicted at the condition of 0.8 % (w/v) of yeast extract, 0.5 M of sorbitol and 3 % (w/v) of ethanol added before induction at 16 ºC overnight, induced by 0.05 mM of IPTG concentration (Table 5.5). To verify the predicted results, a group of experiments were carried out under the optimized levels, demonstrating an activity of 5.541 mmol/L, very close to the prediction of the model (96.2

%), suggesting that there was an excellent agreement between the predicted and experimental data, thus validating the model.

Most authors had studied the optimization of nitrilase production by monitoring the influence of one factor at a time on the experimental response while only one parameter is changed (Banerjee, Kaul and U C Banerjee, 2006; Naik *et al.,* 2008; Liu *et al.,* 2011; Nageshwar *et al.,* 2011; Sohoni *et al.,* 2015), but few studies have utilised statistical approaches to maximize recombinant protein expression. The major disadvantage of this "one-factor-at-a-time" approach is that it does not include the interactive effects among the variables studied. Dubey *et al.* (2011) applied statistical design of experiments to analyze the effect of different concentration of medium components for the expression of *Pseudomonas putida* nitrilase by recombinant *E. coli* cells, including lactose, tryptone and fructose. In the optimized medium, *E. coli* produced nitrilase with activity by 2.2 times higher than that produced in un-optimized medium. Also using trough statistical designs, Kameswaran *et al*. (2014), observed a signifcant effect of yeast extract followed by a feed of fructose and ε-Caprolactam on nitrilase production, resulting in a 16 fold increase in nitrilase productivity when compared to unoptimized conditions. These researchers studied the effect of carbon and nitrogen source, growth factors, metals ions, inducers, pH, temperature, agitation, aeration. However, to date, no systematic studies had been done by using ethanol and/or sorbitol on the medium before induction of nitrilase activity, which has been shown to have a significant positive effect in this study. It seems likely that further improvement in yield should be possible through further variation of ethanol and sorbitol concentrations. Additionally, it would be beneficial to perform omics-based analysis which could lead to a further improved quantitative model and better understanding about the fundamentals of environmental stress on metabolic activities of host cells, which could facilitate the improvement of recombinant protein expression.

5.3.5 Nitrilase enzyme assay using Nessler's colorimetric assay and protein analysis

In 96 well microtitre plates (Sarstedt Ltd) the recombinant *E. coli* expressing *nit*2 were first subjected to nitrilase activity studies with the nitriles of interest (towards 3HBN, 3HGN, 3HPPN, 4HPAN and 2PPN). Nitrile hydrolyzing activity was calculated by

measuring the absorbance of the sample at 425 nm, and relating the absorbance to the standard curve in order to determine the concentration of NH_3 in the sample (Table 5.6).

Table 5.6 Activity screening towards nitriles of interest.

Nitriles	Activity[a] (mmol/L)		
	No optimization (whole cells)	Optimized medium (whole cells)[b]	Nitrilase purified
4-hydroxyphenylacetonitrile (4HPAN)	3.33	5.54	5.29
2-phenylpropionitrile (2PPN)	0.97	-	-
3-hydroxyglutaronitrile (3HGN)	0.50	-	-
3-hydroxybutyronitrile (3HBN)	0.42	-	-
3-hydroxy-3-phenylpropionitrile (3HPPN)	0.76	-	-

[a]Activity assay towards 4-hydroxyphenylacetonitrile determined using Nessler's colorimetric activity (24 h) assay expressed in terms of concentration (mmol/L) of NH_3 produced by using whole cells.

The recombinant strain demonstrated highest activity towards 4-hydroxyphenylacetonitrile substrate. In this case, 4HPAN was chosen for further medium engineering. As demonstrated in this table, under the optimized conditions, nitrilase activity using whole cells of the recombinant *E. coli* and after purification procedures of the recombinant protein were increased by 1.66- and 1.58-fold, respectively. These results therefore supported the predicted values and the effectiveness of the model, indicating that the engineered medium favors the activity of Nit2.

SDS–PAGE was used to analyze the recombinant Nit2 as shown in Figure 5.11. The His-tag fused on the C-terminal was also used to successfully isolate the protein (Figure 5.11 lane 1). Recombinant Nit2 shows increased expression in the optimized medium in comparison to normal condition without optimization, as shown. There is no report available in the literature where ethanol and sorbitol were used to enhance the expression and activity of recombinant nitrilase. Increased expression and activity of the recombinant nitrilase under ethanol and sorbitol treatment is a potentially highly beneficial route to be taken with other nitrilases or indeed other enzymes in general.

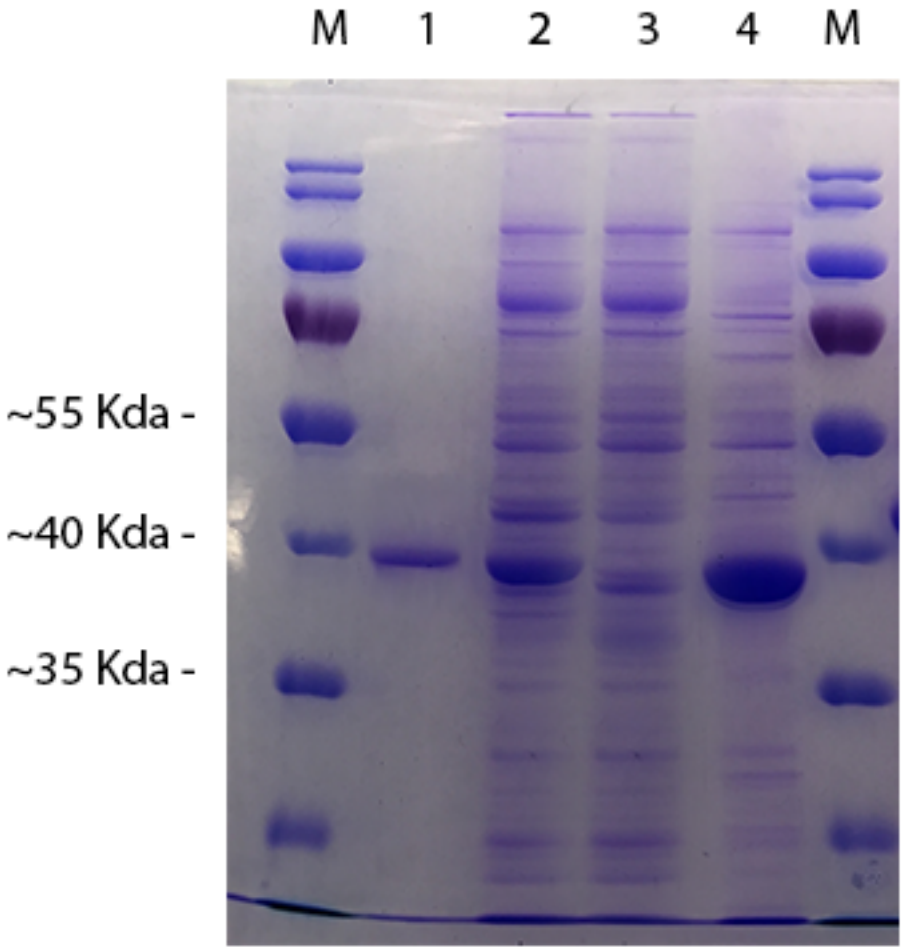

Fig. 5.11 Coomassie blue-stained 12 % (w/v) SDS-polyacrylamide gel for the analysis of Nitrilase 2 expressed in *E. coli* BL21(DE3)pLysS. All samples were induced with 0,05 mM IPTG onvernight at 16 ºC, wherever applicable. Lane 1, His-tag purified Nit2. Lane 2, Nit2 induced with 0,05 mM IPTG onvernight at 16 ºC in non-optimized medium. Lane 3, un-induced control. Lane 4, induced in optimized medium: 0,8 % (w/v) of yeast extract, 0,5 M of sorbitol and 3 % (w/v) of ethanol added before induction at 16 ºC overnight, induced by 0,05 mM IPTG. M: molecular weight markers (NEB).

The purifed Nit2 was also analysed using Native-PAGE gel electrophoresis (Figure 5.12). This technique is used for studying the composition and structure of native proteins, since both the conformation and biological activity of proteins remain intact during this technique (Davis, 1964).

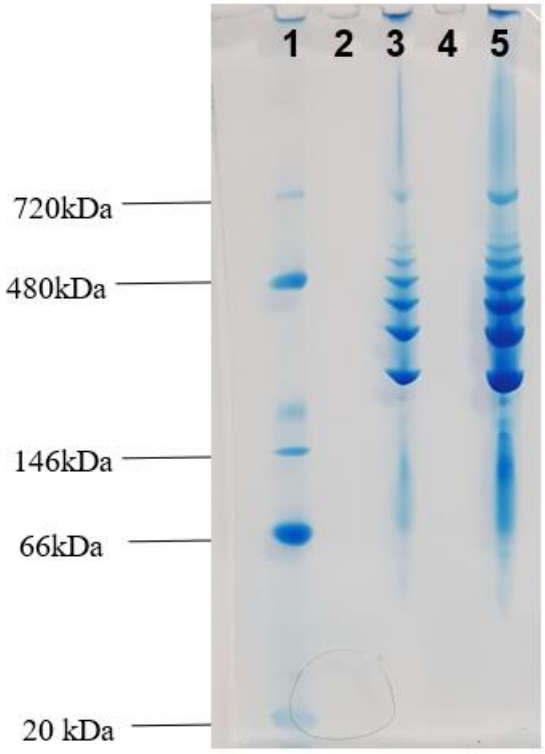

Fig. 5.12 Native-PAGE™ gel analysis of the purified Nit2 post staining with InstantBlue®. Lane 1, NativeMark™ Unstained Protein Standard. Lane 2, N/A. Lane 3, Purified Nit2 enzyme (5 µl). Lane 4, N/A. Lane 5, Purified Nit2 enzyme (10 µl).

Four protein bands within the range of 156 kDa to 440 kDa, assumed to be subunits, were detected as shown in lanes 3 and 5 (Fig. 5.12). These results suggest that 4 subunits within the range approximately of 156 kDa, 264 kDa, 352 kDa and 440 kDa are composed of multiples of dimers, with each monomer measuring approximately 44 kDa each including the His-tag. It is also thought that the largest band, ~704 kDa, is 16 subunits. In addition, 3 faint subunit bands evident within the proposed 480 kDa and 704 kDa range on the Nativemark™ protein ladder were found, as demonstrated in the figure. Thus, it is proposed that these 3 faint bands once formed one of the larger aggregated forms of the Nit2 enzyme, however, through purification steps or inability to self-associate it may have lost one of the 44 kDa subunits from the larger dimer units within the cell during induction. Indeed, it is unknown if this band-laddering of oligomers is present in the cell pre-lysis. If so, this would be due to a lack of formation and aggregation. It also could be explained that the “laddering” is due to the breakdown of the larger intact form

of the enzyme during the his-tag purification process. In fact, whole cell-lysates could be used for assessment to analyse the cellular contents pre-his-tag purification; even so, this may still lead to the “laddering” effect.

It is not elucidated if each band of the laddered oligomers native enzyme is active, or if perhaps only one band is the active subunit. In fact, the nitrilases are generally known to exist in solution as inactive dimers. Its subunit size varies between 30 and 45 kDa (Banerjee *et al*. 2002; O’Reilly and Turner, 2003), which self associate to form active oligomers (Park and Horton, 2019). Notably, Goldlust and Bohak (1989) demonstrated that the nitrilase of *Fusarium oxysporum* f.sp. *melonis* shows many bands with native-PAGE with sizes from 170 to 880 kDa. In like manner, activity assays have shown nitrilase enzymes to be active with 4 to 22 subunits with addition of subunits always taking place in pairs. In this way, each of the bands could be assessed for activity in order to evaluate if the Nit2 enzyme is active in each of the subunit formations.

5.3.6 Temperature optimization

The effects of temperature on purified His-tagged Nit2 activity were investigated at varying temperatures (0-70 ºC) with 4HPAN as a substrate. The optimum temperature of the Nit2 purified protein was determined. Reactions were carried out for 1 hour in 100 mM potassium phosphate buffer at pH 7.0 with 10 mM 4-hydroxyphenylacetonitrile as the substrate. Nitrilase activity at different temperatures was expressed as a relative activity with the highest value in each experiment. The results are shown in Figure 5.13 and Appendix IV. Nit2 activity increased when the reaction temperature ranges from 10 to 45 ºC and reached its optimal temperature at 45 ºC, after which the nitrilase activity sharply decreased, which is probably due to the partial inactivation of the enzyme at higher temperatures. This data agrees well with other reported nitrilases, which have temperature optima from 40 to 55 °C, as typically seen from mesophilic bacterial species (Layh *et al.,* 1998; Kiziak *et al.,* 2005; Banerjee *et al.,* 2006; Pawar *et al.,* 2012; Duca *et al.,* 2014). On the other hand, data from Cabrera and Blamey (2017) show that the thermostable nitrilase from an Antarctic *Pyrococcus* sp was active in the range of 50 to 100 °C reaching its maximum at 90 °C using 2-cyanopyridine as substrate, which exhibits high thermostability after 8 h of incubation at 90 °C. The temperature optimum of this nitrilase is higher than most nitrilases described so far (Shen *et al.*, 2021).

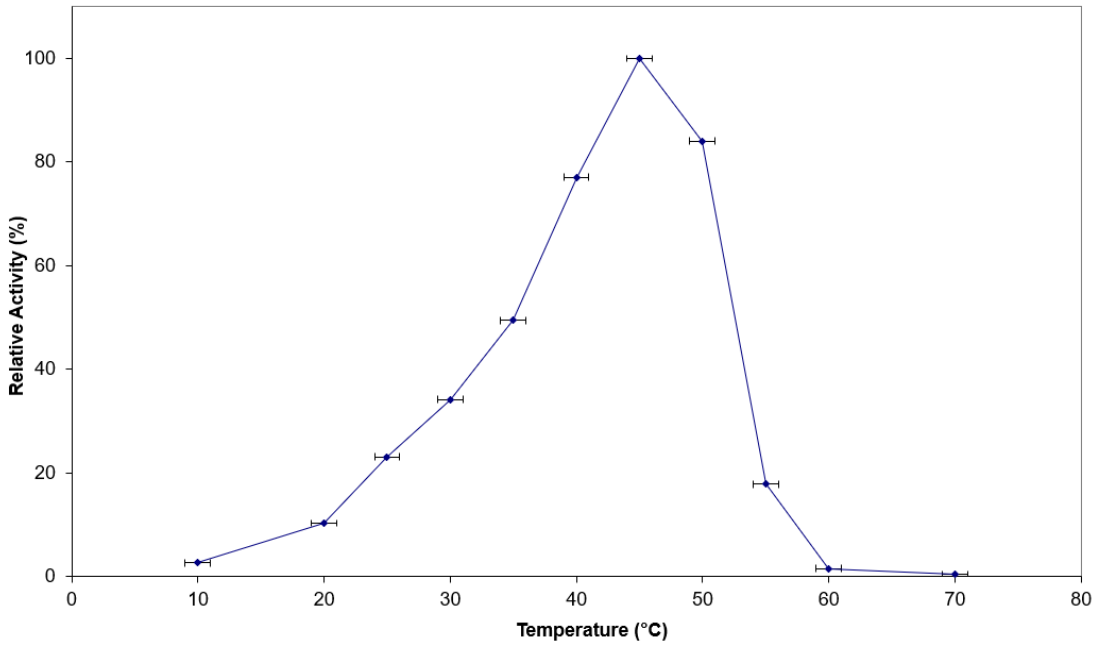

Fig. 5.13 Effects of temperature on the Nit2 purified His-tagged recombinant enzyme activity. Reactions were run for 1 hour in sodium phosphate buffer (100 mM, pH 7.0) at a range of 10 to 70 °C. Activity was determined using the Nesslers microscale colorimetric assay. The highest enzyme activity at 45 ºC was defined as 100 %. Assays were performed in triplicate.

5.3.7 pH optimization

The optimum buffer conditions of the Nit2 purified protein were determined. Nit2 enzymatic activity was measured at various pH conditions in phosphate buffer (100 mM, pH 7.0) with 10 mM 4-hydroxyphenylacetonitrile as the substrate. The results are shown in Figure 5.14 and Appendix IV. The Nit2 had an optimum activity in potassium phosphate buffer at pH 7.5 and retained greater than 80 % activity between pH 6.5 and 7.5. Additionally, the optimal pH of Nit2 is in agreement with that reported for most nitrilases, whose pH is in the range from 7 to 8 (Sunder *et al.*, 2020). For instance, *R. rhodochrous* J1 and *R. rhodochrous* K22 have been found to produce the nitrilase enzyme mostly at neutral pH, but the maximum activity with respect to the specified substrate was observed to be in the pH range 7.0 to 7.5 (Chhiba-Govindjee *et al.*, 2019).

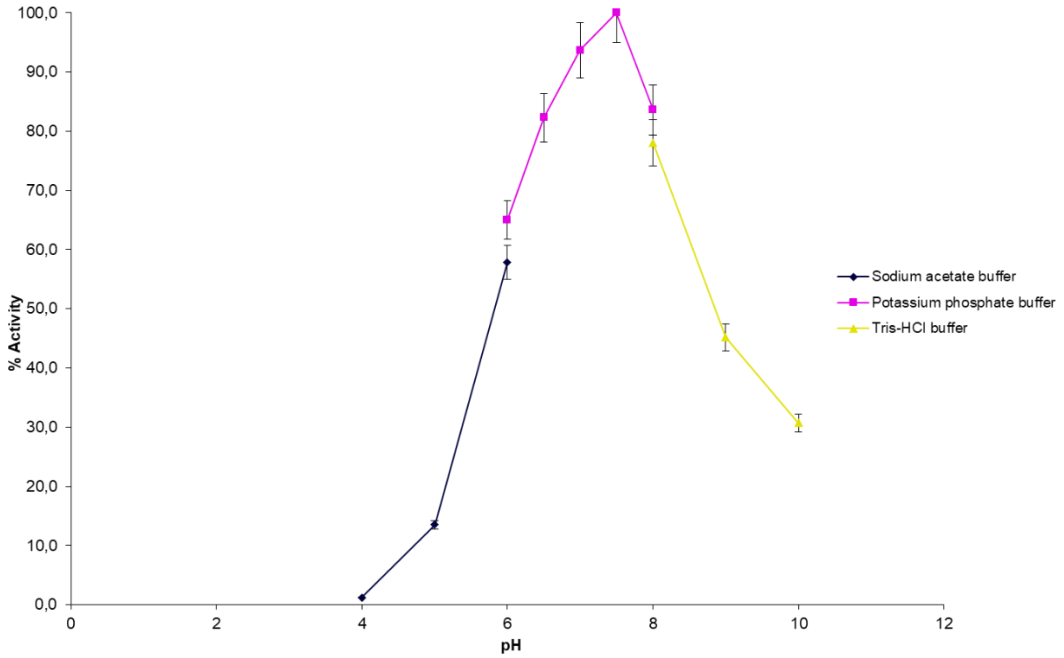

Fig. 5.14 Effect of pH on Nit2 activity. Purified nitrilase was incubated with 100 mM of: (1) sodium acetate buffer (pH 4.0, 5.0 and, 6.0); (2) potassium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5 and 8.0); (3) tris-HCl buffer (pH 8.0, 9.0 and 10.0). Reactions were run for 1 hour at 30 °C with enzyme assays performed in triplicate. Activity was determined using the Nesslers microscale colorimetric assay. The highest enzyme activity at pH 7.5 was defined as 100 %.

In addition, the pH optima of the thermophilic Nit2 is slightly lower than that of other nitrilases, which display maximum activity at pH between 8 and 9 (Layh *et al.,* 1998; Kiziak *et al.,* 2005; Pawar *et al.,* 2012). Rather, it is similar to that of *Alcaligenes faecalis* ATCC 8750 (pH 7.5) (Yamamoto *et al.,* 1992b), *Arthrobacter* sp. J-1 (pH 7.5) (Bandyopadhyay *et al.,* 1986) and *Pseudomonas fluorescens* Pf-5 (pH 7.0) (Kim *et al.,* 2009).

Nit2 activity decreased rapidly as the pH was reduced from the optimum. When the pH was at or below 5, the activity of Nit2 decreased sharply and at pH 4.0 the activity was 83-fold less than that at pH 7.5. A similar profile is observed with biotransformations carried out under alkaline conditions where activity is lost at higher pH values. However, the drop in activity is not as sharp under basic conditions. The enzyme displayed a surprising amount of activity with 45 % relative activity at pH 9 and 30 % relative activity at pH 10.

5.4 Conclusion

In summary, a novel approach to improve the activity of recombinant nitrilase enzyme was developed using whole cells of *E. coli* BL21(DE3)pLysS. This study proved that statistical experimental designs offer an efficient and feasible approach for Nitrilase fermentation medium optimization and explored some factors that affect the solubility expression of recombinant nitrilase in *E. coli*. The conditions for nitrilase activity by *E. coli* were optimized by two-stage of the statistical experimental designs, named Plackett-Burman and Box-Behnken. These experimental methodologies revealed an increase in nitrilase activity by medium engineering. To this author's knowledge, this is the first study concerning the use of ethanol and sorbitol before induction time in order to enhance the activity and expression of nitrilase. The Nit2 produced has potential applications for biotechnology and pharmaceutical industries towards the production of pharmaceutical intermediates using biotransformations. By using this strategy, other recombinant nitrilase enzymes could also be produced. Further studies of the structure and functions of the Nit2 enzyme should be performed, including effect of metal ions and other reagents, kinetic parameters and X-ray crystallography. The results also give a basis for further study with large scale fermentation for production of Nit2.

CHAPTER 6

CONCLUSION AND FUTURE WORK

6.1 Conclusion and future work

The biotransformation of nitriles mediated by microorganisms has attracted considerable attention in academia and industry as a sustainable alternative to the conventional chemical reactions that require drastic conditions of pH, temperature and pressure, use of metal catalysts, high-energy consumption and low selectivity in the process. As a result, the search for microorganisms which contain the enzymes responsible for these biotransformations (nitrilases, NHases and amidases) is crucial. In the first part of this study, a high throughput screening strategy was applied for the investigation of nitrile-hydrolyzing activity towards the production of enantiopure β-hydroxy acids from the environment, towards the expansion of the Pharmaceutical and Molecular Biotechnology Research Centre (PMBRC) isolate library by using soil samples from various parts of the globe and by soil samples collected in Ireland. A range of bacteria were isolated for further study. High-throughput colorimetric activity assays were used to identify those that demonstrate the most promising activity. Gene screening of isolates demonstrating desired activity for the presence of nitrilase, NHases and amidase was performed by conventional PCR.

The past few decades have witnessed the fast development of nitrile-degrading enzymes, both their reaction mechanisms and applications in manufacture of a series of pharmaceuticals and chemicals. The work reported in this thesis consisted of (1) isolation of bacteria with activity towards three pharmaceutically relevant β-hydroxynitriles from environmental samples collected worldwide; (2) development of a high throughput screening strategy for filamentous fungi with potential for nitrile biotransformation; (3) application of functional metagenomics to search for novel nitrile hydrolyzing enzymes using environmental samples collected in Ireland; and (4) formulation of an appropriate production medium using statistical optimization that can substantially increase nitrilase production.

On the other hand, it was necessary to adapt this strategy to filamentous fungi, once it was developed for use in bacterial cells. This work presents a strategy that facilitates the rapid identification of fungal isolates demonstrating nitrile hydrolysing activity. The strategy incorporates toxicity, starvation and induction studies along with subsequent colorimetric screening for activity, further focusing the assessment towards the substrates of interest. This high-throughput strategy uses a 96 well plate system, and has enabled

the rapid biocatalytic screening towards β-hydroxynitriles. *Fusarium solani* strain F3 was identified and found to catalyse the hydrolysis of 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile with remarkably high enantioselectivity under mild conditions, to afford (S)-3-hydroxybutyric acid and (S)-3-hydroxy-3-phenylpropionic acid in 98.03 and >99.9 % *ee* respectively. The biocatalytic capability of this strain, including the variation of parameters such as temperature and time, were further investigated and all results indicate the presence of a highly enantioselective enzyme using the whole mycelial cells. To this author's knowledge, this is the first study concerning the high throughput method for fungal nitrile-metabolism enzyme screening using 96-well microtest plates. Moreover, primers designed for bacteria were used in both bacteria and fungi, yielding amplicons of interest. By using these methods, we have found four promising isolates which are a source of genes for potential nitrile-degrading enzymes.

The second part of this work focused on applying functional metagenomics to search for new nitrile-metabolising enzymes from soil samples. In fact, functional metagenomics is a powerful experimental approach for studying gene function, starting from the extracted DNA of mixed microbial populations.

The use of the biodiverse environment in the search for novel catalysts by microbiological selection techniques is the traditional method for the discovery of new enzymes in the development of biocatalysts for different industrial sectors. A fosmid DNA library was prepared from metagenomic DNA extracted from soil samples collected in Ireland. The resulting *Escherichia coli* clone library was screened using functional selection for nitrile-hydrolyzing enzyme activity using β-hydroxynitriles as substrates; 3-hydroxybutyronitrile, 3-hydroxyglutaronitrile and 3-hydroxy-3- phenylpropionitrile, leading to the identification of 33 clones demonstrating activity. Gene screening of these functionally active isolates for the presence of nitrilase, NHases and amidase was performed by conventional PCR and partial gene sequences were identified. This author believes that by using these molecular approaches, it is possible to build an extraordinary bank of clones for exploring novel nitrile-metabolising enzymes.

The following could be undertaken to elucidate the full sequence of nitrile metabolism-related genes. Firstly, a transposon mutagenesis library could be constructed (using the fosmid DNA from the functional clones). The transposon may interrupt the enzyme at a

different position to that of the known partial sequence, which would allow for additional sequence to be elucidated by identifying mutants with knocked out activity.

In order to improve the production of nitrilase by batch culture of recombinant *E. coli*, this study then focussed on integrating statistics with a microbiology laboratory activity to optimize the fermentation process parameters. Three variables (yeast extract, ethanol and sorbitol), which have significant effects on nitrilase activity, were selected from six variables by Plackett-Burman design. With the regression coefficient analysis in the Box-Behnken design, a relationship between nitrilase activity and three significant factors was obtained, and the optimum levels of the three variables were as follows: 0.8 % (w/v) of yeast extract, 3 % (w/v) of ethanol and 0.5 M of sorbitol. The 3D response surface plots and 2D contour plots created by the Box-Behnken design showed that the interaction between ethanol and yeast extract and that between sorbitol were significant. An average of 5.54 mmol/L of nitrilase activity was attained using whole cells in the validation experiment under optimized conditions, which was 66 % higher than the yield of 3.33 mmol/L before optimization.

Possible future work arising from the bank of isolates described in this work would include further screening of the novel isolate library to detect isolates containing enzymes capable of catalysing other types of nitrile metabolism; such as nitrile reductases and amidases capable of acyl transfer. Nitrile reduction can provide a useful method of access to amines that are ubiquitous in pharmaceutical compounds. The discovery of this enzyme class offers a new sustainable eco-friendly method for the synthesis of amines. It may be possible to isolate for a range of nitrile-degrading enzymes by screening and identifying isolates within the library using a high through-put method operating in a 96-well plate format. One could potentially identify isolates capable of resolving racemic mixtures of nitriles via the selective reduction of one enantiomer. The potential of the isolate library to be embedded in a bioremediation setting/treatment should be investigated also, possible via whole-cell immobilization.

The complete gene sequence of the responsible nitrile-degrading genes should be identified to allow cloning and expression of the enzymes. While conventional sequencing of the partial genes amplified by PCR is possible, complete genome sequencing of each high-performance isolate via commercial services is a faster and more economical approach.

Also, improvement of nitrile-metabolising enzyme activity, stability and selectivity via directed evolution of these cloned genes should be carried out to bring the enzyme performance above all prior benchmarks.

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APPENDIX I

ADDITIONAL CHROMATOGRAMS

Additional Chromatograms

Below are sample chromatograms observed from the crossed enantioselective screening of bacterial isolates towards on 10 mM 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile.

Chiral HPLC Chromatograms: Standard of racemic (R)-3-Hydroxy-3-phenylpropionic acid and (S)-3-Hydroxy-3-phenylpropionic acid:

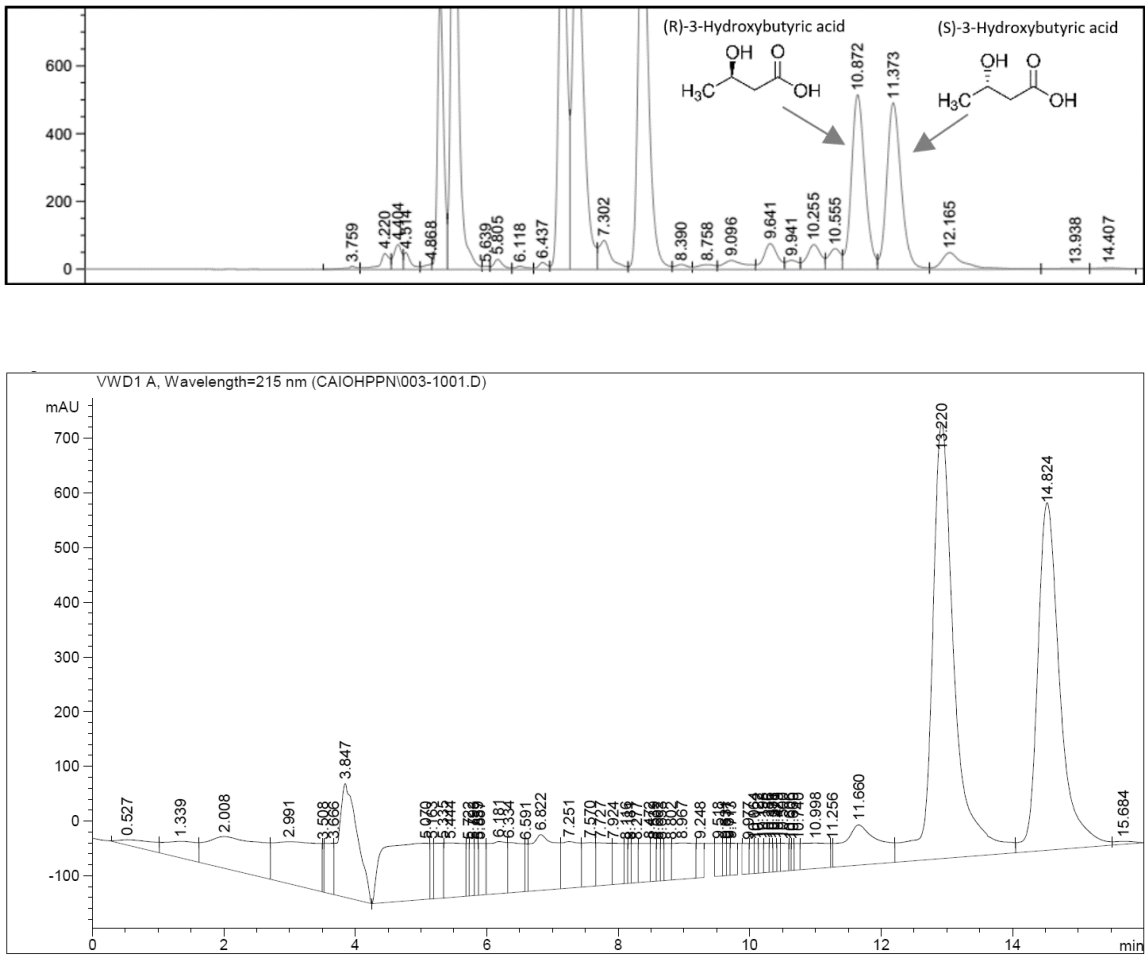


Fig. A.1 Chiral HPLC Chromatograms. Biotransformation of 3-Hydroxy-3-phenylpropionitrile by isolate 1 A. Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm.

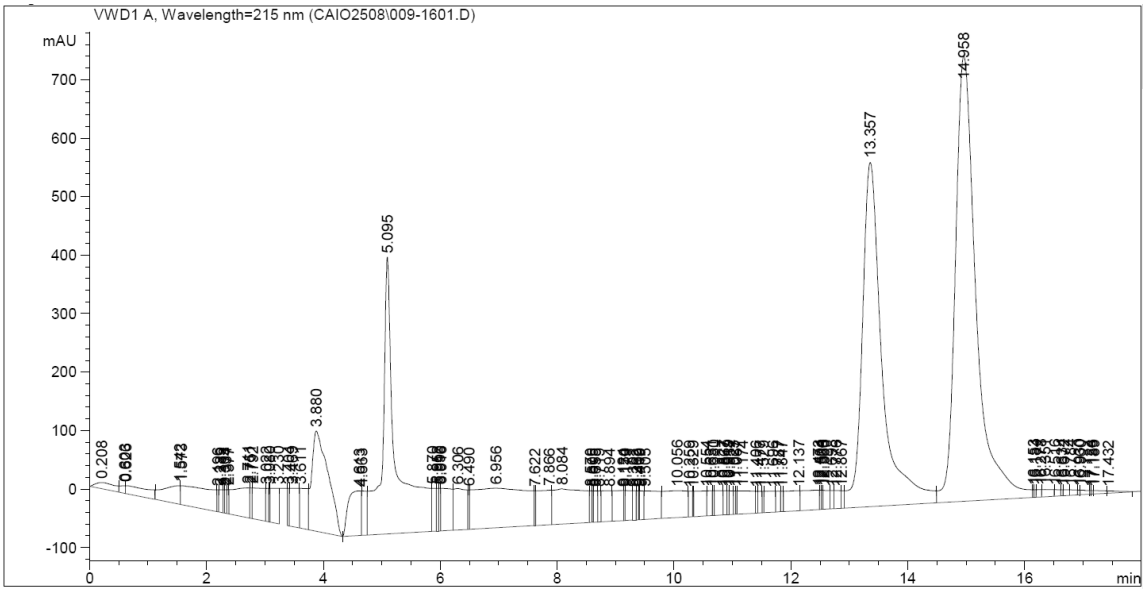


Fig. A.2 Chiral HPLC Chromatograms. Biotransformation of 3-Hydroxy-3-phenylpropionitrile by isolate 2 A. Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 Ml/min and a detection wavelength of 215 nm.

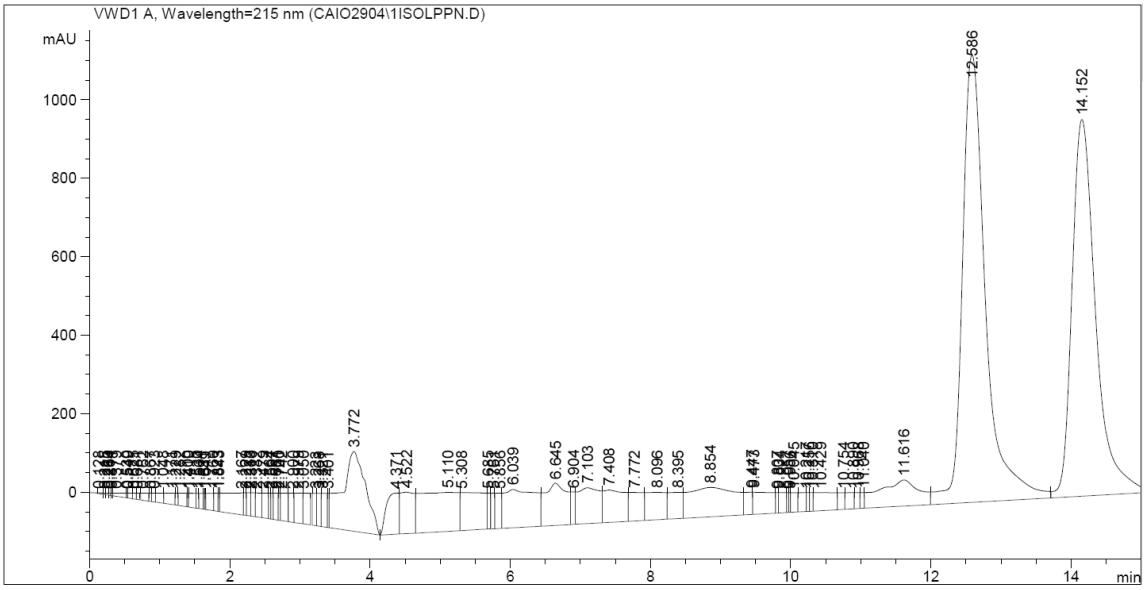


Fig. A.3 Chiral HPLC Chromatograms. Biotransformation of 3-Hydroxy-3-phenylpropionitrile by isolate 46F7A. Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 Ml/min and a detection wavelength of 215 nm.

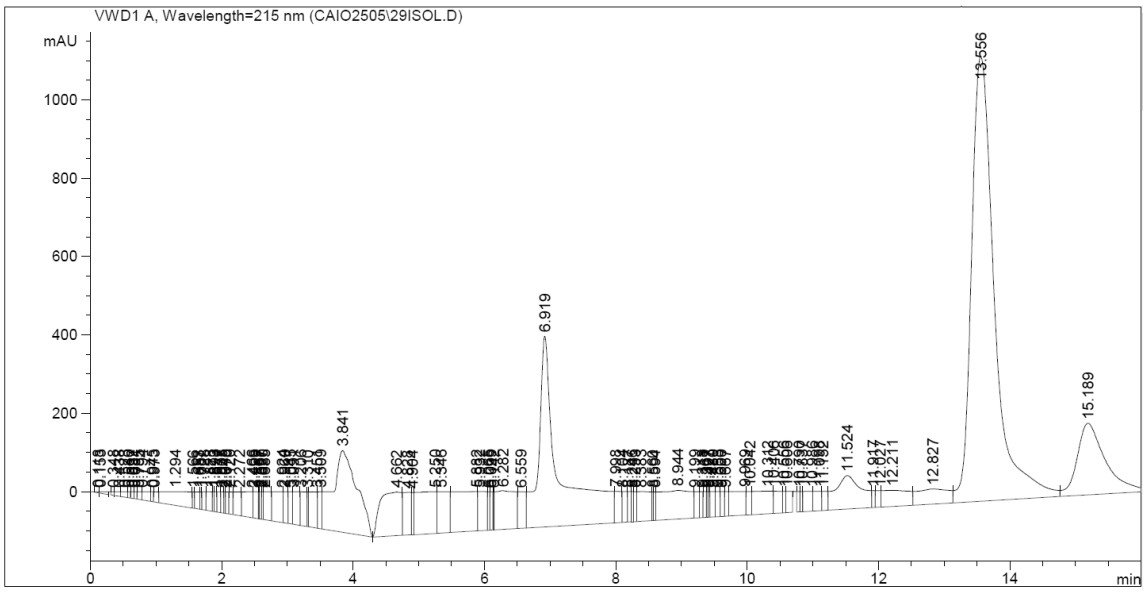
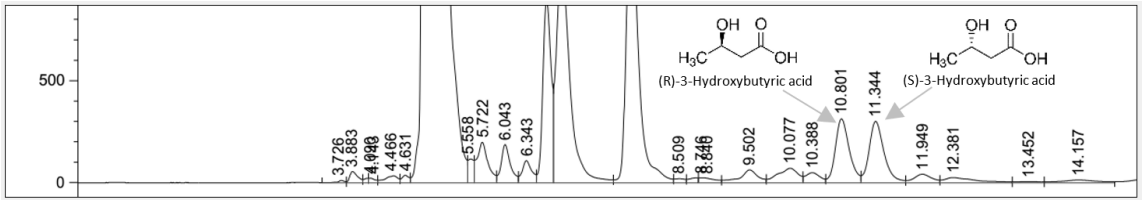


Fig. A.4 Chiral HPLC Chromatograms. Biotransformation of 3-Hydroxy-3-phenylpropionitrile by isolate 29A. Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 Ml/min and a detection wavelength of 215 nm.

Chiral HPLC Chromatograms: Standard of racemic (R)-3-Hydroxybutyric acid and (S)-3-Hydroxybutyric acid:



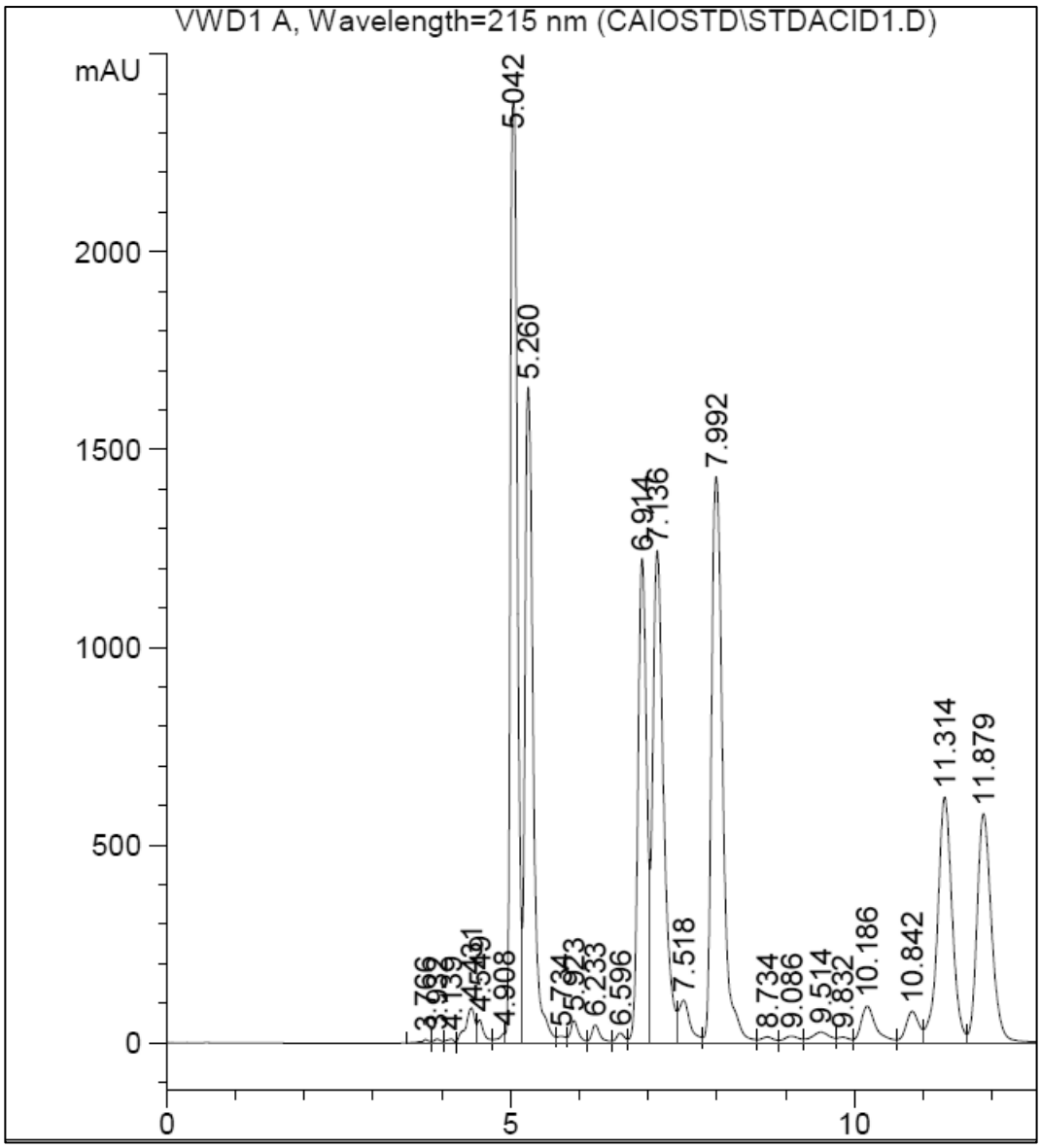


Fig. A.5 Chiral HPLC Chromatograms. Biotransformation of 3-hydroxybutyronitrile by isolate 1C. Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 Ml/min and a detection wavelength of 215 nm.

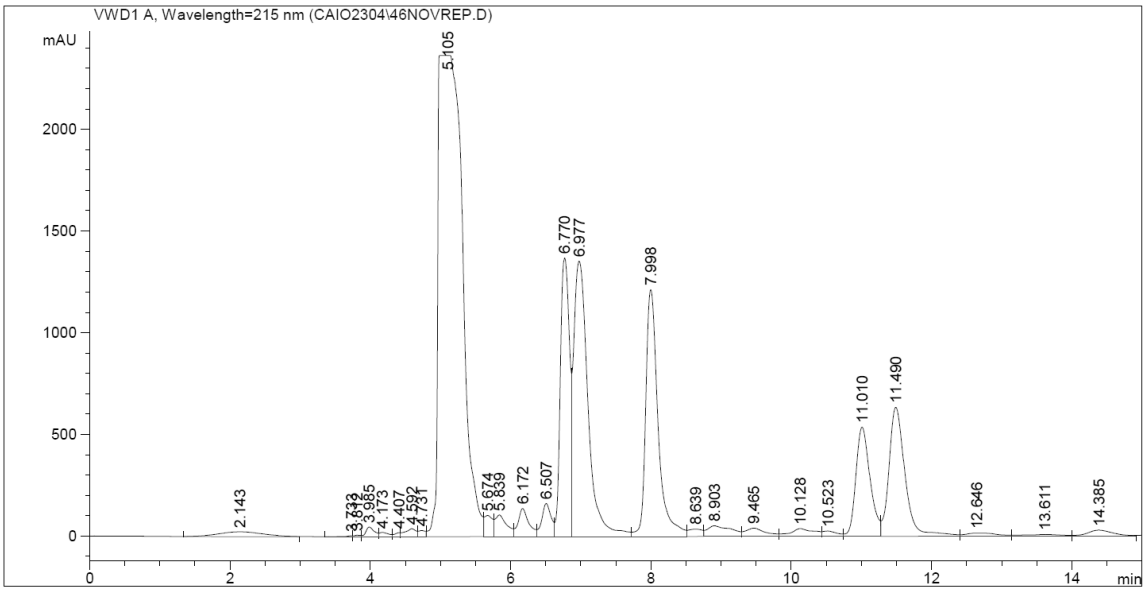


Fig. A.6 Chiral HPLC Chromatograms. Biotransformation of 3-hydroxybutyronitrile by isolate 2C. Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 Ml/min and a detection wavelength of 215 nm.

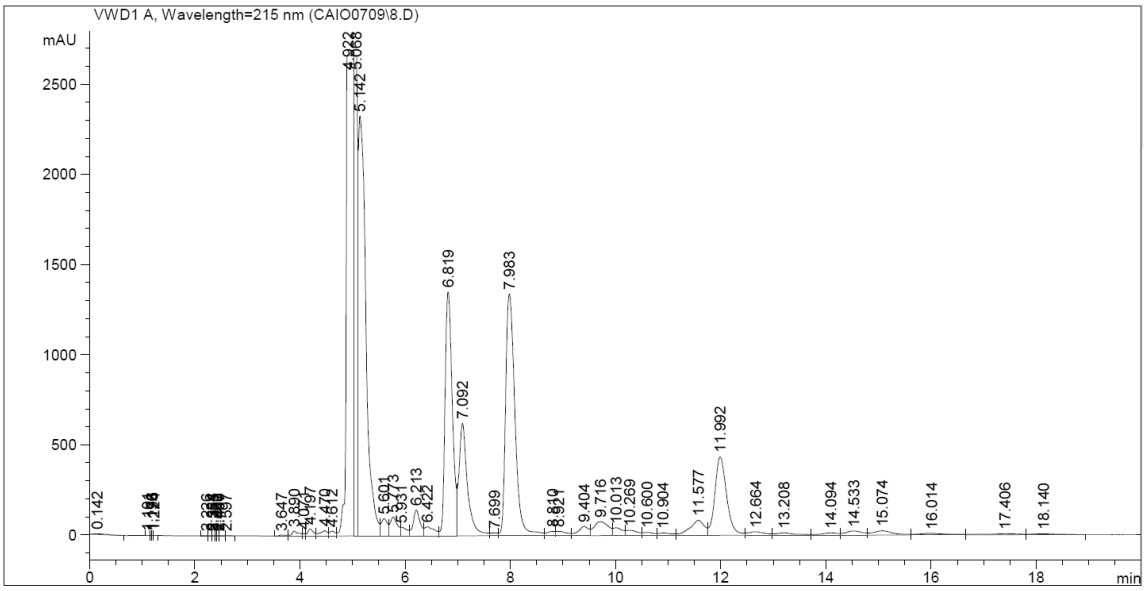


Fig. A.7 Chiral HPLC Chromatograms. Biotransformation of 3-hydroxybutyronitrile by isolate 3C. Conditions of HPLC analysis: Chiralcel AD-H column, mobile phase: 90 % (w/v) hexane, 10 % (w/v) IPA and 0.1 % (w/v) TFA, with a flow rate of 0.8 Ml/min and a detection wavelength of 215 nm.

APPENDIX II

PUBLICATION

Biomaterials and Tissue Technology

oat open access text

Research Article ISSN: 2398-970X

Applying functional metagenomics to search for novel nitrile-hydrolyzing enzymes using environmental samples

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Abstract

A metagenomic approach was used to search for novel nitrile hydrolyzing enzymes from environmental samples. Nitrile compounds are versatile and can be converted into amides, amines, imines, oximes, carboxylic acids, esters and alcohols, encompassing a large group of economically important synthetic intermediates. The pharmaceutical industry particularly requires amides and acids for use as intermediates in the manufacture of many drugs and chemicals. The use of the biodiverse environment in the search for novel catalysts by microbiological selection techniques is the traditional method for the discovery of new enzymes in the development of biocatalysts for different industrial sectors. A fosmid DNA library was prepared from metagenomic DNA extracted from soil samples collected in Ireland. The resulting *Escherichia coli* clone library was screened using functional selection for nitrile-hydrolyzing enzyme activity using ⍺-hydroxynitriles as substrates; 3-hydroxybutyronitrile, 3-hydroxyglutaronitrile and 3-hydroxy-3- phenylpropionitrile, leading to the identification of 33 clones demonstrating activity. Gene screening of these functionally active isolates for the presence of nitrilase, nitrile hydratase and amidase was performed by conventional PCR and partial gene sequences were identified. Further studies to identify complete gene sequences for cloning and expression are underway towards realizing the commercial potential of the associated enzymes.

Introduction

The pharmaceutical industry has several motivations to probe the enormous resource that is uncultivated microbial diversity. Presently, there is a global political pressure to encourage industrial/white biotechnology to substantially impact such industrial production. Thus, the development of novel enzymes as the ideal biocatalyst is very desirable, if not required. In fact, metagenomics seems to provide those new molecules with various functions, but eventually, heterologous expression is required for any new enzymes to become an economic success [1].

One interesting example of novel enzymes are the nitrile-degrading enzymes, which by hydrolysis comprises the most common pathway for this microbial metabolism [2]. In this way, we can find two different enzyme systems to work with [3]. The first is by nitrilases, which convert nitriles directly to the corresponding carboxylic acids, via addition of two molecules of water. On the other hand, the second system is a combination of a nitrile hydratase (NHase), which converts the nitrile to amide through a hydrolysis step via the addition of one molecule of water, and an amidase, that subsequently hydrolyzes the amide to the corresponding carboxylic acid via the addition of another molecule of water [4].

The need of amides and acids for use as intermediates in the manufacture of many drugs and chemicals by pharmaceutical industries is great [5]. These may be obtained by traditional chemical methods, but this approach has problems; the conditions required are harsh/extreme and undesirable by-products are produced [3,6,7]. An alternative to the use of traditional chemical methods is the use of nitrile-metabolising enzymes as biocatalysts.

The efficiency of biocatalysts to perform various chemical reactions has been the main attraction for their use in chemical synthesis because of the selectivity presented by natural biocatalysts, such as enantioselectivity (preferential involvement of one enantiomer over the others in a chemical or enzymatic reaction) [8]. In addition, a wide spectrum of chemical compounds that are accepted as xenobiotic substrates for reactions exist, while mild and environmentally sustainable conditions give these biocatalysts some fundamental characteristics for use in different transformations of biotechnological interest [9].

The use of the biodiverse environment in the search for novel catalysts by microbiological selection techniques is the traditional method for discovery of new enzymes in the development of biocatalysts for different industrial sectors. The use of microorganisms has a particular interest due to the short period of cultivation, the wide variety of metabolic processes/enzymes involved and due to an unlimited number of microorganisms in the environment which are very diverse, thus providing the potential discovery of enzymes with many different applications [10].

There are two main different approaches in the isolation of microbial genes/enzymes from the environment; culture-dependent and culture-independent methods. Both allow the characterization of and access to some of the diverse microbiome within a sample and therefore are considered complementary. It has been estimated that 1% of the microorganisms are detected on plates with culture medium due to selective conditions, depending on the composition of the

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culture media [11,12]. On the other hand, the culture-independent methods indicates a predominance of many uncultivated species [13]. One example of a culture-independent molecular method is metagenomics, which consists of direct extraction of nucleic acids from the environmental sample(s), followed by cloning into a suitable vector, transformation into host cells and screening for the genes and/or functions of interest [14], without going through prior DNA amplification steps.

Currently, uncultured environmental samples can be explored by the metagenomic approach [15]. Most of the well-characterized NHases, amidases and nitrilases, which are largely of bacterial origin, have been obtained by selection methods allowing only the positive strains to grow on a minimum medium with a nitrile as the sole nitrogen source [16-19].

This current study focused on applying functional metagenomics to search for novel nitrile hydrolyzing enzymes using environmental samples. This will allow the cloning, expression and purification of recombinant enzymes, which is the form/stage required by potential industrial partners/customers for screening/scale-up/go-to-market or indeed any industrial exploitation. The nitriles chosen for this study were 3-hydroxybutyronitrile (3HBN), 3-hydroxyglutaronitrile (3HGN) and 3-hydroxy-3-phenylpropionitrile (3HPPN), which are β-hydroxynitriles, which can act as sources of β-hydroxycarboxylic acids by biotransformation - these products could be widely used as chiral precursors for pharmaceutical compounds.

Material and methods

Substrates of interest

Racemic 3HBN and 3HGN were purchased from ENAMINE®, and 3HPPN from Sigma®. All other chemicals were of analytical grade and obtained from Macron Fine Chemicals™ and Acros Organics Chemicals.

Total DNA extraction and metagenomic fosmid library construction

10 soil samples used in this work as sources of metagenomic DNA were obtained from environmental soils collected from terrestrial and aquatic microenvironments in Co. Waterford, Ireland. Total DNA was extracted according to the previously described protocol [20]. The cells were lysed using beads and 20% (x/v) SDS, DNA was then isolated by purification using phenol/chloroform extraction followed by alcohol precipitation. The pellet of DNA was resuspended in TE (10 mM Tris/HCl, 1 mM EDTA, pH 8), treated with RNAse at 37°C for two hours and stored at -20°C.

Cloning of metagenomic DNA into the vector pSMART® FOS Vector (Lucigen®) and packaging recombinant lambda phage by Gigapack III XL packaging extract (Stratagene®) were performed as per the manufacturer's instructions. Briefly, metagenomic DNA samples were subjected to an end-repair reaction to create blunt ends with 5′ phosphate groups for ligation into the blunt, dephosphorylated vector. Fosmids containing the inserts were then packaged with Gigapack III XL and used to infect the Replicator™ FOS strain. Infected cells were spread on YT+CXIS plates (8 g/L bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl and 15 g/L agar) supplemented with 12.5 μg/mL chloramphenicol, 40 μg/mL X-Gal, 0.4 mM IPTG and 5% (w/v) sucrose, and incubated at 37°C overnight for selecting transformants. The titer of the packaged fosmid phage particles was first determined to calculate plating requirements. Clones were transferred to 96-well Megablocks® containing LB broth supplemented with the suitable antibiotic and stored after growth at -70°C in the presence of 20 % (w/v) glycerol.

Fosmid clone selection by functional screening

Individual clones from the metagenomic fosmid library were pre-cultured in 96-well Megablocks® containing 600 μL of TB broth [11.8 g/L bacto-tryptone, 23.6 g/L yeast extract, 9.4 g/L dipotassium hydrogen phosphate (K_2HPO_4; anhydrous), 2.2 g/L potassium dihydrogen phosphate (KH_2PO_4; anhydrous), 0.4 % (w/v) glycerol] supplemented with 8 mL filter-sterilized 50 % (w/v) glycerol and 12.5 μg/mL chloramphenicol, and then incubated at 25°C and 250 rpm for 16 h. After growth, the clones were plated on M9 agar supplemented with 10 mM nitrile substrates and incubated for 6 days at 25°C for nitrile functional screening. All functional screening in solid medium was carried out in triplicate. For long-term storage, cells were maintained frozen at -70°C in 20 % (v/v) glycerol.

Gene screening by conventional PCR

NHase, amidase and nitrilase gene screening were performed by direct colony PCR of functional clones. Table 1 shows the combinations of primers used to amplify the *NHase* and *amd* genes. The complete α subunit genes were amplified using the forward primer NHA-F and the reverse primer NHA-R. The complete β subunit genes were amplified using the forward primer NHB-F and reverse primer NHB-R. The complete αβ genes were amplified using NHA-F and NHB-R. NHase primers were designed previously [19] in order to amplify Fe-type NHase genes. The complete amidase gene was amplified using the forward primer Amd1-F and the reverse primer Amd1-R [21]. Each 25 μL PCR reaction mixture contained 12.5 μL GoTaq®Green Master Mix (Promega), 10 μM each primer and 15-30 ng DNA or cells adjusted to O.D.@$_{600}$ = 0.04. The PCR thermal profile consisted of: 1 cycle of 95°C for 5 min, 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 40 s, followed by 1 cycle of 72°C for 5 min, with the exception of complete αβ gene amplifications which required an extension time of 1.5 min and of complete amidase gene which required an extension time of 2 min.

Nitrilases genes were amplified by using degenerate primers and PCR conditions described previously in Coffey *et al.* [19].

Results

Metagenomic library screening for clones expressing nitrile metabolizing activity

To identify novel genes coding for nitrile hydrolyzing enzymes from new sources, a fosmid metagenomic library was constructed with total DNA isolated from environmental samples collected in Ireland from terrestrial and aquatic microenvironments. A total of 1,2 x 10^4 clones was obtained from the metagenomic library. These clones were individually screened for nitrile activity on solid medium by functional

Table 1. Primers designed for PCR amplification of the α and β NHase subunit genes, the complete αβ genes for *NHase* gene, and for the complete amidase gene (*amd*).

Primers	Sequences	Amplicons
NHA-F	5'-ATGTCAGTAACGATCGACCAC	~600 bp
NHA-R	5'-AGGCAGTCCTTGGTGA CGAT	
NHB-F	5'-ATGGATGGAGTACACGATCT	~600 bp
NHB-R	5'-TCAGGCCGCAGG CTCGAGGT	
Amd1-F	5'-ATACGCGTGAATTCGTGGCGACAATCCGACCTGAC	~1782 bp
Amd2-R	5'-GGTGTTGAGTCGGAGTGGATCTTCGAAACTTCCTAG	

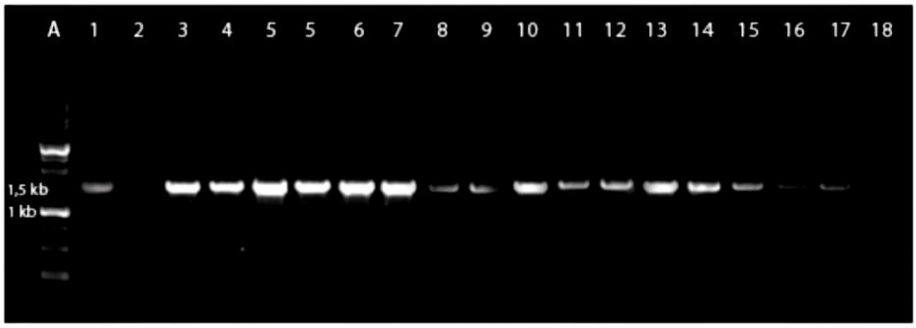

Figure 1. A total of 33 clones were confirmed for the ability to use nitriles as the sole nitrogen source and were submitted to gene screening using conventional PCR. PCR amplification with the amidase primers

screening, indicated by growth using the selected nitrile as the sole source of nitrogen. Of the 1.2 x 10^4 clones screened, 16, 12 and 5 clones showed activity towards 3-hydroxyglutaronitrile, 3-hydroxybutyronitrile, and 3-hydroxy-3-phenylpropionitrile respectively.

PCR screening

A total of 33 clones were confirmed for the ability to use nitriles as the sole nitrogen source and were submitted to gene screening using conventional PCR. PCR amplification with the amidase primers is shown in Figure 1. All the results of the DNA fragments amplifications are shown in Table 2.

Discussion

To construct a metagenomic library to search for new nitrile-metabolizing enzymes, we used DNA fragments representing a mixed microbial soil population obtained in Ireland. A total of 10 environmental samples collected in Ireland from terrestrial and aquatic microenvironments were processed into genomic DNA libraries containing fragments approximately 38-40 kb in size.

We chose a function-based metagenomic approach, which allows the identification of novel nitrile metabolizing enzymes, making it possible to focus gene screening and therefore detect only enzymes that are functional [22].

It is a common practice for enhancing the desired functions in a microbial community to induce the growth of specific microorganisms by applying selective enrichment methods to the sample to produce an increased screening hit rate [23-28]. For instance, Grant et al. [29] used metagenomic DNA of cultures grown in medium containing carboxymethylcellulose as the only carbon source, and they observed that the number of glycosyl hydrolases detected was about four times greater than the number identified in metagenomic libraries obtained using DNA taken directly from environmental samples.

A total of 1.2 x 10^4 clones was obtained from the metagenomic library. The 33 positive clones demonstrating growth on the chosen nitriles were subjected to conventional PCR screening. Our results demonstrated that 48 % of the positive clones contain the complete amidase gene. In contrast, no clones have presented NHase genes of the sequence type targeted by the chosen primers (Fe-type NHase genes with similar homology to those often seen in *Rhodococci*, such as *Rhodococcus erythropolis* strain AJ270 (accession number AJ490527) or strain N771 (accession number AJ716152). It is important to point out that these clones may have a different NHase that the primers do not amplify due to sequence divergence at the primer binding sites. On the other hand, most of them yielded potential PCR products for a nitrilase gene.

Table 2. Numbers of clones yielding PCR products of the expected size of nitrile hydrolyzing genes.

Functional screening	Amidase	Nitrilase	NHase
Clones from 3HPPN	6	19	-
Clones from 3HBN	10	39	-
Clones from 3HGN	_	25	-

In summary, this study consisted of applying functional metagenomics to search for novel nitrile hydrolyzing enzymes using environmental samples collected in Ireland. Gene screening of the positive clones demonstrating the potential presence of nitrilase and amidase genes. Further work to identify complete gene sequences for cloning and expression is underway towards realizing commercial potential. We believe that by using this molecular approach, we may build an extraordinary bank of clones producing novel nitrile-metabolizing enzymes.

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Compliance with ethical standards

Not applicable.

Conflict of interest

The authors declare that there is no conflict of interest of a scientific or commercial nature. The authors have no relevant affiliations to, or financial support from any organization that may have a financial interest in the subject matter.

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APPENDIX III

COMPLETE POSITIVE ISOLATES FOR NITRILE HYDROLYZING GENE SCREENING USING CONVENTIONAL PCR

Conventional PCR screening of isolates for NHase, amidase and nitrilase genes.

			Nitrile Hydratase / Amidase					Nitrilase (Clades)								
ID		Nitrile	α	β	αβ	ami	1A	1B	2A1-20	2A21-37	3A	4A	5A	5B	6A	BURK
1	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	+	+	-
2	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	+	-	+
3	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	+	-	-
4	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	-	-	-
7	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	-	-	-
8	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	-	-	-
9	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
10	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
13	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
15	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
17	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
18	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
19	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
20	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
21	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
22	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	+	-	-

23	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-
25	A	3HBN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
26	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
27	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	-	+	+	+	+
30	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	+	+	+	+
32	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	A	3HBN	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
36	A	3HBN	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
37	A	3HBN	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
38	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
39	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	+	+	-	+	+
40	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
42	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
43	A	3HBN	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
44	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
45	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46F7	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+
47	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	+	+	+	+
49	A	3HBN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-

50	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
51	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	-	-	-	
52	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	-	-	-	
53	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
54	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
55	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	
56	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
57	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
58	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
59	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	
60	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
61	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
62	A	3HBN	-	-	-	-	-	+	-	-	-	-	+	-	-	-	
63	A	3HBN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	
64	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
65	A	3HBN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	
66	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
67	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	-	-	
68	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	-	-	
69	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	
70	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	-	-	-	
71	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	-	-	-	
72	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	
73	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
74	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
75	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
76	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	

77	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
78	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
79	A	3HBN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
80	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
81	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
82	A	3HBN	+	+	+	+	-	+	-	-	-	-	+	-	-	-	-
83	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
84	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-
85	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
86	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
87	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
88	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
89	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
90	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
91	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
92	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
93	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
94	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
95	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
98	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
99	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
100	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-
101	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
102	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
103	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-

104	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
105	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
106	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
107	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
109	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
110	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
111	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
112	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
113	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
114	A	3HBN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
115	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
116	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	-	-
117	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	-	-
118	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
119	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
120	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
121	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
122	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
123	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
124	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
125	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	+	-
126	A	3HBN	+	+	+	+	-	+	-	-	-	-	-	+	-	+

			Nitrile Hydratase / Amidase				Nitrilase (Clades)								
ID		**Nitrile**	**α**	**β**	**αβ**	**ami**	**1A**	**1B**	**2A1-20**	**2A21-37**	**3A**	**4A**	**5A**	**5B**	**6A**	**BURK**
1	B	3HGN	+	-	-	-	-	+	-	-	-	-	+	-	+	-
2	B	3HGN	-	+	+	+	-	-	-	-	-	-	-	-	-	-
3	B	3HGN	+	+	+	+	-	-	-	-	-	-	-	+	-	-
4	B	3HGN	-	+	+	+	-	-	-	-	-	-	-	-	-	-
5	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	-	+
6	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	-	-
7	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	-	-
8	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	+	-
13	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	-	-	+
14	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-
18	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	-	-	-
20	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	-	-
21	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	-	-	-
22	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	-	-
23	B	3HGN	+	+	+	+	-	-	-	-	-	-	-	-	-	-
24	B	3HGN	+	+	+	+	-	-	-	-	-	-	-	-	-	-

25	B	3HGN	+	+	+	+	-	-	-	-	-	-	+	+	-	-
26	B	3HGN	+	+	+	+	-	-	-	-	-	-	-	+	-	-
27	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	-	-
28	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	-	-	-
29	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	-	-	-
30	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	+	-	-
31	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
32	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
33	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
34	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
35	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
36	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-
37	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	+	-	-
39	B	3HGN	+	+	+	+	-	+	-	-	-	-	+	+	+	+
40	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
41	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
42	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
43	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
44	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
45	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
46	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
47	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
48	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
49	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
51	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-

52	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-		
53	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-		
54	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
55	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
56	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
57	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
58	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-		
59	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-		
60	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	+	-	-		
61	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
62	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
63	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
64	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
65	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
66	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-		
67	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
68	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-		
69	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
70	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
71	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-		
72	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-		
73	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
74	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
75	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
76	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-		
77	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-		
78	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-		

79	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	+	-	-
80	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	+	-	-
81	B	3HGN	+	+	+	+	-	+	-	-	-	-	-	-	-	-
82	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
83	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	+	-	-
84	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-
85	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	+	-	-
86	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
87	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
88	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
89	B	3HGN	+	-	-	-	-	+	-	-	-	-	-	-	-	-
90	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	+	-	-
91	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
92	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
93	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
94	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-
97	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-
98	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-
99	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	+	-	-
100	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-
101	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-
102	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-
103	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-
104	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
105	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-

106	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-		
107	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
108	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
109	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-		
110	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	+	-	-		
111	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
112	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-		
113	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
114	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
115	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-		
116	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-		
117	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-		
118	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-		
119	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
120	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
121	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
122	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
123	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
124	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-		
125	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
126	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
127	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
128	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
129	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
130	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
131	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
132	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-		

133	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
134	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-	
135	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-	
136	B	3HGN	-	-	-	-	-	+	-	-	-	-	+	-	-	-	
137	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
138	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-	
139	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
140	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
141	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-	
142	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
143	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
144	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
145	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
146	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
147	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
148	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
149	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
150	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
151	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
152	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
153	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
154	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
155	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
156	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
157	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
158	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
159	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

160	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
161	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
162	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
163	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
164	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
165	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
166	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
167	B	3HGN	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
168	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
169	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
170	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
171	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
172	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
173	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
174	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
175	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
176	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
177	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
178	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
179	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
180	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
181	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
182	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
183	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
184	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
185	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
186	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-

187	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
188	B	3HGN	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
189	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
190	B	3HGN	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
191	B	3HGN	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-
192	B	3HGN	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-

			Nitrile Hydratase / Amidase				Nitrilase (Clades)								
ID		**Nitrile**	**α**	**β**	**αβ**	**ami**	**1A**	**1B**	**2A1-20**	**2A21-37**	**3A**	**4A**	**5A**	**5B**	**6A**	**BURK**
1	A	3HPPN	+	+	+	+	-	+	-	-	-	-	+	-	+	-
2	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	C	3HPPN	+	+	+	+	-	+	-	-	-	-	+	-	-	-
4	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	C	3HPPN	+	+	+	+	-	+	-	-	-	-	+	-	-	+
7	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	C	3HPPN	-	-	-	-	-	-	-	-	-	-	-	-	-	-

APPENDIX IV

OPTIMUM TEMPERATURE AND PH DETERMINATION

Table 1. Temperature optimization of the Nit2 recombinant protein (peak area).

	Temperature (°C)										
	10	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**70**
Replicate 1	0.094	0.135	0.191	0.230	0.309	0.407	0.532	0.475	0.135	0.074	0.052
Replicate 2	0.098	0.139	0.195	0.239	0.302	0.430	0.542	0.467	0.141	0.071	0.057
Replicate 3	0.096	0.145	0.199	0.242	0.299	0.441	0.549	0.451	0.143	0.067	0.062
Average triplicate of assays	0.096	0.140	0.195	0.237	0.303	0.426	0.541	0.464	0.140	0.071	0.057
Standard deviation	0.002	0.005	0.004	0.006	0.005	0.017	0.009	0.012	0.004	0.004	0.005
Relative Activity (%)	2.708	10.347	23.125	34.167	49.444	77.083	100.0	84.028	17.847	1.597	0.417

Table 2. pH optimization of the Nit2 recombinant protein (peak area).

Buffer	Replicate 1	Replicate 2	Replicate 3	Average triplicate of assays	Standard deviation	Relative activity (%)
Sodium acetate – pH 4	0.089	0.091	0.091	0.090	0.001	1.20
Sodium acetate – pH 5	0.19	0.191	0.189	0.19	0.001	13.5
Sodium acetate – pH 6	0.573	0.572	0.577	0.574	0.002	57.9
Potassium phosphate – pH 6	0.495	0.502	0.489	0.495	0.006	65.0
Potassium phosphate – pH 6.5	0.614	0.611	0.613	0.612	0.001	82.3
Potassium phosphate – pH 7	0.651	0.624	0.673	0.649	0.024	93.7
Potassium phosphate – pH 7.5	0.72	0.799	0.67	0.729	0.065	100.0
Potassium phosphate – pH 8	0.649	0.635	0.678	0.654	0.021	83.6
Tris-HCl pH 8	0.71	0.713	0.697	0.706	0.008	78.0
Tris-HCl pH 9	0.425	0.429	0.43	0.428	0.002	45.2
Tris-HCl pH 10	0.29	0.3	0.3	0.296	0.005	30.7