# A STUDY ON THE DIVERSITY AND PRODUCTION OF MICROBIAL EXTRACELLULAR NUCLEASES: POTENTIAL ANTI-BIOFILM ENZYMES

BY

NITHYALAKSHMY RAJARAJAN

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## DEDICATION

"The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires" – William Arthur

**To my Parents and all my great Teachers**

## DECLARATION

I hereby declare that the work presented in this thesis is the original research carried out by me at Newcastle University, except where stated, and has not been previously submitted for a degree or any other qualification at this or any other university.

Nithyalakshmy Rajarajan

Newcastle upon Tyne, UK

## ABSTRACT

Microorganisms have been viewed as planktonic, free living single cells but predominantly they exist as sessile multispecies communities in the natural environment forming 'biofilms'. Biofilms are beneficial for organisms to survive in natural environment as well as for biotechnological applications such as microbial fuel cells and bioremediation. However, biofilms are associated with disease persistence and biofouling and are comprised of adhered microbes within a hydrated matrix rich in polysaccharides, proteins and extracellular DNA (eDNA). eDNA is an important structural component and its degradation by deoxyribonucleases may be a novel approach to eradicate biofilm related problems.

The present work was undertaken in this context to discover and produce microbial nucleolytic enzymes for applications for the control of harmful biofilms. Eighty six out of 260 bacterial isolates which included thermophilic and psychrophilic strains, showed deoxyribonuclease activity. The diversity and function of extracellular nucleases was also investigated throughout the microbial world using bioinformatics tools. Sequence driven analysis suggested that major bacterial lineages contain diverse extracellular nucleases with biological function related to nitrogen, phosphate and carbohydrate metabolism, protection, survival and virulence.

Production optimisation for one specific extracellular nuclease, NucB, from *Bacillus licheniformis* EI-34-6 was carried out. This enzyme was previously known to cleave eDNA causing biofilm dispersal, and may therefore be used commercially to remove biofilms. The understanding of *B. licheniformis* physiology was applied in order to enhance NucB production 10-fold. For further characterisation of the enzyme and to

understand its biological mechanism in breaking down biofilms, NucB was expressed in the SURE expression host *B. subtilis* NZ8900. This allowed a 68-fold increase in protein yield. NucB protein has been purified to high degree purity with specific nuclease activity of 15000 U/mg of protein. Biophysical characterisation showed that the protein was thermally stable and could reversibly refold.

Statistical optimisation of extracellular nucleases production in diverse bacteria grown at different temperatures was demonstrated as a promising methodology for enhancing key enzyme secretion. The effectiveness of biofilm disruption by NucB was successfully tested with different single species biofilms grown on polystyrene, glass, and stainless steel surfaces. Biofilm dispersal efficiency of other microbial nucleases ranged between 60 – 95 % of removal after 1 h.

The results presented in this thesis demonstrate that bacteria were able to produce nucleases across broad temperature range. In context to biofilm dispersal, bioinformatic analysis speculates the ecological implication of secreted diverse microbial extracellular nuclease-like genes were to decide the fate of eDNA and play pivotal role in nutrient cycling of the eco-system. Bioprocess development confirmed process optimisation can reliably produce functional and well-folded recombinant NucB at levels suitable for applications where biofilm removal is needed. Production optimisation of extracellular nucleases from diverse bacteria expanded the availability of different nucleases with wide range of anti-biofilm properties. Evidence is also presented to show that extracellular nucleases can disperse preformed microbial biofilms on different substrata. Microbial extracellular nucleases therefore appear to be a rich and unexplored source of anti-biofilm enzymes.

## LIST OF PUBLICATIONS

Parts of the work described in this thesis have been presented in the following publications

### Peer reviewed papers

1. **Rajarajan, N**., Ward, A.C., Burgess, J.G., Glassey, J., 2012. Use of physiological information and process optimisation enhances production of extracellular nuclease by a marine strain of *Bacillus licheniformis*. *Bioresource Technology*, http://dx.doi.org/10.1016/j.biortech.2012.12.064

2. Jakubovics, N., Shields, R., **Rajarajan, N**., Burgess, J.G., 2013. The critical role of extracellular DNA in microbial biofilms. *Letters in Applied Microbiology*. *Accepted article*, doi:10.1111/lam.12134.

### Conference peer reviewed oral presentations

1. **Rajarajan, N**., Ward, A.C., Burgess, J.G., Glassey, J., 2012. Enhanced production of biofilm dispersing extracellular nuclease by a marine strain of *Bacillus licheniformis*. *The 3rd International biotechnology and biodiversity conference*, Johor Bahru, Malaysia **(Won best oral presentation award)**

2. **Rajarajan, N**., Kennedy, R.O., Ward, A.C., Burgess, J.G., Glassey, J., 2012. Overproduction of biofilm dispersing nuclease, NucB in high cell density fermentation of recombinant *Bacillus subtilis*. *The 9th European Symposium on Biochemical Engineering Science conference*, Istanbul, Turkey.

### Poster presentations

3. **Rajarajan, N**., Kennedy, R.O., Ward, A.C., Burgess, J.G., Glassey, J., 2011. Towards industrial production of biofilm dispersing nucleases from marine bacteria. *Young Researchers Meeting*, London, UK.

4. **Rajarajan, N**., Lamb. H.K., Hawkins, A.R., Ward, A.C., Glassey, J., Burgess, J.G., 2012 Enhanced production, purification and biophysical characterisation of a recombinant biofilm dispersing nuclease, NucB, produced by *Bacillus subtilis*. *The 9th Annual Bioprocess UK Meeting*, Bristol, UK.

5. **Rajarajan, N**., Ward, A.C., Burgess, J.G., Glassey, J., 2013 Enhanced production of NucB from recombinant *Bacillus subtilis*: optimisation, scale up and biofilm dispersal studies. *The 2nd European Congress of Applied Biotechnology*, The Hague, The Netherlands.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| A | Adenine |
| A260 | Absorbance at 260 nm |
| A595 | Absorbance at 595 nm |
| A600 | Absorbance at 600 nm |
| ANOVA | Analysis of variance |
| BLAST | Basic local alignment search tool |
| bp | Base pairs |
| BDCs | Biofilm dispersing compounds |
| bDNase | Bovine deoxyribonuclease I |
| BSA | Bovine serum albumin |
| C | Cytosine |
| CCD | Central composite design |
| CF | Cystic Fibrosis |
| cfu | Colony forming units |
| Conc. | Concentration |
| CV | crystal violet |
| DCW | Dry cell weight |
| DNA | Deoxyribonucleic acid |
| dsDNA | Double stranded DNA |
| DNase I | Deoxyribonuclease I |
| DTT | Dithiothreitol |
| eDNA | Extracellular DNA |
| EPS | Extracellular polymeric substances |

| | |
|---|---|
| EPM | Extracellular polymeric matrix |
| ECM | Extracellular Matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| g | grams |
| h | hours |
| hDNase | human DNase I |
| kbp | kilo base pairs |
| kDa | kilo Dalton |
| LB | Luria Bertani |
| L | litre |
| M | Molar (mol/L) |
| mg | milligram |
| mg/L | milligram per litre |
| mM | millimolar (millimol/L) |
| mm | millimetre |
| min | minute |
| nm | nanometer |
| MEGAN | MEtaGenome Analyzer |
| NucA | Nuclease A |
| NucB | Nuclease B |
| OD | Optical density |
| PAGE | Poly acrylamide gel electrophoresis |
| PB | Plackett-Burman |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PI | Propidium iodide |
| RNA | Ribonucleic acid |

| | |
|---|---|
| rpm | Revolutions per minute |
| RSM | Response surface methodology |
| RT | Room temperature |
| SDS | Sodium dodecyl suphate |
| TE | Tris EDTA |
| U/mg | Units/milligram |
| U/ml | Units/millilitre |
| UV | Ultra violet |
| v/v | volume/volume |
| w/v | weight/volume |
| µg | microgram |
| µl | microliter |
| µM | micromolar (micromol/litre) |

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# Chapter 1 Introduction

## 1.1 MICROBIAL BIOFILMS

Historically, microorganisms have been viewed as planktonic, free living single cells. However, microorganisms in natural, medical and industrial systems are found attached to surfaces in an extracellular polymeric matrix (EPM), as biofilms, a life-style with many advantages over life as a free living microbe (McDougald *et al.*, 2012). Organisms growing within biofilms exhibit distinct phenotypic characteristics at the gene transcription level (Elias and Banin, 2012). As early as the 1930s, Zobell (1931) described the existence of bacteria as sessile communities. This was the first research study to understand the communal behaviour of bacteria attached to surfaces (Lappin-Scott, 1999). After that, the development of a uniform film of organisms on a microscopic glass slide was demonstrated in fresh water (Henrici, 1933; Henrici and Johnson, 1935). However, the word 'biofilm' was only coined in 1978 by microbiologist William Costerton during his studies on the attachment of sessile bacteria (Costerton *et al.*, 1978).

Over the past few decades, these sessile communities were shown to be the cause of many persistent bacterial infections. This is due to their increasing innate resistance to antibiotics and host immune attack (Arciola *et al.*, 2012). Researchers have been working on the opportunistic pathogens such as *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus mutans* which attack and infect any compromised human immune system. These organisms are as models to grow biofilm and identify those factors that are responsible for biofilm formation (Davey and O'Toole, 2000). Additionally, bacterial biofilms are problematic on in-dwelling medical devices such as synthetic joints and catheters (Stephens, 2002; Vertes *et al.*, 2012). Biofilms were also identified to be problematic in water reclamation systems and drinking water filtration systems where pathogenic bacteria and fungi thrive within biofilms and can contaminate water treatment systems (Siqueira *et al.*, 2011). In many industrial settings such as brewing and food, biofilms are found attached to the surface of production platforms and pipe lines which leads to fouling and scale formation (Ludensky, 2003; Gunduz and Tuncel, 2006; Brooks and Flint, 2008;

Storgards, 2009). To counteract these challenges, it is necessary to review microbial development of biofilms, to understand biofilm structure and the role of extracellular polymeric substances and to implement urgently needed novel biofilm control strategies.

### *1.1.1 Development and growth of biofilms*

Microbes are able to form biofilms when there is flow of nutrients and a surface to which they can adhere (O'Toole *et al.*, 2000) and also in many cases unfavourable environmental conditions will result in biofilm formation (Harmsen *et al.*, 2011). Microbes undergo a transition from planktonic to sessile community-based survival in which they interact with friendly and unfriendly neighbours of various species. Biofilm development can be categorised into five stages (Figure 1.1).

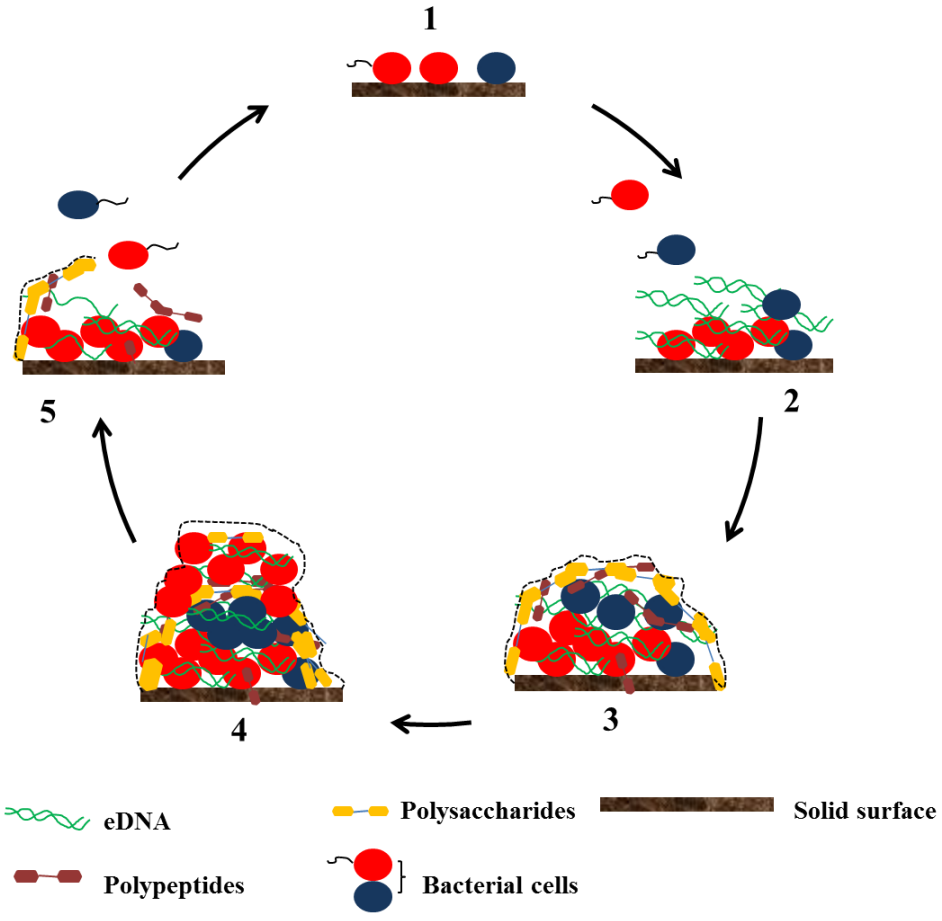



**Figure 1.1 The stage-wise biofilm development cycle 1. Individual free living cells attaching surface as monolayer, 2. EPS produced by cells, microcolony formation and sessile growth, 3. Early development of biofilm with build-up of EPS matrix, 4. Complex biofilm architecture and maturation & 5. Dispersal and release of single cells. Modified from Rendueles and Ghigo (2012)**

Stage 1 (Initial attachment of planktonic cells) – the free living cells attach to the surface as monolayer, where there is a reversible interaction between cell and surface mediated by various non-specific covalent bonds or electrostatic forces. Stage 2 (Microcolonisation and sessile growth) – cells form a multilayer, covered by host and tissue-specific adhesins such as extracellular DNA (eDNA). Stage 3 (Biofilm developed with EPS matrix) – the cells growing into the sessile microcolony lead to biofilm development with EPS components such as polysaccharides and polypeptides which makes the process irreversible, Stage 4 (Maturation) – biofilm attains complexity in its architecture; and Stage 5 (Biofilm dispersal and cell detachment) – dispersal of the biofilm and cells detach and are dispersed into other new environments.

### *1.1.2 Biofilms in the natural environment*

Microorganisms are most prevalent in biofilms in natural environments such as hydrothermal vents (Guezennec *et al.*, 1998) and hot springs (Davey and O'Toole, 2000; Boomer *et al.*, 2009). When a clean surface is submerged into the sea, it attracts bacteria that attach and colonise the surface forming a biofilm. This was observed in *E. coli* and *V. cholera* which showed surface attachment in marine habitats, ranging from free floating aggregates to the surfaces of the ballast tanks of ships (Shikuma and Hadfield, 2010). In a natural habitat, mixed-species contribute to mixed biofilms that compete, co-operate and communicate with each other for their survival under fluctuating and harsh conditions with exogenous stresses such as extreme temperatures and pH, lack of nutrients and UV exposure (Foster and Bell, 2012). Hence, mixed-species interactions may cause alteration in the physiological and regulatory elements within biofilm dwelling bacteria, and this can eventually lead to better adapted strains. These strains adapt to other difficult conditions, including chemical warfare agents secreted by predators, natural product inhibitors and the ability to be motile within the biofilm and to escape from the biofilm (Matz *et al.*, 2008). This is due to their plasticity and horizontal gene transfer ability. Gene transfer enhances the associated bacteria carrying the mobile genetic elements of the same or different bacterial origin which alter their phenotype. Thus, mixed-species biofilms are a dominant life-style in nature. Multi-species biofilms are also prominent in the human host, for instance in the oral cavity and the lungs of cystic fibrosis (CF) patients (Gustave *et al.*, 2012).

### *1.1.3 Biofilms from a clinical perspective*

Many advanced technologies used in medical treatments are able to solve complex therapeutic problems. However, the invasive nature of these techniques creates other challenges including the potential for biofilm formation, for example on medical devices and catheters. Biofilm derived infections are rarely resolved by host immune defence mechanisms and are difficult to eradicate. Growth within a biofilm can lead to up to a thousand fold increased resistance to antibiotic treatment compared to the same planktonic organisms (Davey and O'Toole, 2000). More than 60% of nosocomial infections such as those associated with pneumonia, Hickman catheters, mechanical heart valves, urinary catheters , vascular grafts, and contact lenses are caused by biofilms of *P. aeruginosa, S. aureus*, *S. epidermidis* and *E. coli* (Guggenbichler *et al.*, 2011; Arciola *et al.*, 2012).

Device related infection was first reported as the consequence of biofilm formation in clinical infections due to host inflammatory response (Habash and Reid, 1999). Bacterial biofilms on the skin or in the oral cavity enter the blood stream and colonise implanted valves (Ramage *et al.*, 2006). Guggenbichler et al., (2011) recently reviewed the causes, frequency and preventive measures required for nosocomial infections associated with implantable medical devices. Other infections have been reported, such as from dental plaque, where acidogenic Gram positive *Streptococcus mutans* and *Streptococcus salivarius* can cause tooth decay and periodontitis can be caused by other Gram negative oral bacteria such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Mohammed *et al.*, 2013). Studies on the oral cavity by Kolenbrander (2000) intriguingly describe specific interspecies interactions. A study by Jakubovics *et al.* (2008) on a systemic search of *Streptococcus gordonii* regulated genes by DNA microarray showed co-aggregation with *Actinomyces naeslundii* in response to interaction in multi-species dental plaque biofilm.

In cystic fibrosis patients, the presence of biofilms was noticed in the airway that demonstrated the severity of the lung infection caused by *P. aeruginosa* colonisation (Gustave *et al.*, 2012). Eventually, biofilms alter their phenotype causing different responses and antibiotic therapy has no effect on them (Hall-Stoodley *et al.*, 2004). This was also exemplified in an opportunistic pathogen *Acinetobacter baumannii*, where

clinical isolates from a biofilm mode of growth produced a high mortality rate in an insect model, *Galleria mellonella*, compared to planktonic cells and showed reduced sensitivity to antibiotics (Wand *et al.*, 2011). Hence, from a clinical perspective biofilms are a persistent cause of many human infections and therefore detrimental. These microbial communities within biofilms can change the composition of extracellular polymeric substances (EPS) and it is crucial to understand biofilm structure and the composition of the biofilm matrix.

### *1.1.4 Biofilm structure and the role of the extracellular polymeric matrix (EPM)*

A biofilm is comprised of mostly water (97 %), microbial cells (archaea, bacteria and eukaryotic microbes such as yeast or fungi) embedded in an extracellular polymeric matrix (EPM). Their behaviour is strongly influenced by variables such as ecological diversity and nutrient concentrations (Davey and O'Toole, 2000). EPM is made up of polysaccharides, proteins, nucleic acids and lipids and is considered the "house of biofilm cells" (Flemming *et al.*, 2007). EPM acts as the main factor providing favourable conditions for cells to thrive in a biofilm microenvironment. For example, it supports growing cells with nutrients and maintains the stability and density of the biofilm.

Biofilms form a monolayer when they come in contact with a surface enabling cell-surface interactions. At this stage biofilms are very pervasive both in natural environments and in interactions between the pathogen and human host (Flemming and Wingender, 2010). There are various adhesive structures that enable microbial attachment to surfaces such as the flagellum, pili, synthesized adhesins, and specific adhesions (Flemming *et al.*, 2007; Izano *et al.*, 2008). Czaczyk (2007) described the various matrix constituents and their function, enabling the formation of biofilms. Additional information on the availability of anti-biofilm compounds that could be used to prevent biofilm formation or disperse existing biofilm, in various environments was provided by Karatan (2009) and Flemming (2010).

Exopolysaccharides are known as the main structural component anchoring the cells within the biofilm onto the attached surface. There are several extracellular polysaccharides which are diverse structurally and functionally and they can be available in many forms including cell-bound capsular polysaccharides, unbound 'slimes' and the O-antigen component of lipopolysaccharide. The capsular and lipopolysaccharrides have enormous complexity in their structure and have a strong association with pathogenicity (Branda *et al.*, 2005). Extracellular polysaccharides are composed of chained monosaccharides linked by glycosidic bonds forming homo or heteropolymers. The best characterized polysaccharides in EPS matrices are: alginate, which is a heteropolysaccharide produced by *P. aeruginosa* and *Azotobacter vinelandii*; and β- 1, 6-*N*-acetyl-D-glucosamine (PGA or PNAG) produced by *Aggregatibacter actinomycetemcomitans, E. coli, S. epidermidis, S. aureus* and *Yersinia pestis* (Karatan and Watnick, 2009). Diversity of exopolysaccharides in biofilms, their structural and functional properties, and the interaction of polysaccharides within biofilms provide protection for cells contributing to single and multiple species within the biofilm microenvironment (Sutherland, 2001). However, their production is not required for initial attachment and they appear to be released during the irreversible attachment stage. This was demonstrated in *P. aeruginosa,* when a non-motile subpopulation required two extracellular polysaccharides (Pel and Psl) and biosurfactants to form micro-colonies, after initial attachment (Yang *et al.*, 2012a). Hence, the main function of this component in biofilms is constructive and in multilayer formation.

Extracellular proteins such as biofilm associated protein A (BapA): a large cell-surface protein, have been shown to be required for biofilm formation of *Salmonella enterica* serovar *enteritidis*. The expression of Bap A is coordinated with that of genes coding for proteinaceous appendages such as curli fimbriae and cellulose (Latasa *et al.*, 2005). Bap multidomain proteins were also reported to promote the adhesion of *S. aureus* but prevent entry into epithelial cells (Valle *et al.*, 2012). However, in another study the importance of Bap was shown in addition to eabling biofilm persistence of *A. baumannii* in medical relevant surfaces. Bap also play a role in adherence to both normal human bronchial epithelial cells and normal human neonatal keratinocytes (Brossard and Campagnari, 2012). Other protein compounds that represented the best studied structural proteins in biofilm matrices include glucan binding proteins of *Streptococcus mutans* and lectin like proteins of *Azospirillum brasiliense*, LecA and

LecB of *P. aeruginosa*, extracellular protein TasA of *Bacillus subtilis* and autotransporter proteins (Flemming and Wingender, 2010). These observations are similar to polysaccharides were no other protein compound except for Bap is found in multiple species biofilm formation. The structural composition is also very heterogeneous in nature as some involve other complex structure, such as glucans or lectins (Merritt *et al.*, 2003; Tielker *et al.*, 2005).

An important constituent other than sugars and proteins in the biofilm matrix is extracellular DNA (eDNA). It has an adhesive function to increase the initial attachment of individual cells within the matrix (Kreth *et al.*, 2009) and maintains the increasing mass of growing biofilms. The presence of eDNA as a major structural component in a biofilm matrix was first demonstrated in *P. aeruginosa* (Whitchurch *et al.*, 2002) with the function of a cell-to-cell inter-connecting compound, enabling efficient attachment of cells and strengthening of the biofilm matrix (Flemming and Wingender, 2010). eDNA is essential for initial attachment and it appears to be released during the reversible attachment stage. Studies on bacterial biofilms also showed the mechanism of DNA release into the extracellular space is likely to be favoured by quorum sensing and iron signalling during micro-colony formation (Yang *et al.*, 2012b).

Functions of eDNA in the natural environment show that it acts as an antimicrobial agent and a reservoir for gene transfer leading to increased diversity and microbial evolution (Johnsborg *et al.*, 2007). It is a rich source of nutrients in oligotrophic environments (Mulcahy *et al.*, 2010). Also eDNA is transformed by archaea and bacteria which develop natural competence as shown in *Streptococcus* sp., *Neisseria* sp., and *Pseudomonas* sp., (Zafra *et al.*, 2012). These functions are related to surface attachment and biofilm formation in single and mixed species biofilms (Dominiak *et al.*, 2011). Therefore, eDNA is considered as an essential and universal biofilm matrix component. Goodman et al., (2011) also highlighted eDNA as a common key component in many pathogenic biofilms, and focused on the immune response targeting a common family of nucleoid associated proteins as a potential universal strategy for treating biofilm related diseases. Table 1.1 lists studies that have reported the presence of eDNA in single and mixed microbial populations in natural, clinical, industrial and laboratory biofilms in chronological order. eDNA is clearly emerging as a major component of the macromolecular scaffold of many different biofilms .

**Table 1.1 The widespread presence of extracellular DNA (eDNA) in biofilm formation**

| Name of species | Biofilm studied | Evidence of eDNA as a key component | Reference |
|---|---|---|---|
| *Pseudomonas aeruginosa* | Alginate biosynthesis study revealed majority EPM as eDNA | eDNA presence confirmed colorimetrically showing peak absorbance at 260 nm and DNase I treatment | (Whitchurch *et al.*, 2002) |
| *Streptomyces coelicolor* A3 | Biofilm model system showing mycelial network and pellet formation | Pellets were disintegrated into fragments by DNase I treatment confirming eDNA presence | (Kim and Kim, 2004) |
| *Pseudoalteromonas tunicata* | Marine biofilm | eDNA released due to autolysis observed within cell debris by LIVE/DEAD Bac-light staining | (Mai-Prochnow *et al.*, 2004) |
| *Pseudomonas aeruginosa, P. putida, Rhodococcus erythropolis and Variovorax paradoxus* | Single and mixed biofilms | eDNA extracted and compared with cellular DNA using randomly amplified polymorphic DNA (RAPD) | (Steinberger and Holden, 2005) |
| *Pseudomonas aeruginosa* | Biofilms formed by opporuntistic pathogen | eDNA visualised by fluorescence micrograph using DDAO staining dye | (Allesen-Holm *et al.*, 2005) |
| *Rheinheimera baltica* | Fresh water bacterial biofilm | Epiflourescence micrographs showed filamentous network of eDNA as structural component during biofilm formation | (Bockelmann *et al.*, 2006) |
| *Staphylococcus aureus, Staphylococcus epidermidis* | Biofilm in nosocomial infections | eDNA presence revealed by confocal laser scanning microscopy (CLSM), quantified using real-time PCR and treated using DNase I | (Rice *et al.*, 2007) |
| *Escherichia coli* | Biofilm model to study role of DNA in bacterial aggregation | Exogenous DNA addition increased percentage bacterial adhesion and it was dependent on concentration of DNA, studied using YOYO-1 fluorescent dye eDNA labelling | (Liu *et al.*, 2008) |
| *Enterococcus faecalis* | Opportunistic pathogen causing implant infection due to persistant biofilms | High concentrations eDNA laced among live bacteria present in microcolony visualised by CLSM | (Thomas *et al.*, 2008) |
| *E. coli, Haemophilus influenzae, Klebsiella pneumoniae, P. aeruginosa, S. aureus, Streptococcus* | Effect of DNase and antibiotics treatment on biofilms | eDNA was extracted and quantified with an average size of 30 kb in the biofilm matrix and treated using DNase I | (Tetz *et al.*, 2009) |

| | | | |
|---|---|---|---|
| *pyogenes and Acinetobacter baumannii* | | | |
| *Streptococcus gordonii, Streptococcus sanguinis* | Biofilms of oral commensals | eDNA quantified by real-time PCR and identified by fluorescence micrograph using DAPI staining dye | (Kreth *et al.*, 2009) |
| *Bacillus cereus* | Pellicle biofilm in natural environment | eDNA detected on glass wool fibres by CLSM using propidium iodide and DAPI dyes | (Vilain *et al.*, 2009) |
| *Rhodovulum* sp., *Acinetobacter* sp., *S. aureus ATCC 25923*, *P. aeruginosa PAO1* and *E. coli* K-12 | Biofilms developed for conducting eDNA extraction | eDNA extracted from biofilms using filtration and treatments with gylcanase, dispersin B, proteinase K and analysed by RAPD | (Wu and Xi, 2009) |
| *Acidovorax temperans* | Activated sludge biofilm | By attempting to isolate polysaccharides from biofilm, eDNA was isolated and DNA role is cell attachment was confirmed by treatment with and without DNase I | (Heijstra *et al.*, 2009) |
| *Aeromonas hydrophila* | Role of TapY1 and PlsB in biofilm formation | High concentration eDNA found in supernatant of tapY1 mutant culture and in pelleted cells of wild type cultures | (Khan, 2009) |
| *Nitrosomonas oligotropha, Nitrospira, Thauera, Curvibacter, Competibacter. Comamonas denitrificans* | Nitrifiers and Denitrifiers in waste water treatment plants | eDNA consituted 9-50% to the extracellular polymeric matrix extracted from bacterial biofilms | (Andersson *et al.*, 2009) |
| *Helicobacter pylori* | Biofilm in gastroduodenal pathogenic infection | eDNA was detected and characterised in biofilm matrix by RAPD analysis, its role in attachment was evaluated by DNase I treatment | (Grande *et al.*, 2010) |
| *Neisseria meningitidis, Neisseria gonorrhoeae* | Obligate human pathogens forming meningococcal and gonococcal biofilms | Reduced autolysis in mutants developed and reduced DNA secretion indicated eDNA presence during formation of gonococcal biofilms. Menigococcal biofilms showed eDNA presence in CLSM and DNase I treatment | (Lappann *et al.*, 2010) |
| *Shewanella oneidensis* MR-1 | Marine biofilm | eDNA was extracted and measured fluorometrically using PicoGreen dye and two extracellular nucleases associated to cell envelope showed eDNA degradation in biofilms | (Godeke *et al.*, 2011) |

| | | | |
|---|---|---|---|
| *Bordetella bronchiseptica,* | Persistant biofilm colonization mammalian respiratory tract | DNase I addition during initial biofilm growth inhibited biofilm formation indicated DNA as structural component and eDNA shown by DDAO staining appeared in CLSM | (Conover *et al.*, 2011) |
| *Vibrio cholerae* | Diarrhoeal disease causing pathogenic biofilms | Extracellular nuclease Xds and Dns deleted mutants increased biofilm formation indicated eDNA presence. eDNA extracted, quantified and visualised by agarose gel analysis | (Seper *et al.*, 2011) |
| *Listeria monocytogenes* | Persistant biofilm in food processing plants | Cultures without high molecular weight eDNA demonstrated no adherence to surface and treatment using DNaseI resulted in dispersal of biofilms | (Harmsen *et al.*, 2011) |
| *Acinetobacter baumannii* | Multidrug resistant pathogenic biofilm | eDNA present in membrane vesicles (MV) were found released by transmission electron microscopy and atomic force microscopy. Biofilm augmentation assay using eDNA showed 200% biofilm formation and inhibtion shown by DNaseI treatment | (Sahu *et al.*, 2012) |
| *Borrelia burgdorferi* | Causative agent of Lyme diseases | Biofilm aggregates stained with DDAO dye detected eDNA in CLSM | (Sapi *et al.*, 2012) |
| *Candida albicans* | Opportunistic pathogen, biofilm in implant infections. | DNaseI treatment dispersed existing biofilms visualised by scanning electron microscopy, which also increased biofilms susceptibility to antifungal treatments | (Martins *et al.*, 2012) |
| *Streptococcus pneumoniae* | Human pathogen | eDNA visualised by fluorescent labelling demonstrated using CLSM | (Domenech *et al.*, 2012) |
| *Myxococcus xanthus* | Natural biofilm formed by soil bacterium | eDNA visualised in membrane chamber system through up-right fluorescence microscopy using PI dye | (Hu *et al.*, 2012) |
| *Coagulase-negative staphylococci, Staphylococcus auerus and alpha-haemolytic streptococci* | Bacterial biofilms associated with Chronic Rhinosinusitis | eDNA extracted and quantified using Picogreen fluorescence dye and visualised by CLSM. Bbiofilms treated with NucB showed dispersal confirmed eDNA presence | (Shields *et al.*, 2013) |
| *Streptococcus intermedius* | Biofilms formed commensal bacterium | eDNA presence and co-localisation visualised by DDAO dye staining, DNaseI treatment on biofilm growth inhibited biofilm formation | (Nur *et al.*, 2013) |

| | | | |
|---|---|---|---|
| *Salmonella enterica* serovar *typhimurium* | Biofilm formed by enteroinvasive bacterial pathogen | Biofilms cultivated in flow chambers and glass surfaces stained with Toto-1 which showed accumulation of eDNA fibres extending from an aggregate | (Johnson *et al.*, 2013) |
| *Aspergillus fumigatus* | Biofilms associated with adaptive antifungal resistance | Fluorescence microscopy and quantitative PCR demonstrated eDNA presence. RAPD analysis showed eDNA identical to genomic DNA | (Rajendran *et al.*, 2013) |
| *Bdellovibrio bacteriovorus* | Biofilm formed by predatory bacterium | Nucleic acid stained by Hoechst 33372, a dye that stain DNA and visualised by phase contrast and fluorescent microscopy | (Lambert and Sockett, 2013) |
| *Reinheimera* sp. F8, *Pseudomonas*, *Serratia*, and *Microbacterium* | Biofilm formation by environmental isolates and eDNA adhesion | eDNA visualised by fluorescence microscopy and quantified using PicoGreen labelling | (Tang *et al.*, 2013) |
| *Burkholderia cenocepacia* | Biofilm associated to cystic fibrosis | eDNA visualised using immunolabelled light microscopy and staining with film tracer FM1-43 | (Novotny *et al.*, 2013) |
| *Gardnerella vaginalis* | Biofilm related to bacterial vaginosis | eDNA extracted and quantified using UV-Vis spectroscopy and eDNA degraded DNase treatment | (Hymes *et al.*, 2013) |

The origin of eDNA in biofilms is unknown but may arise as shown in Figure 1.2. Cell lysis provides a simple mechanism for releasing DNA into the biofilm (Barnes *et al.*, 2012) as shown in Figure 1.2A. In some cases, active mechanisms for initiating the lysis of a proportion of a cell population contribute to eDNA release (Grande *et al.*, 2010). The existence of autolysis mechanisms has been known for many years, but their importance in releasing DNA and modulating biofilm formation is only beginning to be appreciated. Gram negative bacteria release eDNA either via cell lysis by prophage induction or it is produced through membrane vesicles and secreted into the extracellular space and it acts as a scaffold for twitching cells to migrate and form mushroom layers in biofilms (Spoering and Gilmore, 2006).

As shown in Figure 1.2B, microbial fratricide is a process by which a proportion of the cells in a population of micro-organisms, upon induction, release a factor that kills their siblings. In the case of *E. faecalis*, fratricide occurs in response to a quorum sensing signal at high cell densities (Thomas *et al.*, 2008).

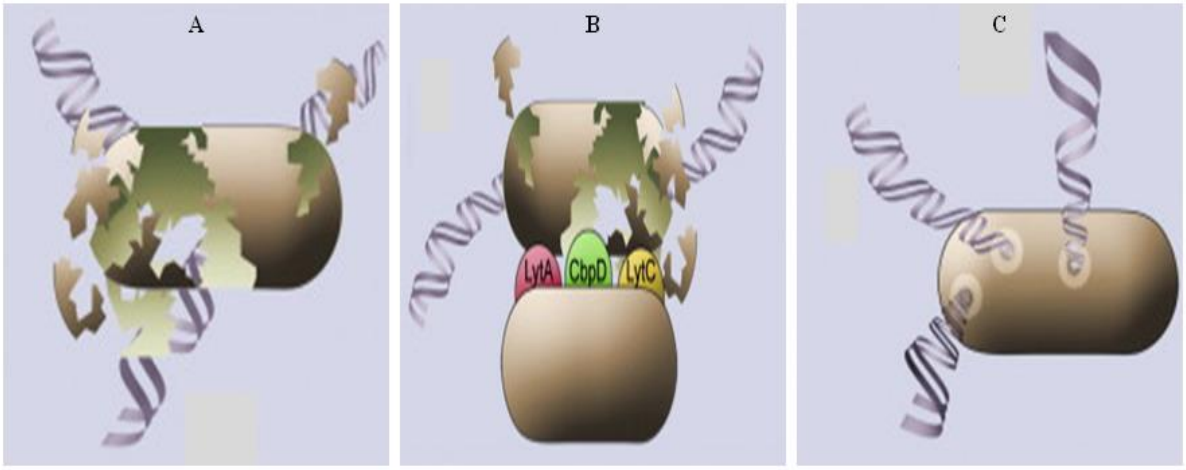



**Figure 1.2 A. Passive release of DNA from bacteria that have died from natural causes, B. Capture of DNA by a fratricide mechanism. Competent *S. pneumoniae* cells kill and lyse non-competent sister cells present in the same environment. They produce murein hydrolases that lyse the cell and release DNA, C. Active release of DNA in *N. gonorrhoeae* mediated by a type IV secretion system related to bacterial conjugation. Modified from Johnsborg *et al.* (2007)**

The active release of DNA fragments via lysis-independent mechanisms has been described in bacteria as shown in Figure 1.2C. For example, many Gram-negative organisms produce membrane vesicles that contain DNA either in the lumen or on the outer surface (Dorward and Garon, 1990; Schooling *et al.*, 2009). In *Neisseria gonorrhoeae*, an unusual type IV secretion system (T4SS) provides an alternative mechanism for the secretion of DNA into the extracellular milieu (Hamilton *et al.*, 2005). Unlike most T4SSs, the *N. gonorrhoeae* system does not require cell-cell contact to function. *N. gonorrhoeae* biofilms are stabilised by an extensive matrix of eDNA (Steichen *et al.*, 2011). At present, the relative contributions of cell lysis, outer membrane vesicle production and the T4SS to the overall matrix composition are unknown (Jakubovics *et al.*, 2013). Further investigations in this area will be important not only for the understanding of the function of eDNA in biofilms, but also for the accurate interpretation of data from studies that employ DNA amplification methodologies to analyse microbial biofilm communities.

## 1.2 BIOFILM DISPERSAL

Intense interest has been shown by researchers investigating the initial stages and the importance of biofilm formation for the survival of organisms in the natural environment. As shown in Figure 1.1, detachment of cells is the final stage of biofilm

development cycle can be caused by external perturbations and by internal biofilm processes which is normally viewed as control measure for biofilm removal. However, biofilm dispersal is an essential step for active biological dispersal, bacterial survival and disease transmission. Natural dispersal occurs in most biofilm environments and changes the structure within the biofilm to initiate dispersal in response to a number of factors including an increase in cell density, hydrodynamics and a reduction in nutrient availability (Stoodley *et al.*, 1999b). The natural dispersal of large numbers of cells from a biofilm is also capable of initiating an infection (Hall-Stoodley *et al.*, 2004). However, this is the least-studied biofilm process and there is still a significant lack of understanding of how this process occurs within a biofilm (Stoodley *et al.*, 2001). Hence, understanding the mechanism of biofilm dispersal is an important step towards identifying and producing biofilm dispersing compounds (BDCs) that have the potential to control biofilms.

### *1.2.1 Biofilm dispersal strategies and mechanism*

Biofilm detachment can be caused by external pressure, such as increased liquid shear, or from an internal biofilm process such as enzymatic degradation of the extracellular polymeric matrix (EPM). The initial target for biofilm dispersal is to break EPM and allow the release of the organism(s) to their planktonic state.

There are three distinct biofilm dispersal strategies described in literature: Strategy 1 is swarming / seeding / hollowing dispersal in which individual cells are rapidly released from hollow cavities that are formed inside the biofilm into the bulk fluid or surrounding nutrient environment. Strategy 2 is clumping / sloughing dispersal in which cells aggregate and large portions of biofilm in later stages of biofilm formation detach and shed as clumps or emboli. Strategy 3 is surface / erosion dispersal in which biofilm structures move across surfaces over the course of biofilm formation (Lappin-Scott and Bass, 2001; Jackson *et al.*, 2002; Stoodley *et al.*, 2002; Mai-Prochnow *et al.*, 2004; Boles and Horswill, 2008).

The mechanisms of dispersal are broadly classified as active or passive. Active means the dispersal is initiated by the bacteria themselves (Boyd and Chakrabarty, 1994; Vats and Lee, 2000; Boles and Horswill, 2008) and passive means the dispersal is mediated

by external forces that may involve fluid shear, mechanical abrasion, human intervention and predator grazing (Stoodley *et al.*, 1999c; Lawrence *et al.*, 2002; Dusane *et al.*, 2008; Chambliss-Bush, 2012).

Strategy 1 is always an active process, strategies 2 and 3 can be either active or passive. Previously, studies have detailed the molecular systems, biology behind the formation and dispersal mechanisms of biofilms. Studies have also emphasised the heterogeneity of the biofilm community and indicated the possible gene, signal and regulatory network that may be of interest for further investigation on biofilm dispersal in problem related areas (Davey and O'Toole, 2000; Hall-Stoodley *et al.*, 2004; Karatan and Watnick, 2009). This research led to search for important factors that can be further investigated and used biotechnologically as biofilm dispersing compounds.

### *1.2.2 Importance of biofilm dispersing compounds*

Microbes have evolved self-dispersal mechanisms to escape from existing biofilms. In a competing environment they can also deliberately release dispersal compounds to avoid the biofilms of the competitors. The possible agents which might be released by microbes in either situation could be signaling molecules or enzymes that degrade matrix compounds. For instance, marine bacterium *Pseudoalteromonas tunicata* was reported to produce an autocidal protein, AlpP, which kills the subpopulation of cells during biofilm development and the active dispersal cells take the ecological advantage of this event for colonisation to new surfaces (Mai-Prochnow *et al.*, 2006). This approach evolved directly or indirectly to repress subpopulation or other multi-species biofilms and this may be an effective method of manipulating biofilm development.

Table 1.2 shows possible genes and gene products involved in biofilm dispersal mechanisms in known organisms and their dispersal spectrum, which may also include other bacterial and fungal species. It also highlights the importance of matrix degrading enzymes (Boyd and Chakrabarty, 1994; Vats and Lee, 2000; Dow *et al.*, 2003; Kaplan *et al.*, 2004a; Mann *et al.*, 2009) and quorum sensing signals that involve regulation of cyclic-dimeric GMP. These are the most powerful tools for biofilm eradication and can have applications from medical to industrial settings.

**Table 1.2 Gene/Gene products involved in active or passive biofilm dispersal mechanism**

| Microorganism | Genes/ gene products responsible for dispersal | Signals/ targets/ active mechanism for dispersal | Dispersal spectrum | Reference |
|---|---|---|---|---|
| ***Matrix degrading enzymes*** | | | | |
| *Pseudomonas aeruginosa* | Alginate lyase | Mucoid matrix-enzymatic degradation | Self | (Boyd and Chakrabarty, 1994) |
| *Streptococcus mutans* | Surface protein releasing enzyme (SPRE) | Surface adhesin P1 -enzymatic degradation | Self | (Vats and Lee, 2000) |
| *Actinobacillus actinomycetemcomitans* | Dispersin B/ N-acetyl glucosaminidase | Hydrolyse glycosidic links in matrix by enzymatic reaction | *S. aureus* and range of G-ve bacteria | (Kaplan *et al.*, 2004b) |
| *Marinomonas mediterranea* | Lysine oxidase (LodA) | $H_2O_2$ production | Range of G-ve bacteria | (Mai-Prochnow *et al.*, 2008) |
| *Staphylococcus aureus* | Thermonuclease & *cidA* – positive regulator of murein hydrolase activity | DNA enzymatic degradation (e-DNA) and cell lysis | *Pseudomonas* sp., *Vibrio* sp. , *Staphylococcus* sp. and *E. coli* | (Mann *et al.*, 2009) |
| *Bacillus licheniformis* EI-34-6 | DNase, NucB | DNA degradation | Self, G+ve, G-ve bacteria | (Nijland *et al.*, 2010) |
| *Staphyloccocus aureus* | Nuclease, Nuc1 | eDNA degradation | G+ve and G-ve | (Tang 2011) |
| *Streptococcus salivarius* | Exo-β-D-fructosidase, fruA | Sucrose digestion | *Streptococcus mutans* | (Rendueles and Ghigo, 2012) |
| *Streptococcus intermedius* | Arginine deiminase | Down regulation of fimbri fimA and mfa 1 | *Porphyromonas gingivalis* | (Rendueles and Ghigo, 2012) |
| ***Surfactants/ Fatty acids*** | | | | |
| *Bacillus subtilis* | Surfactin, sfp locus | Alteration of cell surface interactions | *Salmonella enterica, E. coli, Proteus mirabilis and P.* | (Mireles *et al.*, 2001) |

| | | | | |
|---|---|---|---|---|
| | | | *aeruginosa* | |
| *Pseudomonas aeruginosa* | Rhamnolipids rhlA/ | Surfactant induced | Self | (Davey *et al.*, 2003) |
| *Xanthomonas campesteris* | Cis-11-methyl-2-dodecenoic acid & *Man A* | Enzymatic/ DSF & c-di-GMP regulation | Self | (Dow *et al.*, 2003) |
| *Pseudomonas aeruginosa* | Cis-2-decenoic acid | DSF-like / Fatty acid methyl | G+ve, G-ve & *C. albicans* | (Davies and Marques, 2009) |
| *Bacillus pumilus* S6-15 | 4-Phenylbutanoic acid | Reduced hydrophobicity and EPS | G+ve and G-ve | (Nithya *et al.*, 2011) |
| *Candida sphaerica* | Lunasan | Anti-adhesive biosurfactant | Self | (Luna, 2011) |
| ***Quorum sensing molecules*** | | | | |
| *Escherichia coli* | CsrA induction | Regulates flagellar biosynthesis and quorum sensing signal | Self | (Jackson *et al.*, 2002) |
| *Pseudomonas putida* | Periplasmic protein and transmembrane protein | Starvation induced dispersal c-di-GMP | Self | (Gjermansen *et al.*, 2005) |
| *Pseudoalteromonas tunicata* | Autolytic protein (AlpP) | Biofilm inhibitor and autotoxic cell lysis | G+ve & G-ve bacteria | (Mai-Prochnow *et al.*, 2006) |
| *Pseudomonas aeruginosa* | Biofilm dispersal locus (Bdl A)-Chemotaxis sensory regulator | Phosphodiesterase activity & c-di-GMP level | Diverse bacteria | (Morgan *et al.*, 2006) |
| *Shewanella oneidensis* | yhjH | Phosphodiesterase activity & c-di-GMP / sloughing | Self | (Thormann *et al.*, 2006) |
| *Pseudomonas aeruginosa* | Nitric oxide and Tobromycin Phosphodiesterase | Nitrosative stress / c-di-GMP quorum sensing | Self, G+ve, G-ve and *C. albicans* | (Barraud *et al.*, 2006) |
| *Actinosporanfium vitaminophylum* and *Streptomyces* sp., | Pyrrolomycins C, D, F1, F2a, F2b, F3 | Antibiofilm activity | Staphylococcal biofilms | (Schillaci *et al.*, 2008) |

| | | | | |
|---|---|---|---|---|
| *Staphylococcus aureus* | Aureolysin & AIP | Agr- quorum sensing system | Self | (Boles and Horswill, 2008) |
| *Pseudomonas aeruginosa* | Iso propyl-β- D-thiogalactoside (IPTG) | Affinity with lecA , stop cell-cell interaction | Self | (Karatan and Watnick, 2009) |
| *Pseudomonas aeruginosa* | N-butyryl –L-homoserine lactone | LasI/R and RhlI/R quorum sensing signals | Self | (Karatan and Watnick, 2009) |

The role of quorum sensing in biofilm formation and/or dispersal is ambiguous. Notably, cell to cell communication appeared to encourage biofilm formation robustly in *Pseudomonas aeruginosa* and has an impeding effect on attachment of *S. aureus* biofilms (Boles and Horswill, 2008). Similarly, indole, an interspecies communication molecule, has been shown to encourage biofilm formation in *P. aeruginosa* but inhibits *E. coli* biofilm development (Lee *et al.*, 2007). You et al., (2007) showed that extracts of marine *Streptomyces albus* could disperse the mature biofilms of several marine *Vibrio* species and this was due to the attenuation of quorum sensing signal systems. Advances in cell signalling suggests that it not an incidental but a controlled function (Stoodley *et al.*, 1999a). Therefore, the development of a generally applicable antibiofilm compound based on quorum sensing mechanism must be difficult.

EPS- degrading enzymes, such as alginate lyase produced by *P. aeruginosa,* that degrades alginate, and dispersin B produced by *A. actinomycetemcomitans,* that degrades Poly-N-acetyl glucosamine (PNAG) contribute to bacterial detachment from the matrix (Boyd and Chakrabarty, 1994; Kaplan *et al.*, 2004b). The oral bacterium *Streptococcus intermedius* produces hyaluronidase, an enzyme that degrades the glycosaminoglycan hyaluronan (HA) found in the extracellular matrix of connective tissue (Pecharki *et al.*, 2008). These enzymes have been known for decades in biofilm matrix degradation. However, there is no single polysaccharide that is present in all the biofilms and also the number of species reported containing one particular polysaccharide such as alginate or PNAG is limited. Henceforth, the applicability of these enzymes as antibiofilm enzymes is not universal and demands additional enzymes for effect biofilm removal.

Marine environment is rich and unexplored source for bioactive compounds. In this

research there was access to marine sources such as deep-sea sediments and seaweeds which can be used to look for novel microbial enzymes. Hence, continuous investigation of available marine resources hypothesised to provide a fresh and stable enzyme. Recently, a seaweed associated marine strain of *B. licheniformis* biofilm produced an extracellular DNase, NucB in its supernatant. This is an enzyme that breaks the eDNA in the biofilm matrix and this strategy was used to disperse any established microbial biofilms (Nijland et al., 2010). Another recent study showed that the *S. aureus* secreted nuclease Nuc1 has the ability to degrade nucleic acids and dissolve preformed biofilms of several bacteria, including *P. aeruginosa*, *Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis* (Tang 2011). Further, studies revealed that eDNA degradation by nucleases may hold promising potential in preventing or eradicating biofilms.

***1.2.3***

### *1.2.4 Role of nucleases in biofilms dispersal*

Many bacteria produce extracellular DNase enzymes, which are anchored to the cell wall or secreted into the extracellular milieu. The ability of different strains to produce DNases does not appear to correlate with reduced dependence on eDNA for biofilm formation (Shields *et al.*, 2013). However, studies with isogenic mutants have provided evidence that extracellular nucleases keep the growth of biofilms in check. In *Shewanella oneidensis*, the disruption of genes encoding two extracellular nucleases, ExeS and ExeM resulted in altered biofilm formation and the accumulation of eDNA (Godeke *et al.*, 2011). Disruption of a third nuclease-encoding gene, *endA*, had little effect on biofilm structure (Heun *et al.*, 2012). Initially (since about 2000) eDNA degradation was shown only by bovine pancreatic DNase I and human DNase I whereas in recent years reports on microbial nucleases are increasing. These nucleases are secreted within a biofilm environment capable of degrading eDNA in a biofilm matrix as shown in Figure 1.3. Enhancing biofilm dispersal have been demonstrated in many genera such as *Bacillus*, *Bdellovibrio*, *Shewanella*, *Pseudomonas*, *Staphylococcus*, *Vibrio* and *Listeria* (Nijland *et al.*, 2010; Godeke *et al.*, 2011; Harmsen *et al.*, 2011; Seper *et al.*, 2011; Tang 2011; Lambert and Sockett, 2013). Hence search and discovery of microbial nucleases would be a promising eDNA target based dispersal approach in this emerging research area.

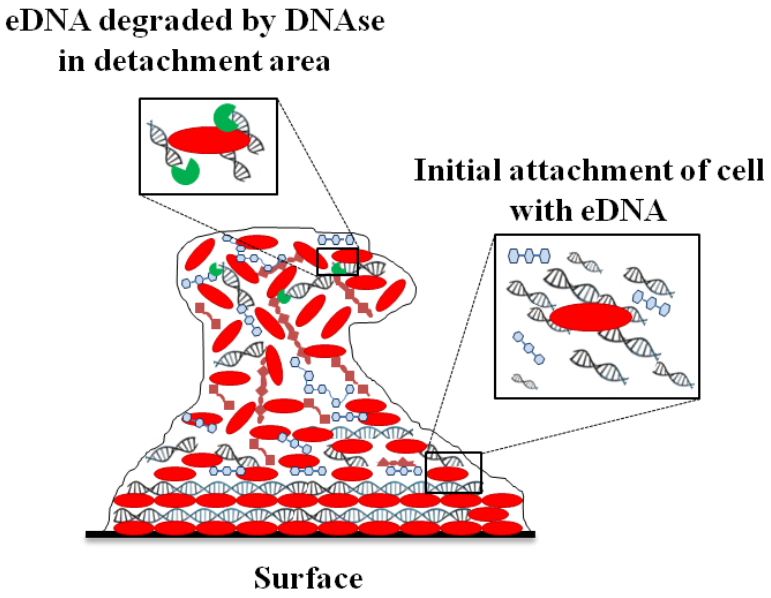



**Figure 1.3 Pictorial representation of the cells within a biofilm matrix. The cells release extracellular DNA (right bottom magnified area, showing eDNA as double helix spiral structures) to attach initially on to solid surface, following which other matrix producing cells start accumulating. In an aged biofilm, some cells produce matrix degrading enzymes which includes DNase (Top left magnified area, showing DNase as green pacman structure) which breaks down the eDNA and allow cells to disperse. Modified from (Lembre *et al.*, 2012)**

This novel antibiofilm approach forms the fundamental basis of research carried out in this thesis. Nucleases belong to a general enzymatic group of hydrolases and cleave the phosphodiester bonds of nucleic acid molecules (Kumar and Kannan, 2011). They are widespread in prokaryotes and eukaryotes, and diverse, based on localization, structure, function and mode of action (Yang, 2011). Nucleases functions were widely understood inside a cell related to DNA replication and repair, RNA maturation, restriction and molecular applications (Marti and Fleck, 2004). Nevertheless, limited information is available on the diversity and importance of extracellular nucleases in the natural environment.

In the early days of research, several different types of extracellular nucleases have been identified and produced by diverse groups of microbial communities (Alexander *et al.*, 1961; Heins *et al.*, 1967; Nestle and Roberts, 1969; Porschen and Sonntag, 1974). However, this was studied extensively in cultured microorganisms and very little is known about these systems and their roles in unculturable taxa. To extend our knowledge in this research area, in Chapter 2 data mining was conducted across the

whole genomes and metagenomes available in the public databases and diversity of extracellular nucleases was studied.

Though there are studies which provide information about enzymes involved in biofilm dispersal studies their production and scale up strategies have not been reported yet. The lack of understanding in development technology is the missing link for any new bio-product (Zaborsky, 1999). The main reason behind this the scale up of process from a laboratory scale bioreactor and then to industrial scale is a challenging task and needs understanding on process characterisation. Also, it is necessary to achieve growth conditions that will express the enzymes at optimum level (Egan *et al.*, 2008). Notably, biomass concentration and the expression level of bioactive products in marine microorganisms were minimal, when such fermentations were compared to conventional production (van der Wielen and Cabatingan, 1999). Thus, the natural marine environment results in low harvesting possibilities when organisms are rare or not living under ambient conditions. Hence, one section of the research carried out in this thesis involves bioprocess development of microbial nucleases which have potential biotechnological applications.

## 1.3 BIOPROCESS DEVELOPMENT FOR OPTIMISED NUCLEASE PRODUCTION

Many products for the pharmaceutical and industrial application are nowadays produced by biotechnological processes, increasingly replacing existing chemical processes (Thiry and Cingolani, 2002). Ideas for innovative products and new ways for their production on a small scale have to be transformed into complete processes at a large scale (Kennedy and Krouse, 1999). Production costs, working volume, effective scale-up, product yield, waste, downstream processing and many other points have to be optimised before any process can be used effectively in industry. This involves consideration of critical process parameters at the process level (Chu and Robinson, 2001). In recent years, this has been extended to optimisation at cellular levels involving gene transcription and proteomics (Nijland *et al.*, 2007). In this thesis, the focus is placed on the optimisation and scale-up of upstream processes of nuclease production which includes process parameters, growth and cell physiology driven approach, media design as well as high cell density culture conditions.

Process optimisation is a topic of central importance in production of microbial enzymes. With particular regard to biotechnological production processes, in which even small improvements can be decisive for commercial success, process optimisation is presently an undisputed component of the agenda of any commercial concern (Queiroga *et al.*, 2012). In bioprocessing, improvements in the productivity of the microbial metabolite are achieved, in general, via the manipulation of nutritional and physical parameters and by strain improvements as the result of mutation and selection (Albermann *et al.*, 2013). These measures can alter the product yield significantly. Statistical methodologies are generally regarded as advantageous as they reduce the number of experimental runs necessary to understand the effect of an essential variable (Cho *et al.*, 2009; Kumar *et al.*, 2010). Thus, in this thesis design of experiment tools, Placket-Burman (P-B) design and Central composite design (CCD) using statistical software packages such as Minitab 16.0 and Design Expert 8.0 described in Chapter 3, were used to find the essential media components and their optimum levels for enhanced microbial nuclease production.

The key behind bioprocessing high value bioactive compounds is the fundamental understanding of the biology and molecular activity of the gene/ gene product of interest (Sameera, 2011). Literature studies showed the involvement of host expression systems which are already well-characterised and therefore reducing the cost and time requirement for the development of an economically viable production strategy. The cultivation and further downstream processing for formulation and commercialization is simplified by choosing a suitable heterologous host system that can over-express the bioproduct and therefore maximise its yield (Terpe, 2006; Lefevre *et al.*, 2007). Powerful tools for engineering the genetic and cellular components in hosts enable researchers to choose and construct better expression systems (Shojaosadati *et al.*, 2008).

### *1.3.1 Bacillus subtilis as a heterologous host expression system*

*Bacillus subtilis*, a rod shaped Gram positive bacterium, naturally produces various proteins into the medium at high concentrations (Fu *et al.*, 2007). It is considered as a GRAS (generally recognised as safe) organism by FDA compared to *E. coli*, a Gram

negative bacterium (Nijland *et al.*, 2007). Its major advantage is that the *B. subtilis* outer membrane has no lipopolysaccharides (LPS), endotoxins, commonly produced by *E. coli* which are pyrogenic in humans and other mammals (Terpe, 2006). Another disadvantage of *E. coli* is that it does not naturally secrete high amounts of proteins (Mergulhao *et al.*, 2005). Producing secreted protein is advantageous to simplify downstream processing steps. Thus, major scientific research on recombinant protein production and commercialisation exploits *B. subtilis* as a cell-factory for secreted heterologous proteins of interest (Pohl *et al.*, 2010)

There are numerous studies and reviews on heterologous protein secretion using this host system highlighting a number of possible bottlenecks which need optimisation of the expression host-vector construction. (Wong, 1995; Vuolanto *et al.*, 2001; Li *et al.*, 2004; Fu *et al.*, 2007; Wu *et al.*, 2007). For instance, over-production of a specific protein or metabolic pathway can lead to the death of the host and therefore a strong inducible promoter was developed for *B. subtilis*. Subtilin inducible (Bongers *et al.*, 2005) or IPTG inducible promoters (Shojaosadati *et al.*, 2008) are the most commonly used promoters of this kind. In addition, advanced molecular genetics and genetic engineering technology are readily available for *B. subtilis* which will increase the possibility of constructing heterologous expression systems for enhanced production of nucleases and scale-up to commercialisation.

### *1.3.2 Strategies for optimisation of cultivation conditions*

For any production system development the key requirement is to achieve high volumetric productivity (gram of product per litre in unit time) and significant cost cutting strategies. In engineering principles, volumetric productivity is proportional to final cell density and specific productivity. Thus high volumetric productivity is very much feasible if a production system can be developed aiming to use high cell density cultivation techniques and therefore will have a positive impact in decreasing other miscellaneous costs on energy consumption, equipment handling, waste water management and man power as well as enhanced downstream processing (Vuolanto *et al.*, 2001; Wu *et al.*, 2007). As discussed in section 1.3.1, heterologous host expression systems for BDC using *B. subtilis* can make these conditions possible and high cell density cultivation may be very simple and inexpensive.

Besides strain development, designing a suitable cultivation method is crucial to attaining high cell density and efficient BDC productivity (Shojaosadati *et al.*, 2008). Therefore, another important research focus in this study is to look into the cultivation parameters such as: medium composition, inoculum, pH, temperature, aeration, agitation and shear stress for optimisation from shake flask to reactor scale. Optimisation of parameters using statistical design of experiments such as Plackett-Burman and response surface methodology (Johnson *et al.*) plays a significant role in the production of various biotechnological products (Park *et al.*, 1992; Vuolanto *et al.*, 2001; Lau *et al.*, 2004; Chen *et al.*, 2007b; Wu *et al.*, 2007). Cultivations can be performed using batch, fed-batch or continuous cultivation methods. However, the continuous mode of operation introduces potential problems in the manufacture of pharmaceutical products using recombinant systems due to potential issues with strain stability. On the other hand, batch cultivations are relatively simple and robust, but reaching high cell-density is possible only in the fed-batch mode of operation, under controlled conditions and there is requirement to monitor the batch to batch variability for reproducibility (Thiry and Cingolani, 2002). Notable high cell density production systems achieved using *B. subtilis* as host are summarised in Table 1.3.

**Table 1.3 High cell density cultivation of recombinant bio-products and their production strategy using *B. subtilis* expression system**

| **Product** | **Cell density** | **Productivity** | **Cultivation mode** | **References** |
|---|---|---|---|---|
| Phytase | 56 g/l | 47.7 U/ml | Fed-batch (Glucose concentration controlled) | (Vuolanto *et al.*, 2001) |
| Staphylokinase | OD 28.4 | 255 mg/L | Fed-batch (sucrose feeding at 100 g/h) | (Kim, 2001 #554) |
| Amylase | 17.6 g/l | 41.4 U/ml | Fed-batch(exponential feeding) | (Huang *et al.*, 2004) |
| Penicillin G acylase | OD 60.1 | 1960 U/ml | Fed-batch (pH stat) | (Zhang *et al.*, 2006) |

| | | | | |
|---|---|---|---|---|
| Riboflavin | -- | 16.36 g/L | Fed-batch (glucose limited) | (Wu *et al.*, 2007) |
| β-galactosidase | 184 g/L | 157 g/L | Fed-batch (pH stat) | (Shojaosadati *et al.*, 2008) |
| Subtilisin | OD 65 | 6.19 U/ml | Fed-batch (exponential feeding) | (Shojaosadati *et al.*, 2008) |
| Phytase | 35.6 g/L | 28.7 U/ml | Fed-batch (controlled glucose concentration) | (Shojaosadati *et al.*, 2008) |

Eventually, in this thesis high cell density batch cultivation of recombinant *Bacillus subtilis* was carried out to achieve increased nuclease production for further characterisation studies. Nevertheless, there are common problems encountered in production experiments such as the choice of cultivation medium suitable for the particular strain. Such a study will only be possible for lab scale as it is not cost effective in industrial settings. Therefore, in this thesis, challenges of media design have been addressed that can be scaled up for industrial production of nucleases.

## 1.4 RESEARCH AIMS AND OBJECTIVES

The extracellular DNA is a target for an extracellular nuclease enzyme that can lead to either dispersal or inhibition of biofilms suggesting that it may provide extremely useful and novel therapies. There is a gap existing in the knowledge of extracellular nucleases diversity, their functional role, production and characterisation. Accordingly, this central research theme was addressed in this thesis in order to develop microbial nuclease enzymes as potential anti-biofilm compounds.

The main research objective of the thesis was to investigate the diversity of microbial extracellular nucleases and to develop bioprocess systems that enhance the production of extracellular nucleases to use in biofilm dispersal application.

The aims of this study are:

- To investigate the extracellular nuclease producing capability of bacteria isolated from diverse environments and to determine the diversity of microbial populations with extracellular nucleases activity using sequence driven data mining.
- To develop cultivation systems that enhance the production of the extracellular nuclease, NucB, produced by the marine isolate *B. licheniformis*
- To further enhance the production of NucB using the recombinant expression host, *B. subtilis*, and to purify and characterise the enzyme.
- To optimise production of nucleases in diverse bacteria.
- To investigate the efficacy of extracellular nucleases as potential anti-biofilm enzymes.

Thus, to meet out these objectives the thesis has been structured to review in chapter 1 the current and emerging biofilms related problems and the importance of microbial extracellular nucleases as emerging solution for biofilm dispersal which is the main research theme of this thesis.

Chapter 2 then investigated the diversity of microbial extracellular nuclease producers both by culturable and sequence driven approach and highlighted the ecological implications of nuclease secretion.

In order to address the objective on the bioprocess development of nuclease production chapter 3 elaborated the ways of improving NucB, an extracellular nuclease production in marine strain of *B. licheniformis* using physiological understanding of strain behaviour and process optimisation technique.

In chapter 4 the production of NucB was enhanced using high cell density cultivation of recombinant *B. subtilis* and further characterised for biofilm related applications. Further to increase the knowledge on nuclease production process chapter 5

concentrated on production optimisation of nuclease in diverse bacteria such as *Bacillus* sp.NR-AV-5, *Streptomyces* sp.NR-Sr-1 and *Bacillus* sp.NR-T-2.

Chapter 6 demonstrated the potential for extracellular nucleases to disperse single species biofilms tested on various surfaces and

Chapter 7 concluded the overall results obtained in this thesis with suggested future work.

# Chapter 2 Diversity of extracellular nucleases across microbial communities

## 2.1 INTRODUCTION

In Chapter 1, the structural constitution and role of extracellular polymeric matrix of biofilms was reviewed in section 1.1.4, providing the details of already existing polysaccharides, proteins and the recent studies of eDNA in single and mixed species biofilms. Also, the importance of biofilm dispersing compounds was reviewed in section 1.2.2 and the discovery of dispersal compounds including the matrix breaking enzymes was presented in Table 1.2. It was suggested that a mixture of matrix degrading enzymes will be advantageous to completely remove biofilms due to the complex and varied nature of biofilms.

Nevertheless, in biofilm matrix where eDNA is present, DNase 1 is most widely used as shown in Table 1.1. This supports the argument that eDNA is the key matrix component which has a universal structure and its mechanism of degradation does not differ in any biofilms. Hence, eDNA can be an easy target compared to other matrix components to deal with the major problems of biofilms. In recent years, there had been several studies (reported in Chapter 1, section 1.2.3) which showed the microbial nucleases having a role in biofilm matrix and dissolution action on preformed biofilms and also in the eradication of biofilm formation. These studies hypothesise that microbial extracellular nucleases will be useful as an antibiofilm compound and hence, it is important to understand the diversity of microbial extracellular nucleases and their functional role in biofilms.

However, only 1 in 100 bacteria from natural environment is culturable and to increase the rate of discovery of novel biomolecules in recent years metagenomic approach has been employed. It enables to attain positive and novel hits of enzymes available from the dataset of sequences of microbial genomes to include 'unculturable' taxa (Elend *et al.*, 2006). Mining for genes of novel enzymes through sequence-based database searches using bioinformatics tools will have greater advantage and it will be less time consuming than function-based methods (Kennedy *et al.*, 2008). Another advantage of this approach is that, it will allow understanding of the diversity of extracellular nucleases available across the microbial community.

Hence, this chapter aims to investigate the diversity of culturable extracellular DNase producers and also to explore the microbial nuclease diversity by mining the currently available microbial genome and metagenome databases.

## 2.2 MATERIALS AND METHODS

### *2.2.1 Seaweed sample collection and scanning electron microscopy*

Marine environment has been a rich and unexplored source of bioactive compounds. For this reason, in this research study marine sediments and seaweeds were chosen to isolate and identify nuclease producers. The seaweeds *Fuscus vesiculosus* and *Palmaria palmata* were collected by hand at low tide on the coast of Newcastle, Cullercoats and Boulmer beach. Collected samples were transferred in sterile bags on ice. The algal surface was washed in fresh water and bacterial sample was taken from the surface with a sterile swab. Sections of seaweed were cut with a sterile scalpel from holdfast, apical tips and growth nodes.

For scanning electron microscopy, the seaweed samples were fixed in 2% (v/v) glutaraldehyde at 4°C overnight. Specimens were rinsed twice in 0.2M phosphate buffer and dehydrated through a series of ethanol washes as follows: 25%, 50% and 75% (30 min each) and finally in 100% absolute ethanol until dried. Samples were then taken to Electron Microscopy Research Services, Newcastle University were it was dried in a critical point dryer (Bal-tec), mounted on aluminium stubs and sputter coated with gold (performed by service staff). Biofilms were then visualised using a scanning electron microscope (Cambridge Stereoscan 240).

### *2.2.2 Isolation of bacterial strains and growth conditions*

A sterile swab was used to rub the seaweed surfaces and streak onto the Difco marine agar plates. Also, Mid-Atlantic ridge deep sea sediments formerly been collected by Dove marine laboratories boat crew in sterile containers for the research work performed by researcher Dr. Jinwei Zhang which was stored in 50ml sterile falcone tubes at -20°C were diluted appropriately and plated onto Difco marine agar plates. Pure

single colonies were isolated using aseptic techniques and streaked in fresh marine agar plates. Fermented soya (Natto) was purchased from Chinese food store and a swab was rubbed on the beans and streaked into Luria Bertani (LB) agar. Culture collection strains were used from Dove microbial lab collection and soil isolates were obtained from Dr. Gabriel Uguru, Researcher in School of Biology, Newcastle University. In order to check nuclease producing ability of organisms at different temperature ranges which may have high degree of stability and nuclease activity the organisms were grown at various temperatures. Psychrophiles were grown at 16°C for 5 days, mesophiles at 25°C or 37°C and thermophiles at 45°C for 2 days. Bacterial strains were purified through the fourth generation. For growth, strains were cultured in Marine broth or LB broth and incubated in an orbital shaker at 200 rpm.

### *2.2.3 Cultivation and identification of extracellular DNase producing bacteria*

Cultured strains were screened at their growth conditions for extracellular DNase activity using DNase test agar (BD 263220) containing methyl green dye. The production of an extracellular DNase was inferred by the presence of a clear halo in the DNase agar around the colonies. Cell free supernatants of positive hits were used to check whether the activity was observed in the supernatant and not only in the sedentary attached phase.

### *2.2.4 PCR amplification and 16S rRNA gene sequencing*

Genomic DNA was extracted from the isolates using bacterial genomic DNA extraction kit (Invitrogen, UK) according to manufacturer's instructions. 16S rRNA gene amplification using universal primers 27 F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT3') (Lane et al., 1991) was conducted by subjecting the mixture to the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min and sequencing After the amplification, PCR product was purified using PCR purification kit (Invitrogen, UK). 20 µl of each purified DNA fragments were then sequenced directly by Geneius labs, Newcastle University campus, UK.

### *2.2.5 Sequence analysis and data processing*

The 16S rRNA gene sequences of isolates were generated using DNABASER tool and compared with the sequences in the Ribosomal database 2 (RDB-2). Phylogenetic analysis was performed using the MEGA5 after obtaining the multiple alignments of data available from public databases by ClustalX 1.83. Phylogenetic tree was generated using neighbour-joining method with bootstrap score of 1000 replicates.

### *2.2.6 Generating dataset using bioinformatics resources and sequence databases*

Sequence based screening was used for gene mining of extracellular nuclease homologues. Function specific dataset for nuclease gene(s) of interest was constructed for sequence based gene analysis. The databases used to generate these datasets are The Enzyme database (Bairoch, 2000), KEGG database (Zhang and Wiemann, 2009) and UNIPROT protein database (Apweiler *et al.,* 2004). Metagenomic datasets and finished bacterial genomic datasets available through the IMG/M database (Markowitz *et al.,* 2012). By searching through the list of 'genes with extracellular nucleases' from every metagenome on the IMG/M database, extracellular nuclease homologues were retrieved. Then, to gain better insight into the diversity and representation of putative nucleases in the metagenomes and genomes, the databases of translated sequences from 43 environmental metagenome projects and 2136 bacterial genomes were downloaded.

### *2.2.7 Extracellular nucleases diversity analysis using bioinformatics tools*

Dataset of extracellular nucleases generated from section 2.2.6 were BLAST-searched (biological information such as DNA or aminoacid sequences having higher local similarity against the sequences of homologues, if similarity greater than 99% acceptable as same gene sequences) for nucleases through BLAST2GO project (Gotz *et al.,* 2008) and the function and biological process were analysed. The taxonomic distribution and diversity analysis was performed using MEtaGenome ANalyzer, MEGAN 4 (Huson *et al.,* 2007).

## 2.3 RESULTS AND DISCUSSION

### *2.3.1 Identification of bacterial strains*

Diverse epiphytic microorganisms growing on the surface of the hold fast, apical tip and growth nodes of *Fucus vesiculosus* and *Palmaria palmata* were observed using scanning electron microscopy (Figure 2.1). In addition to the bacterial epiphytes, diatoms and filamentous blue green algae were present on the surfaces. These results suggest biofilm mode of growth and development is preferred by bacterial and high organisms in natural marine environment. The abundance of end-attached rod-shaped bacteria forming chains was observed and bacteria with tufts were also seen. The diverse epiphytes were all inter-linked and strongly bound to the surface of the seaweed. A visible extracellular polymeric matrix layer was observed in Figure 2.1 E (circled area) which might be possibly secreted by the bacteria to protect themselves against harsh conditions.

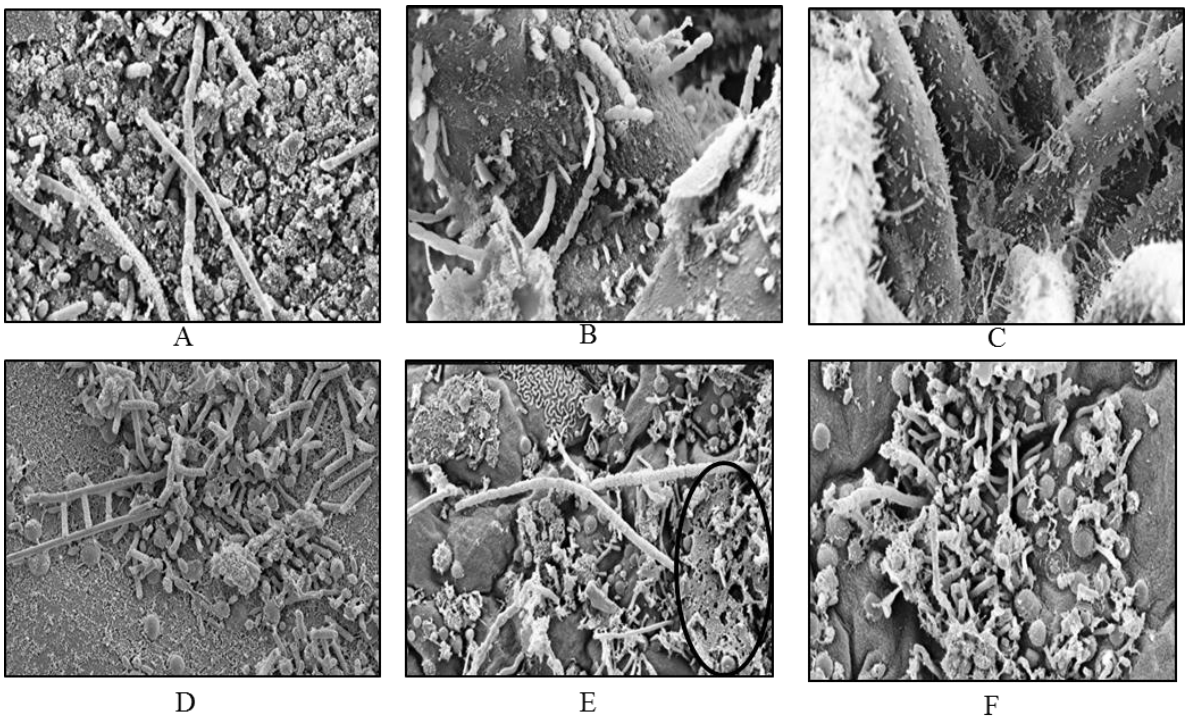



**Figure 2.1 Scanning electron micrograph of *Fucus vesiculosus* (top lane) and *Palmaria palmata* bottom lane seaweed surfaces. A and D are samples of hold fast, B and E are samples of apical tips; C and F are samples of growth nodes**

Hence, the surfaces of marine macroalgae can provide a suitable water-solid interface for microbial colonisation. Previously, seaweed surface associated bacteria were studied

as they have the ability to produce bioactive molecules (Wiese *et al.*, 2009) and it was shown that chemical interactions occur between macroalgae and bacteria to protect each other from predators (Goecke *et al.*, 2010). As a mode of biofilm dispersal, these organisms might have the ability to produce extracellular enzymes to disperse themselves to explore new niches. Hence, it is hypothesised here that the seaweed associated bacteria can produce DNase and the bacterial epiphytes along with other environmental strains were studied.

### *2.3.2 DNase production by culturable bacterial isolates*

Two hundred and seventy five isolates were screened for DNase activity at different temperatures as shown in Table 2.1. The diversity of psychrophiles, mesophiles and thermophiles was higher in deep sea sediments compared to seaweed associated bacteria.

**Table 2.1 Total number of strains screened for DNase activity from various environments grown at different temperatures**

| **Strain origin** | **Range of Growth Temperature (°C)** | | | | | |
|---|---|---|---|---|---|---|
| | 15 | 25 | 35±2 | 45 | > 50 | Total |
| Mid-Atlantic Ridge sea sediment (~2400m depth ) | 59 | 40 | 30 | 23 | 2 | 154 |
| Sea weed associated | 11 | 48 | 32 | 5 | - | 96 |
| Natto food | - | - | 1 | - | - | 1 |
| Culture collection | 9 | - | 1 | - | - | 10 |
| Soil | - | 6 | 8 | - | - | 14 |
| Total | 79 | 90 | 64 | 28 | 2 | 275 |

It is reported in the literature that deep sea sediments are rich source of bacteria and diverse bacterial population. It can supply species that can grow at high temperature and this may be due to dispersal of organisms from hydrothermal vents and deep sea circulation which is transit route for these thermophilic organisms (Dobbs and Selph., 1997). These observations are already seen by researchers where they isolated salt tolerant, pressure tolerant and temperature tolerant bacteria to produce useful

biomolecules (Stach *et al.*, 2003; Zeng *et al.*, 2006; Ver Eecke *et al.*, 2012) and enzymes such as alkaline and acidic proteases (Dang *et al.*, 2009; Zhou *et al.*, 2009). Similarly in current study the majority of bacteria isolated from deep sea sediment were capable of growing at psychrophilic, mesophilic and thermophilic conditions. Hence, further investigation of DNase producers proceeded on isolated bacteria.

Isolated bacteria were streaked onto DNase agar plate and bacteria which showed a zone of hydrolysis around the colony indicated that the DNase activity of these strains was extracellular. This method was easy and quick method used previously to analyse DNase production (Chun et al., 2013) and in this present study it was used to successfully screening DNase producing culturable bacteria. Further, the cell free supernatants of the bacterial strains were tested in wells of DNase agar plates, to identify zones of clearance in the agar as shown in Figure 2.2.

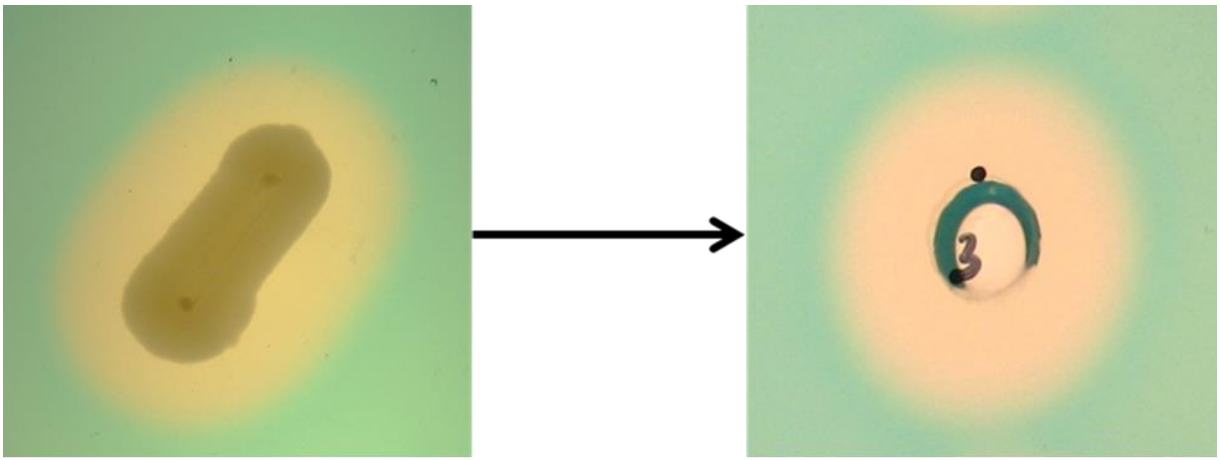



**Figure 2.2 Indication of a zone of hydrolysis of DNA in DNAse methyl green agar. A: Bacterial cell checked for DNase activity; B: Cell free supernatant of bacteria checked for DNase activity.**

Out of 275 environmental isolates, 86 strains displayed DNase activity and they were subjected to further characterisation. The taxonomic identification of these strains was performed using MEGA 5 and the number of psychrophiles, mesophiles and thermophiles that belong to each bacterial genus producing DNase enzymes is summarised in Table 2.2. Psychrophilic organisms showed zone of DNase degradation after 5-day incubation. To the author's knowledge this is the first report of psychrophilic bacteria belonging to genera *Planococcus* demonstrating DNase activity.

In general, the number of psychrophiles showing DNase activity was greater than mesophiles and thermophiles. This may be due to the fact that most bacterial isolates were from marine environment. In literature, there are only reports on the production of amylases, lipases, cellulases and chitinases by psychrophilic organisms (Buchon *et al.*, 2000; Tutino *et al.*, 2002). The study reported here detected DNase producing bacterial isolates thus providing insights into the diversity of hydrolytic enzyme production by culturable psychrophilic bacterial lineages.

Most environmental isolates able to produce DNase belonged to Gram positive genus *Bacillus* (39%) and all the thermophiles identified belonged to *Bacillus* group. This is interesting as the *Bacillus* bacterial group is well known as an enzyme producer and is used widely in industrial processes for commercial production of enzymes (Tang *et al.*, 2004). The next highest hits belonged to genera *Pseudoalteromonas* (22%) and this result agrees with those from previous studies in producing extracellular enzymes (Tutino *et al.*, 2002; Zeng *et al.*, 2006; Bian *et al.*, 2012).

**Table 2.2 Taxonomic identification of the strains that are able to produce DNase enzymes**

| Genus | Psychrophiles | Mesophiles | Thermophiles | Total |
|---|---|---|---|---|
| *Bacillus* | 5 | 6 | 23 | 34 |
| *Lactobacillus* | - | 1 | - | 1 |
| *Vibrio* | 2 | 3 | - | 5 |
| *Marinomonas* | 1 | 1 | - | 2 |
| *Pseudomonas* | - | 5 | - | 5 |
| *Pseudoalteromonas* | 11 | 8 | - | 19 |
| *Serratia* | 1 | - | - | 1 |
| *Exiguobacterium* | 2 | - | - | 2 |
| *Halomonas* | 1 | - | - | 1 |
| *Planococcus* | 2 | 1 | - | 3 |
| *Paenisporosarcina* | - | 1 | - | 1 |
| *Idiomarina* | 1 | - | - | 1 |
| *Arthrobacter* | - | 1 | - | 1 |
| *Shewanella* | 2 | - | - | 2 |
| *Streptomyces* | - | 3 | - | 3 |

| | | | | |
|---|---|---|---|---|
| Non-identified | 5 | - | - | 5 |
| Total | 33 | 30 | 23 | 86 |

This is also the first time DNase activity by mesophilic *Paenisporosarcina* sp. is reported. In addition to *Pseudoaltermonas* high proportion of mesophilic DNase producers were affiliated with the more commonly represented genera *Pseudomonas* and *Vibrio*. This result supports the previous reports of species of *Pseudomonas* producing extracellular deoxyribonucleases to utilise DNA as a nutrient source and *Vibrio* producing DNases for horizontal gene transfer of DNA as part of natural transformation (Blokesch and Schoolnik, 2008; Mulcahy *et al.*, 2010). The versatile ability of isolates belonging to these genera to produce extracellular enzymes suggests they play important role in extracellular DNA degradation in diverse environments.

The zone of hydrolysis for DNA degradation was measured to analyse the natural DNase production capacity of the environmental isolates belonging to these genera as shown in Figure 2.3. In the present study, quantifiable enzyme production was achieved during the stationary phase which corresponds to other enzyme production processes such as amylases and proteases (Gangadharan *et al.*, 2008; Zhou *et al.*, 2009). On the basis of quantitative DNase assay (section 2.3.3), thermophilic and mesophilic bacterial isolates showed high DNase activity compared to psychrophilic producers. Thermostable enzymes are more versatile than thermolabile as they have higher operational stability and a longer shelf life at elevated temperatures (Alvarez-Macarie *et al.*, 1999; Devi *et al.*, 2010). Therefore, there is a need for organisms for the production of thermostable nucleases which can be widely used for industrial biofilm removal applications. The most interesting and diverse producers were further studied for optimising DNase production in Chapter 5.

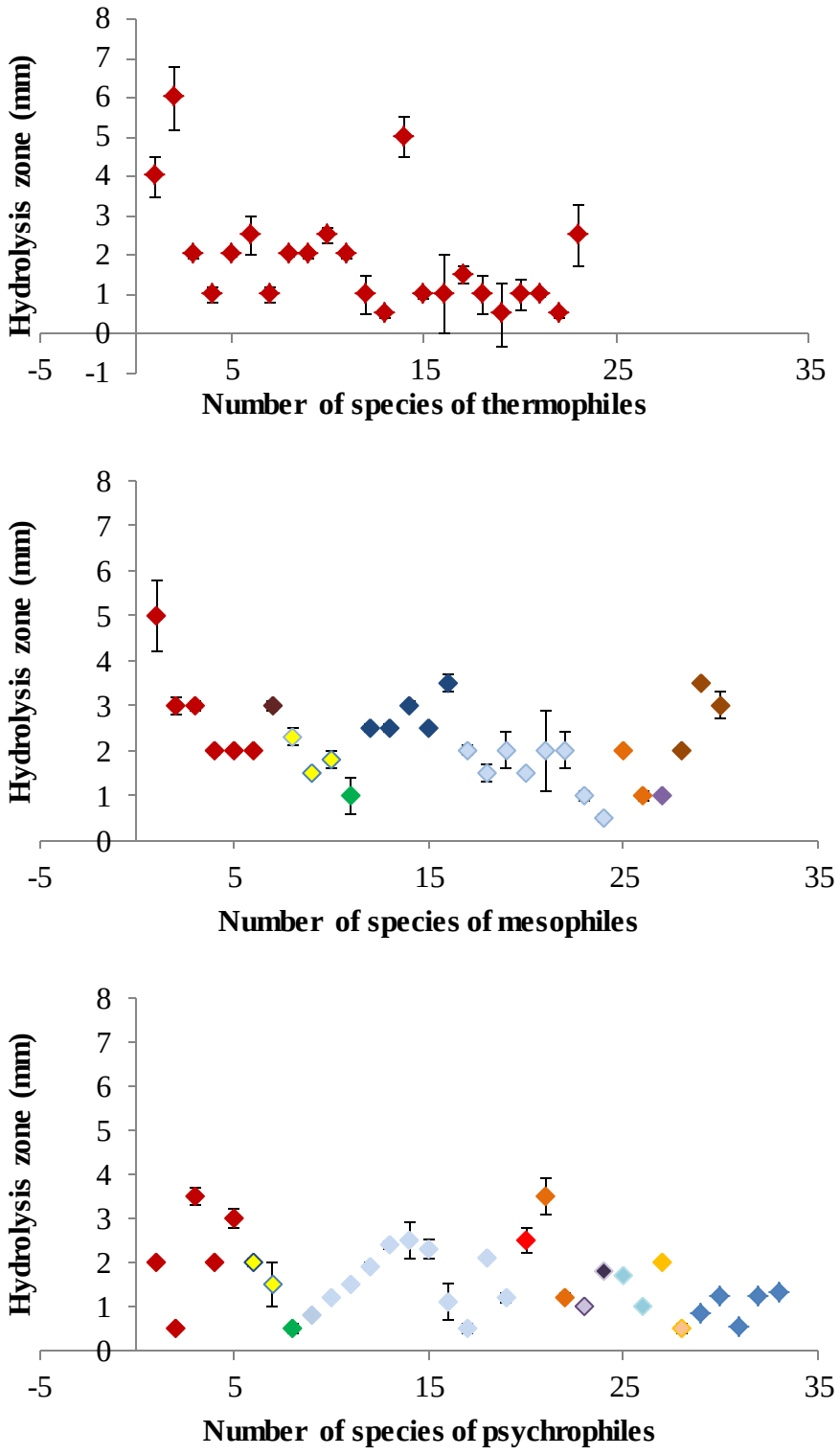



**Figure 2.3 DNase activity of mesophiles, psychrophiles and thermophiles as measured by the hydrolysis zone around the cell culture supernatant., Colour coding represents each bacterial genera as follows: *Bacillus* - dark red, *Vibrio* - yellow, *Marinomonas* - green, *Pseudomonas* - dark blue, *Pseudoalteromonas* - light blue, *Planococcus, Planomicrobium, Paenisporosarcina* - orange, *Serratia* - bright red, *Arthrobacter* - purple, *Streptomyces* - brown, *Halomonas* - light purple, *Idiomarina* - dark purple, *Shewanella* - light blue, *Exiguobacterium* - light orange and non-identified - blue.**

### *2.3.3 Sequence driven identification, diversity and distribution of microbial extracellular nuclease-like genes in the genome and metagenome database portal.*

As a preliminary study, nuclease-like genes were surveyed as described in section 2.2.6 across prokaryotic and eukaryotic organisms and tabulated in Appendix A, Table A.1. From Table A.1 microbial genes which showed characterised diverse nucleases activity and produced secreted protein were chosen as reference genes for further search. This includes NucB, a sporulation specific extracellular nuclease from *B. subtilis*, NucH, a thermonuclease from *S. aureus*, Endo I, a periplasmic nuclease from *E. coli*, NucA, a DNA/RNA non-specific nuclease from *Serratia marcescens*, S1P1 nuc, an extracellular nuclease from *Penicillium melinii* and *Aspergillus oryzae*, Dns, an extracellular deoxyribonucleases from *Aeromonas hydrophila* and End A, a DNA entry nuclease from *Streptococcus pneumonia*.

The MetaBIOME database has been used by researchers previously to identify and explore commercially useful enzymes from metagenomes (Sharma *et al.*, 2010) and as new tool for exploiting novel genes that are involved in marine microbial metabolism (Kennedy *et al.*, 2008). Similarly, in the present study the IMG/M database which comprises of both microbial whole genomes and metagenomes was explored for nucleases. The IMG/M database has the advantageous over other databases previously used for this purpose of exploring currently available microbial genomes and metagenomes with simple method to search for genes of interest. In addition, translated protein sequence retrieval enabled easier analysis. A thorough bioinformatics survey of draft and completed bacterial genome and metagenome extracted 538 microbial nuclease-like gene reads. Additional search for putative extracellular nucleases obtained 324 microbial nuclease-like gene reads.

Each nuclease-like gene read retrieved the 20 highest hits of similar gene sequences through BLAST2GO (Appendix A, section A.1 presented in CD) and the biological processes involving these enzymes were elucidated by gene mapping (Appendix A, Table A.2). The microbial diversity was analysed using MEGAN 4 and the taxonomic distributions are shown in Figure 2.4. This result suggests nuclease-like enzyme positive genotypes were found in the majority of bacterial lineages. The phylum level

distribution is shown in Appendix A, Figure A.1. Microbial diversity of extracellular enzymes using a sequence based approach has been successfully studied earlier for esterases (Elend *et al.*, 2006) and multiple extracellular enzymes (Zimmerman *et al.*, 2013). Hence, this study is one of the few studies reporting microbial diversity of extracellular nucleases. The sequence driven analysis suggests that microbial communities' possess genes that are able to produce diverse extracellular nuclease-like enzymes.

To understand the biological process involving the genes presented in this study, KEGG analysis was performed in MEGAN and outcomes are shown in Appendix A, Figure A.2. Also, a SEED-based functional analysis of the gene reads defined the specific processes extracellular nucleases are implicated (Figure 2.5). The biological functions were similar to the functions generated by BLAST2GO. Some of these are shown in Appendix A, Section A.2 and A.3 (attached in CD). BLAST2GO gene mapping showed nucleases are involved in several complex processes. However, based on the context of the thesis, it is important to highlight only the key roles of nucleases in the environment.

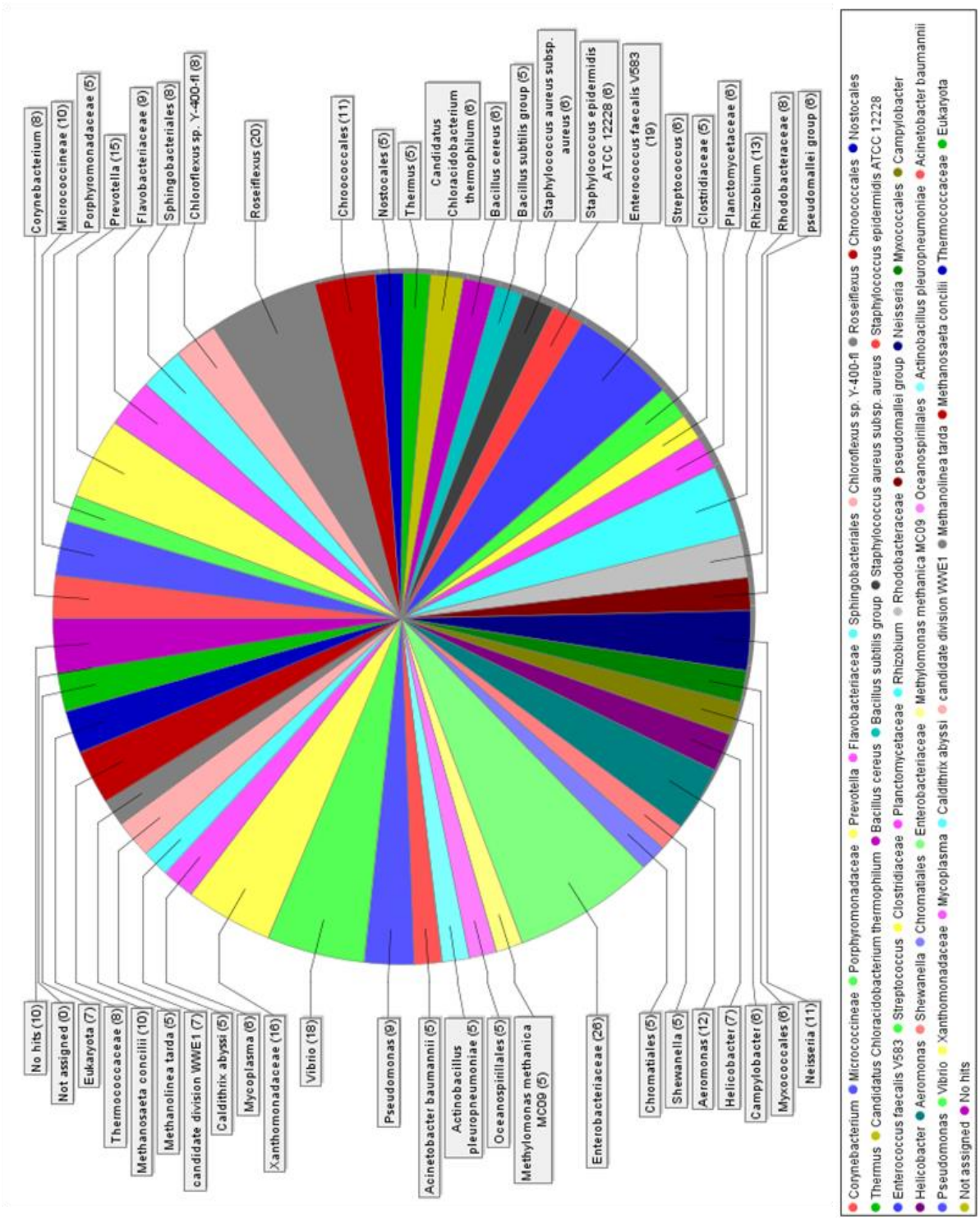



**Figure 2.4 Taxonomic distribution of microbial diversity containing nuclease-like genes. List of taxa encoding nuclease-like genes in the bacterial genome and metagenome database portals. Each colour represented individual taxa and the number in the bracket denotes the number of gene reads.**

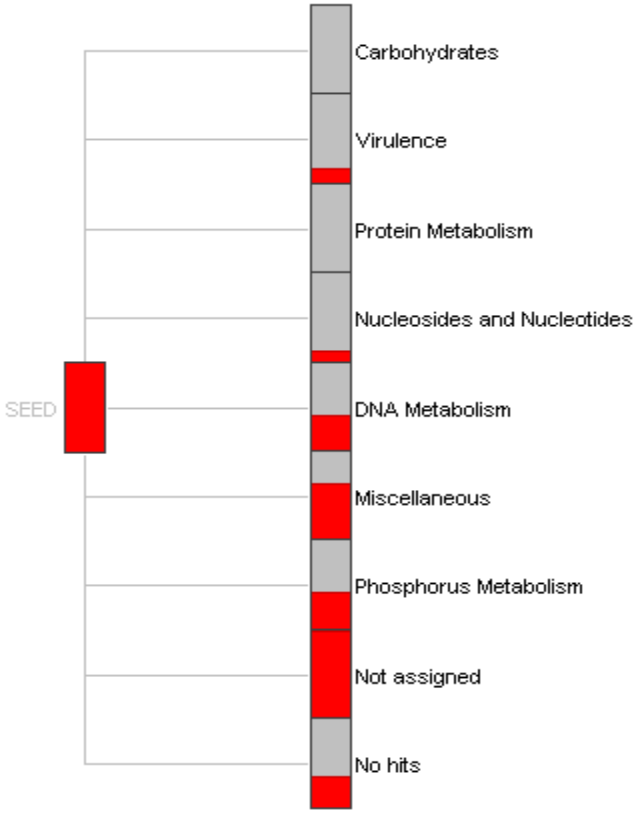



**Figure 2.5 SEED-based functional analysis. Part of the SEED-based analysis of the nuclease-like gene reads obtained from bacterial genome and metagenome. Each item represents a functional role in SEED and the red colour strength indicates the number of reads assigned for each function.**

Primarily nucleases are used by microbes to metabolise nitrogen, phosphorous, carbohydrates, nucleic acids, small molecules such as tRNA, and nucleobase containing compounds. Previously it was reported, that nucleases digest DNA that can be used as sole nutrient source by biofilm forming bacteria *Pseudomonas aeruginosa* and *Shewanella oneidensis* (Mulcahy *et al.*, 2010; Godeke *et al.*, 2011). Another study showed that the balance of extracellular DNA in the marine environment was regulated by nucleases and that was important for the functioning of deep sea ecosystems (Dell'Anno and Danovaro, 2005). The results of the current study of the role of nucleases on cellular metabolism of nitrogen and phosphorous suggest that nucleases decide the fate of extracellular DNA available in the natural habitat and break it down to nitrogen and phosphorous sources that can be readily assimilated by microorganisms.

A second important role of extracellular nucleases was the response to stress. Secretion of nucleases during stress stimulus helps spore formation and release. This functional role was supported by the previous report on NucB secretion during mother cell lysis

and spore formation by *B. subtilis* (Hosoya *et al.*, 2007). Spore formation is a last resort for the *Bacilli* group to withstand stress conditions and harsh environmental conditions. While studying the mechanisms of biofilm formation by *Bacillus* it was shown that spores formed were concentrated on top layer of biofilms embedded in aged biofilm cells (Vlamakis *et al.*, 2013). Therefore, nuclease secretion is hypothesised to help these spores get dispersed into new environments to grow and survive.

Another crucial role played by nucleases is as a virulence factor. This involves DNA entry nucleases which are proposed to lead to DNA entry by competent cells and enable virulence and pathogenesis. Pathogenesis leads to intracellular stress which leads to programmed cell death (Mittler and Lam, 1997). This is a response to invading pathogens, and extracellular nucleases cause DNA degradation and DNA fragmentation. Hence, nuclease facilitates DNA recycling from dead cells as source of nutrient availability in the extracellular space for other organisms. Previous report on virulence development and secretion of nucleases was observed pronounced biofilm formation by *A. baumannii* and *S. aureus* (Wand *et al.*, 2011; Mitchell *et al.*, 2013). This suggests that the biological function of nucleases as a virulence factor helps microbes evade the host, and adapt to the immune system causing cell death and DNA release which is associated with the biofilm development.

## 2.4 SUMMARY

In this chapter, using culture based approach 86 out of 275 bacterial isolates were identified as diverse nuclease producers grown at different temperatures. These results demonstrate the ecological relevance of the bacterial community from various environments and highlight its diversity as representative isolates from several distinct bacterial genera recovered were able to produce DNases. Sequence driven bioinformatic analysis of 43 metagenome and 2136 bacterial genome retrieved 862 extracellular nuclease-like gene reads. Analysis of the reads showed there were 45 different taxa capable of producing secreted nucleases which covers the majority of bacterial lineages. The function based analysis of the sequence reads, facilitated identification of the ecological implications and function of extracellular nucleases. The pivotal role was to decide the fate of eDNA and therefore nuclease secretion involves nutrient recycling, protection, survival and virulence.

# Chapter 3 Use of physiological information and process optimisation enhances production of extracellular nuclease by a marine strain of *Bacillus licheniformis*

## 3.1 INTRODUCTION

Previously a secreted bacterial nuclease, NucB, was reported as an alternative novel biofilm dispersing enzyme (Nijland *et al.*, 2010). NucB was hypothesised to be released as an important enzyme for biofilm degradation and its mechanism of action on dispersing preformed biofilms was shown to be very effective (Shakir *et al.*, 2012). This could potentially be extended to break-up the ubiquitously present eDNA, leading to loosening of the EPS matrix and the complete removal of the majority of biofilms. NucB is a robust and relatively small protein (~12 KDa) that can potentially migrate through complex biofilms more efficiently for eDNA degradation than DNase 1 and other nucleases. This has stimulated commercial interest in this enzyme as a broadly applicable antibiofilm technology and therefore the research reported in this chapter focused on improving the productivity of NucB.

The secretion of a deoxyribonuclease in *Bacillus subtilis* during sporulation has been known for some time (Akrigg and Mandelstam, 1978). This enzyme was sequenced and designated NucB, a sporulation specific extracellular nuclease. It is part of the sigE regulon which is involved in DNA degradation after mother cell lysis (Vansinderen *et al.*, 1995; Hosoya *et al.*, 2007). Subsequently, supernatant obtained from a marine strain of *Bacillus licheniformis* EI-34-6 grown as an air-membrane surface culture was observed to disperse biofilms of Gram positive and Gram negative bacteria (Yan *et al.*, 2003; Nijland *et al.*, 2010). The active compound was later identified as NucB, an extracellular nuclease that degraded eDNA resulting in biofilm removal (Nijland *et al.*, 2010). Based on the available literature, manganese (Mn) has been known to stimulate sporulation since the 1960s (Weinberg, 1964). However its precise role is still unclear, although it may activate enzyme systems necessary for sporulation (Kolodziej and Slepecky, 1964). Given the role of Mn in stimulating sporulation, it may trigger increased expression of NucB and thus can be used to enhance the production of this enzyme.

One important option to improve protein production without genetic modification is the optimisation of medium composition. The formulation of cultivation media is important as it affects product concentration and volumetric productivity as well as the overall process economics (Kennedy and Krouse, 1999). Optimising one factor at a time is tedious and laborious and it does not enable the identification of potential interactions between the investigated factors. The most effective and reliable approach to optimisation is using statistical methods that can account for the interaction of variables in generating a process response (Gangadharan *et al.*, 2008). Hence, the optimisation of cultivation media is carried out done in two steps in the research reported here. A Plackett - Burman (P-B) design is initially applied to identify key factors from a number of variables. This helps to identify significant and non-significant variables using a limited number of experiments. The optimum levels of the key variables and the interactions between them are then estimated by response surface methodology (RSM) using a Central Composite Design (CCD) (Khuri and Mukhopadhyay, 2010). Previous studies of extracellular proteome analysis of *B. licheniformis* DSM13 demonstrated strong induction of NucB secretion during phosphate limited conditions at the start of sporulation (Hoi *et al.*, 2006). Therefore interest was shown in understanding the effect of phosphate on the large scale production of NucB.

So far, *B. licheniformis* has been widely reported as an attractive microbial system for industrial production of various enzymes such as alkaline protease, amylase, chitinase, tannase and keratinase. However, our study in this chapter is the first report on desirable ways to produce a novel biofilm dispersing extracellular deoxyribonuclease, NucB. Specifically, it is one of the few studies to have demonstrated the importance of exploiting the physiological understanding as a novel insight on the regulation of enhanced NucB production. The objective of this study was therefore to understand the interdependence of the various components of the growth medium including manganese and phosphate, the two important factors which affect *B. licheniformis* cell physiology related to the dynamics of the synthesis of the NucB.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Bacterial strain and growth conditions*

A *B. licheniformis* strain EI-34-6, a seaweed associated marine isolate was used in this study (Yan *et al.*, 2003). Luria Bertani (LB) broth (Sigma, UK) was used to prepare a standard inoculum. Cells were inoculated from LB agar plates into 500 ml baffled Erlenmeyer flasks containing 100 ml LB broth and cultivated in an orbital shaker at 200 rpm and 37 °C. After 12 h, the optical density was determined and appropriate production medium was inoculated to an initial absorbance of 0.1 at 600 nm.

Preliminary study on NucB production was carried out by growing the cells with LB, LB with 5 µM $MnSO_4$ and re-suspension medium (Akrigg and Mandelstam, 1978). Samples were taken over time to analyse nuclease activity. For NucB production studies, *B. licheniformis* cells were grown in flasks containing LB medium supplemented with 0, 5, 25, 50, 100 and 200 µM $MnSO_4.H_2O$. The cultures were cultivated in an orbital shaker at 200 rpm and 37 °C for 48 h. $MnSO_4$ (100 µM) was also added to culture medium 0, 6, 10, 14, 18 and 24 h after inoculation and nuclease activity was measured 48 h post inoculation to determine whether the stage of growth of organism at the time of addition of Mn has any influence.

### *3.2.2 Plackett-Burman (P-B) optimisation of medium composition*

Medium components previously reported to affect *Bacillus* biomass, sporulation and enzyme production were studied. Yeast extract, corn steep liquor, hysoy, $MgSO_4$, $KH_2PO_4$, $Na_2HPO_4$, $NH_4Cl$, $(NH_4)_2SO_4$, soluble starch, glycerol and glucose were identified as eleven significant independent factors. A P-B design was used as first step to analyse their effect on NucB production. The low (-) and high (+) concentrations of the media components are shown in Table 3.1.

**Table 3.1 Placket-Burman experimental plan for choosing significant factors of NucB production optimisation by *B. licheniformis* EI-34-6.**

| Factors | Components | Units | Low (-) | High (+) |
|---|---|---|---|---|
| A | Hysoy | g/L | 12.5 | 37.5 |
| B | Yeast extract | g/L | 24 | 72 |
| C | $KH_2PO_4$ | g/L | 1.5 | 4.5 |
| D | $Na_2HPO_4$ | g/L | 3 | 9 |
| E | $NH_4Cl$ | g/L | 0.5 | 1.5 |
| F | $MgSO_4$ | g/L | 0.13 | 0.38 |
| G | Soluble starch | g/L | 2 | 20 |
| H | Glycerol | g/L | 2 | 20 |
| I | $(NH4)_2SO_4$ | g/L | 0.5 | 5 |
| J | Glucose | g/L | 2 | 20 |
| K | Corn steep liquor | g/L | 2 | 20 |

The design of the experimental runs was generated in Minitab 16 (Minitab Inc., USA), and conducted as indicated in Table 3.4 and individual variables were evaluated for their main effects and p-values with nuclease activity as response.

### *3.2.3 Optimisation of key components using response surface methodology (RSM)*

Central composite design was used for three significant factors with six replicates of the centre point. The factors and their coded and uncoded values of the factors at five levels (-1.63, -1, 0, 1, 1.63) are listed in Table 3.2.

**Table 3.2 Factors and the concentration levels used for CCD optimisation study.**

| | Concentration (g/L) /levels | | | | |
|---|---|---|---|---|---|
| **Factors** | **-1.63** | **-1** | **0** | **1** | **1.63** |
| **Yeast extract ($X_1$)** | 8.1 | 24 | 48 | 72 | 87.2 |
| **Corn steep liquor ($X_2$)** | 3.7 | 10 | 20 | 30 | 36.3 |
| **Hysoy ($X_3$)** | 4.6 | 12.5 | 25 | 37.5 | 45.4 |

A total of 20 experimental runs were necessary to estimate the NucB activity. The second order polynomial coefficients were calculated using the Design-Expert 7.5

statistical software package (Stat-Ease, Inc., Minneapolis, USA) including regression analysis. Validation experiments were performed which included three points outside the coded levels of the predicted model. For each variable, the quadratic models were represented as contour plots to visualise the main and interactive effects.

### *3.2.4 Measurement of growth, sporulation and phosphate concentration.*

Cell growth was measured by reading optical density at 600 nm ($OD_{600}$) and by dry weight (g/L) measurements after drying cell pellets at 80°C to a constant weight. Culture samples (1 ml) were heated at 80°C for 20 min. Serial dilutions were then prepared and plated on LB agar plates to determine viable spore counts (Monteiro et al., 2005). Plates were incubated for 24 h at 37°C and the developed colonies were counted, statistically analysed and expressed as $\log_{10}$ CFU/ml. Cells and cell free supernatants were freeze dried and mineral contents (% wt) was evaluated using Scanning Electron microscopy with Energy Dispersive X-ray (SEM-EDX) analysis as previously done by Machnicka (2006) and used to quantitatively determine phosphate concentration in the samples. All the experiments were performed in triplicate.

### *3.2.5 Nuclease assays*

Nuclease activity of *B. licheniformis* EI-34-6 was determined using a modified nuclease assay (Nestle and Roberts, 1969). Culture samples (1.5 ml) were centrifuged at 13,400g for 10 min, cell pellets were discarded. The supernatants were filtered through 0.2 µm pore size Minisart filters (Sartorius, UK) and nuclease activity determined in the cell-free supernatant. The reaction mixture contained 1 mg/ml of salmon sperm DNA (Sigma, UK) in Tris-HCl (50 mM, pH 8.0), BSA (0.1 mg/ml), 5 mM $MnSO_4.H_20$ and 0.125 ml of enzyme source, incubated in water bath at 37°C. After 30 min the insoluble DNA was precipitated with 0.5 ml of cold 4 % perchloric acid and supernatant containing soluble oligonucleotides was estimated by measuring the optical density (OD) at 260 nm of the supernatant. One unit of nuclease activity is defined as the amount of enzyme that causes an increase of 1.0 OD at 260 nm after 30 min at 37 °C. Assays were carried out in triplicate.

## 3.3 RESULTS AND DISCUSSION

### *3.3.1 Effect of manganese on sporulation and NucB production*

Using recombinant microorganisms to increase the production of enzymes may be unstable and unacceptable for many food grade applications and companies. For that reason media improvement may be a more appropriate alternative for the overproduction of enzymes. Secreted metabolites and enzymes are an important component for the survival of microbes occupying harsh environments. In the environment, the last resort used by *Bacillus* to grow, compete and survive nutrient exhaustion is sporulation (Grossman and Losick, 1988). In the current study, enhanced production of extracellular nuclease NucB by *B. licheniformis* EI-34-6 cultivation was achieved by considering physiological conditions such as sporulation, medium optimisation and phosphate availability.

When *B. licheniformis* EI-34-6 was grown in LB, the maximum NucB production, expressed as extracellular nuclease activity, was 37 U/ml. The growth of the cultures in LB, LB supplemented with 5 µM $MnSo_4$ and resuspension medium (Akrigg and Mandelstam, 1978) were similar (Figure 3.1A). However, the medium containing manganese (Mn) increased NucB production (Figure 3.1B). The production of NucB was initiated at the transition from log phase to stationary phase which is typical of extracellular enzyme synthesis in *Bacillus* (Gangadharan *et al.*, 2008).

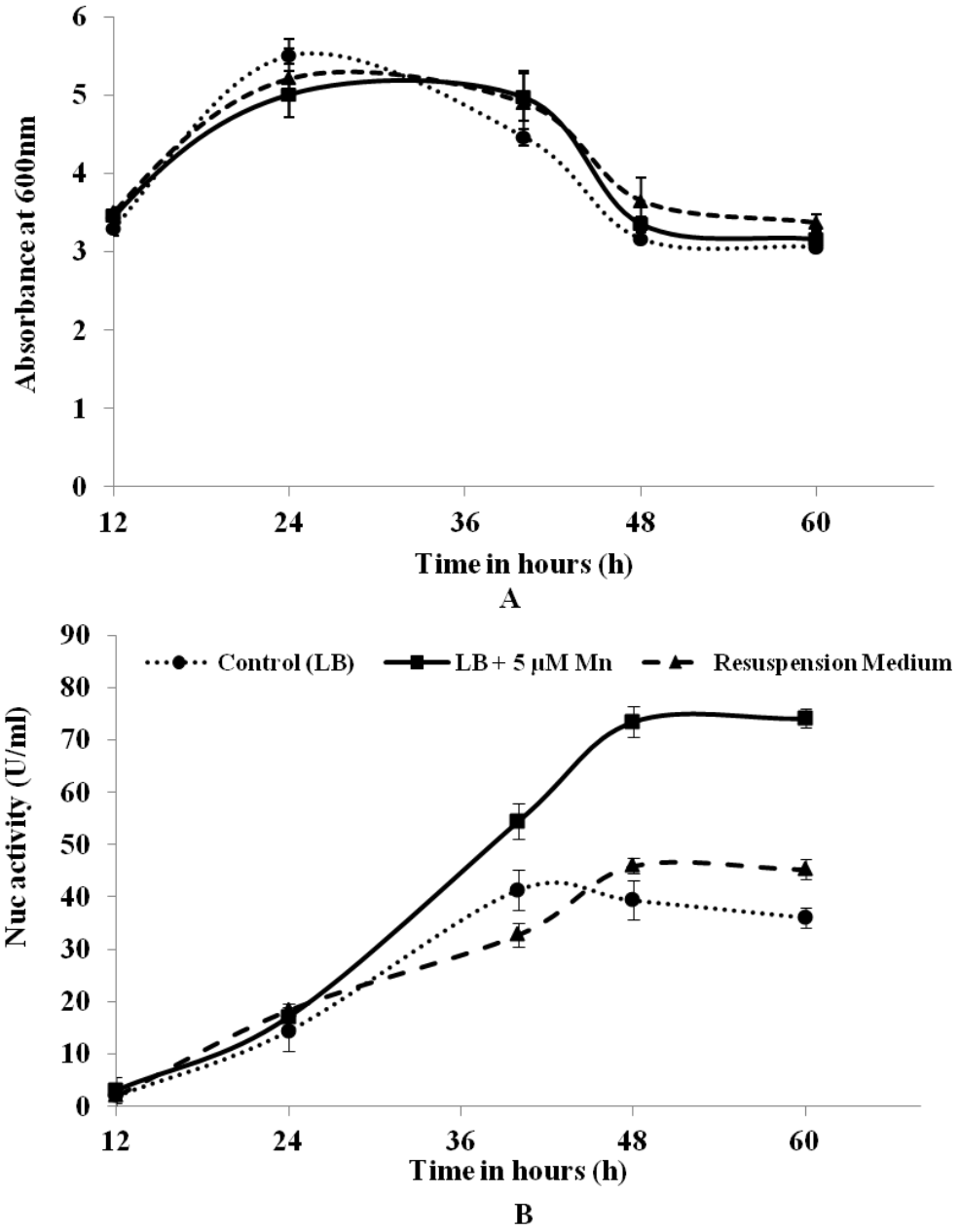
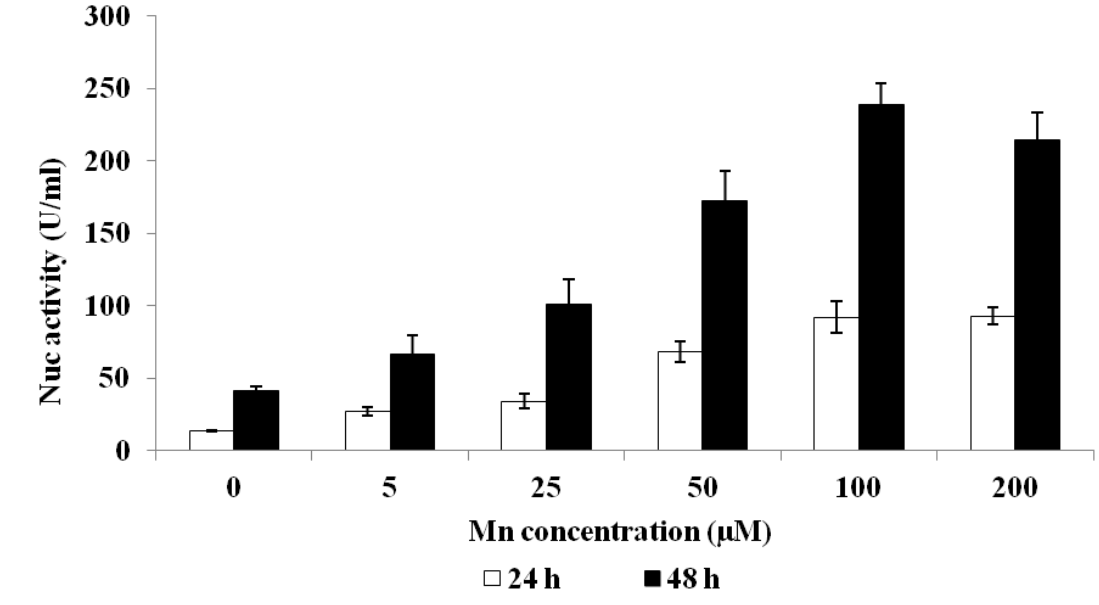


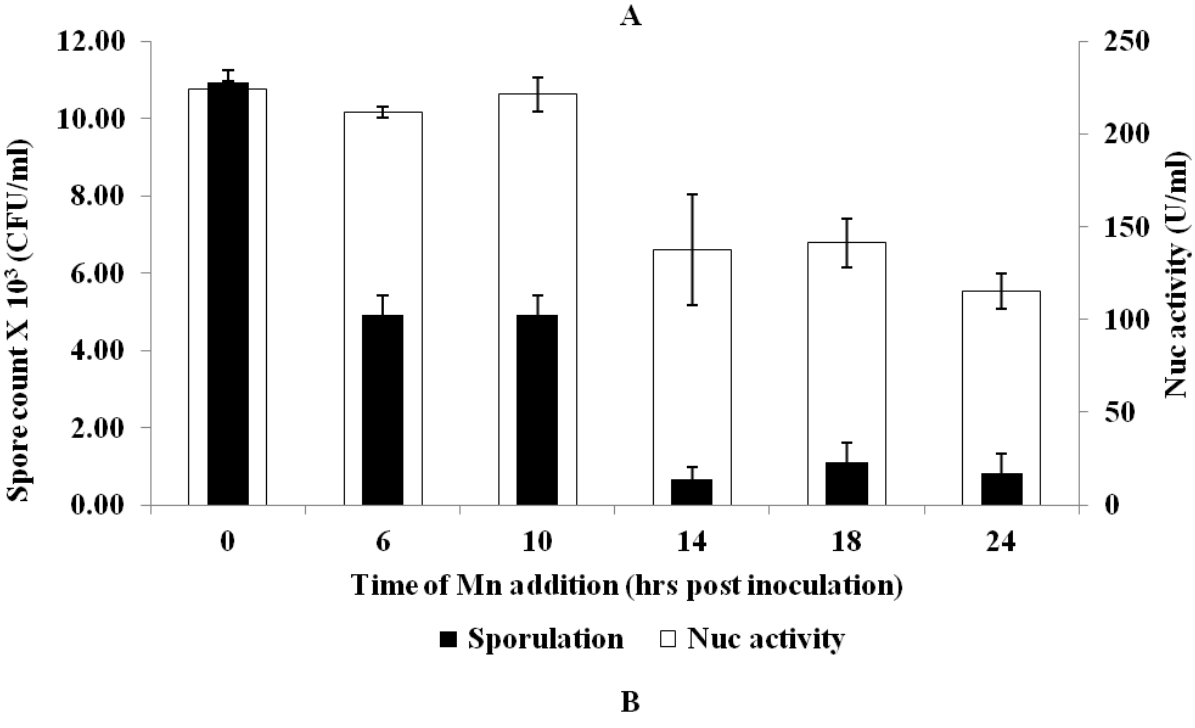

**Figure 3.1 Time trajectory of growth (A) and NucB production (B) by *B. licheniformis* EI-34-6 in control (LB only), LB with 5 µM $MnSO_4.H_2O$ and Resuspension medium (see section 3.2.1 for detailed conditions).**

Nuclease activity showed a steady increase with increase in Mn concentration from 5 µM up to 100 µM and no further increase at 200µM. At 48 h, an even more marked increase in production was observed up to 100µM Mn which supported the highest NucB production (Figure 3.2A). Hence this concentration was used in subsequent experiments. The effect of the time of addition of Mn on the sporulation and the nuclease activity at 48 h was determined. LB medium without Mn addition showed a

sporulation count ~1 X $10^3$ CFU/ml and a nuclease activity of 47 U/ml at 48 h (Figure 3.2a). The maximum increase in sporulation and nuclease activity occurred when Mn was added within 10 h post inoculation. Both nuclease activity and sporulation was decreased when Mn added after 10 h post inoculation (Figure 3.2B).




A




B

**Figure 3.2 The effect of Mn addition on NucB production A: Various concentrations of $MnSO_4.H_2O$ added to LB medium at time of inoculation. B: Addition of 100 µM $MnSO_4.H_2O$ at various times post inoculation.**

The results presented here are in line with observations reported in the literature. Divalent Mn has been shown to be an essential metal ion for sporulation, biosynthesis of metabolites and structures of bacillus spore formers (Stockel *et al.*, 2009). Also, natural sources, such as dry sea lettuce and pineapple extracts which are rich in manganese have been shown to increase sporulation in *B. subtilis* natto sp. (Mitsuboshi *et al.*, 2007). Additionally, Mn acts as a cofactor for enzymes involved in diverse metabolic pathways including nucleotide metabolism. Its supplementation induces sporulation, which directly activates sporulation specific gene expressions (Hoover *et al.*, 2010). Since NucB was identified as a sporulation specific extracellular nuclease (Vansinderen *et al.*, 1995), its synthesis was hypothesised to be linked to sporulation. It was also reported that when partial cell lysis occurs, mother cell-derived DNA is released into the cell supernatant and is readily digested by NucB indicating it being a major extracellular DNase secreted during sporulation (Hosoya *et al.*, 2007). To date manganese has been reported to affect the spore production in *Bacillus* species (Table 3.3). However, this is the first study that showed Mn addition during cultivation of *B. licheniformis* EI-34-6 increases the NucB production 5-fold from 37 U/ml to 225 U/ml with and without 100 μM Mn addition which also corresponds to 5 − 10-fold CFU/ml increase in sporulation.

### *3.3.2 Medium optimisation for NucB production*

Previous studies on *B. subtilis* have shown that the efficiency of sporulation was cell density dependent (Grossman and Losick, 1988). When sporulation was induced at low cell density it resulted in a low proportion of spores whilst induction at high cell density resulted in an increased concentration of spores and efficient sporulation. It is therefore important to maximise the cell density prior to the induction of sporulation. This can be achieved by optimising the media components and in this study P-B design was used as the first step to determine the key components, which showed positive significant effects on cell density and NucB production.

A comprehensive study of a wide variety of nutrient factors reported on sporulation, high cell density cultivation or extracellular enzyme production in the literature for genus *Bacillus* was performed (Table 3.3).

**Table 3.3 Literature search of growth media components using *Bacillus* species in the production studies**

| Organism | Study | Key Growth components/conditions | References |
|---|---|---|---|
| **Starvation conditions** | | | |
| *Bacillus licheniformis* DSM13 | Proteome expression for carbon and nitrogen starvation | Minimal medium with starvation condition for glucose – 0.08%, nitrogen – 1.0 mM | (Voigt *et al.*, 2006) |
| *B. licheniformis DSM 13* | Proteome expression for phosphate starvation | Minimal medium with phosphate starvation condition (0.15 mM) | (Hoi *et al.*, 2006) |
| *B. cereus ATCC 14579* | Glutamate medium starvation | 20 mM (high) and 2.5 mM (low) glutamate advantage carbon and nitrogen not depleted before complete sporulation | (de Vries *et al.*, 2005) |
| **Sporulation** | | | |
| *B. licheniformis* | High yield spore production (1.7 X $10^{11}$ spores) | SSF medium – Rice straw powder, wheat bran, glucose, peptone, yeast extract, $KH_2PO_4$ and CaO | (Zhao *et al.*, 2008b) |
| *B. licheniformis* B36 | Spore production (1.2 X $10^{11}$ spores) | Netting bag solid bioreactor | (Zhao *et al.*, 2008a) |
| *B. cereus* NRRL 100132 | High yield spore production (1 X $10^{10}$ CFU/ml) | Corn steep liquor | (Lalloo *et al.*, 2009) |
| *B. licheniformis, B. subtilis* and *B. coagulans* | Optimum growth medium (7 X $10^9$ CFU/ml for *Bacillus licheniformis*) | Soybean meal, yeast extract and $MgSO_4$ for *B. subtilis* and Soluble starch, yeast extract, $(NH_4)_2SO_4$ for *B. licheniformis* | (Cho *et al.*, 2009) |
| *B. sphaericus* | Large scale production of spores (3.7 X $10^9$ spores) and 4.6 g/L Biomass | Soybean based culture medium | (Prabakaran *et al.*, 2007) |
| *B. megatarium* | HCD growth | Corn steep liquor vs Chemically defined medium (CDM) | (Zhang *et al.*, 2011) |
| *B. subtilis* | Induced spore release | Sodium sulphate | (Burnett *et al.*, 1986) |
| *Bacillus species* | Manganese supplement for Spore induction | 10 mg/L $MnSO_4.xH_2O$ | (Stockel *et al.*, 2009) |
| *B. subtilis natto* sp. | High spore formation | NBF + 0.1 mM/L $Mn^{2+}$ | (Mitsuboshi *et al.*, 2006) |
| *B. subtilis (natto)* | Sporulation of vegetative cells | Extracts of dry lettuce, raw and dry green laver, poppy seeds as high | (Mitsuboshi *et al.*, 2007) |

| | | | |
|---|---|---|---|
| | (90% ) | sources of $Mn^{2+}$ | |
| *B. thuringienis* | Increased sporulation | $(NH_4)_2SO_4$ as inorganic nitrogen source | (Wan *et al.*, 2003) |
| **Production of metabolites** | | | |
| *B. subtilis* natto sp | Nattokinase production in HCDC, (77 g/L DCW) | Glucose-peptone ratio feed, pH stat FB | (Cho *et al.*, 2010) |
| *B. licheniformis* ZJUEL31410 | Elastase production | Corn steep flour, casein, glucose, $K_2HPO_4$, $MgSO_4.7H_2O$ | (Chen *et al.*, 2007c) |
| *B. licheniformis* BC98 | Antifungal molecule production | Yeast nitrogen base without aminoacids, casein hydrolysate glucose, $KH_2PO_4$ | (Patel *et al.*, 2004) |
| *B. licheniformis and B. subtilis natto* | Poly-G-glutamic acid production | Fed-Batch by pH-stat, $MnSO_4$ | (Kedia *et al.*, 2010) |
| Recombinant *B. subtilis* | Phytase production in HCDC (56 g/L DCW) | Yeast extract and peptone feeding and phosphate starvation 0.3 mM ≥ Pi ≥ 0.1 mM | (Vuolanto *et al.*, 2001) |
| *B. subtilis* R14 | Bioactive compounds | Balanced C/N ratio with Glucose and $(NH_4)_2SO_4$, condition for C/N depletion at same time, hence improve sporulation effect. | (de Carvalho *et al.*, 2010) |
| *B. subtilis* 168 | DNase production | Casein hydrolysate medium and Resuspension medium for sporulation | (Akrigg and Mandelstam, 1978) |
| *B. firmus* VKPACU1 | Ribonuclease production | Minimal salt medium with $Mn^{2+}$, Glucose and tryptone | (Kumar *et al.*, 2010) |
| *B. cereus* ZH14 | Ribonuclease production | Sucrose, yeast extract, $MgSO_4.H_2O$ and $KNO_3$ | (Zhou *et al.*, 2010) |
| *Bacillus* sp. RKY3 | Alkaline protease | Corn starch, yeast extract, corn steep liquor and inoculum size | (Reddy *et al.*, 2008) |
| *B. firmus* | Cyclomaltodextrin glycanotransferase | Corn starch, yeast extract, pharmamedia | (Gawande *et al.*, 1998) |
| *B. amyloliquefaciens* ATCC 23842 | Alpha amylase | Wheat bran, Oil cake, $MgSO_4$, $NH_4NO_3$, $KH_2PO_4$, $CaCl_2$, starch and maltose | (Gangadharan *et al.*, 2008) |

Based on this data from Table 3.3 eleven independent factors (Table 3.1) potentially influencing NucB production were selected for P-B design experiments. The components with selected high and low levels are shown in Table 3.4 along with nuclease activity as response.

**Table 3.4 Plackett–Burman experimental design matrix for NucB production[a]**

| RUN | A | B | C | D | E | F | G | H | I | J | K | Nuc activity ( U/ml)[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | + | - | + | + | - | + | - | - | - | + | + | 246 |
| **2** | - | + | + | - | + | - | - | - | + | + | + | 332 |
| **3** | + | - | - | - | + | + | + | - | + | + | - | 204 |
| **4** | + | - | + | - | - | - | + | + | + | - | + | 253 |
| **5** | + | + | + | - | + | + | - | + | - | - | - | 313 |
| **6** | + | + | - | + | - | - | - | + | + | + | - | 327 |
| **7** | - | - | + | + | + | - | + | + | - | + | - | 140 |
| **8** | + | + | - | + | + | - | + | - | - | - | + | 356 |
| **9** | - | + | - | - | - | + | + | + | - | + | + | 376 |
| **10** | - | + | + | + | - | + | + | - | + | - | - | 280 |
| **11** | - | - | - | - | - | - | - | - | - | - | - | 181 |
| **12** | - | - | - | + | + | + | - | + | + | - | + | 187 |
| **13** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 141 |

[a] As described in the Materials and Methods, *B. licheniformis* EI-34-6 was grown in shake flasks containing the various media (pH 7.0) as indicated in the table. 48 h post inoculation the cells were sampled for the determination of Nuc activity. [b] The production yield in terms of volumetric Nuc activity for NucB production in culture broth.

The estimated main effects with their p-values are shown in Figure 3.3. Any factor with a $p < 0.05$ was considered to have a significant influence on the response at 95% confidence level. In this study, yeast extract ($p < 0.0001$), corn steep liquor ($p = 0.003$) and hysoy ($p = 0.022$) had significant positive effects on NucB production. Though there were a number of carbon and mineral sources included in the design, the main effects show that those factors either have a negative or a minimal effect on nuclease activity response (Fig 3.3).

This may be due to the fact that the synthesis of extracellular enzymes could be subject to carbon catabolite repression as previously seen in *B. firmus* (Gawande *et al.*, 1998). Also, the presence of carbon substrates such as soluble starch may promote the secretion of amylases and proteases that could have a negative effect on NucB yield.

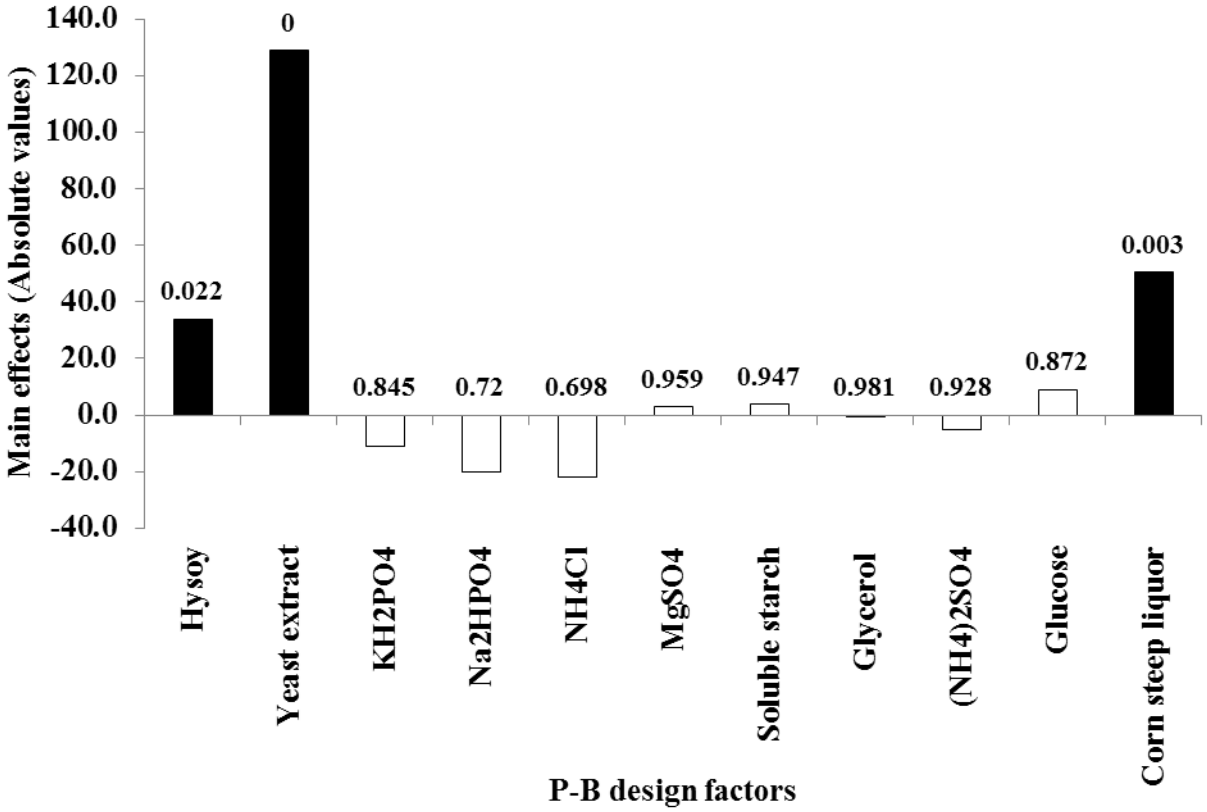



**Figure 3.3 The extent of positive or negative effects of the eleven factors on the NucB activity by *B. licheniformis* EI-34-6 and the corresponding p-values (number above each bar) of the factors showing their significance (if P < 0. 05) based on Analysis of variance (ANOVA) using P-B design study. The black bars indicate the positively significant factors taken for next level optimisation.**

The presence of phosphate in the culture medium was shown to enhance protease production in *B. firmus* (Moon and Parulekar, 1993). On the other hand, phosphate had an inhibitory effect on sporulation in *Anabena doliolum* (Pandey *et al.*, 1991) and on phosphatase activity in cyanobacteria (Pandey, 2006). Also, when 0.5 g/L phosphorous concentration in growth medium was reached, the manganese dependent peroxidase production by *Phanerochaete chrysosporium* decreased greatly (Liang *et al.*, 2012). Therefore literature results are in line to results reported in this chapter, where phosphate had a negative effect on NucB production. Therefore the three significant variables were investigated further by RSM using a CCD to identify their optimum levels and interactions. The experimental and predicted responses of NucB production are presented in Table 3.5.

**Table 3.5 CCD matrix for the experimental design with observed and predicted responses for medium optimisation for the NucB production[a].**

| Run order | $X_1$ | $X_2$ | $X_3$ | Nuc activity (U/ml)[b] | |
|---|---|---|---|---|---|
| | | | | Observed | Predicted |
| 1 | 0 | 1.63 | 0 | 342 | 340 |
| 2 | 0 | 0 | 1.63 | 337 | 349 |
| 3 | 0 | -1.63 | 0 | 238 | 250 |
| 4 | 0 | 0 | -1.63 | 261 | 258 |
| 5 | 1.63 | 0 | 0 | 408 | 424 |
| 6 | -1.63 | 0 | 0 | 280 | 274 |
| 7 | 0 | 0 | 0 | 333 | 312 |
| 8 | 0 | 0 | 0 | 298 | 312 |
| 9 | -1 | -1 | 1 | 247 | 254 |
| 10 | 0 | 0 | 0 | 311 | 312 |
| 11 | 0 | 0 | 0 | 265 | 312 |
| 12 | -1 | 1 | -1 | 238 | 256 |
| 13 | 1 | 1 | -1 | 361 | 346 |
| 14 | 1 | -1 | 1 | 374 | 348 |
| 15 | -1 | 1 | 1 | 372 | 360 |
| 16 | -1 | -1 | -1 | 222 | 211 |
| 17 | 1 | -1 | -1 | 338 | 342 |
| 18 | 0 | 0 | 0 | 344 | 312 |
| 19 | 0 | 0 | 0 | 330 | 312 |
| 20 | 1 | 1 | 1 | 410 | 413 |

[a]As described in the Materials and Methods, *B. licheniformis* EI-34-6 was grown in shake flasks containing the various media (pH 7.0) as indicated in the table (the levels indicated here correlate to the concentrations shown in Table 3.2 for each nutrient factor). 48 h post inoculation cells were sampled for the determination of Nuc activity. [b]The production yield in terms of volumetric Nuc activity for NucB production in culture broth.

By applying regression analysis, a quadratic model (Eq. (3.1)) was fitted to the data as follows:

$$\text{Nuc activity (U/ml)} = 338.96 + 34.49\,X_1 + 22.19\,X_2 + 21.90\,X_3 - 10.11\,X_1X_2 - 9.29X_2X_3 + 15.20\,X_3X_1 - 10.95\,X_1^2 - 16.65\,X_2^2 - 14.20\,X_3^2 \quad \text{Eq (3.1)}$$

where, Nuc activity (U/ml) is the response variable and $X_1$, $X_2$ and $X_3$ are the coded values of yeast extract, corn steep liquor and hysoy, respectively.

Statistical testing of the model was performed by Fischer's statistical test for analysis of variance (ANOVA) is shown in Table 3.6. The model F-value was 2.57 with a low probability value ($P > F = 0.0596$) and the F-value for "lack of fit" was 2.99 with a high probability value ($P > F = 0.1213$). The high model F-value and $P > F$ below 0.1

indicate the model terms are significant. The "lack of fit" P > F greater than 0.1 implies it is non-significant and thus the model provides a good representation of the experimental data. The non-significant interactions are shown in the table and equation to consider the actual model without leaving out the variables and their interactions.

**Table 3.6 Analysis of variance (ANOVA) for the selected quadratic model (Eq. 3.1) of CCD. Df represents degrees of freedom.**

| Variables | Sum of squares | df | Mean square | F-value | p-value (Prob > F) |
|---|---|---|---|---|---|
| Model | 42860.67 | 9 | 4762.30 | 2.57 | 0.0596 |
| Yeast extract ($X_1$) | 18381.25 | 1 | 18381.25 | 9.92 | 0.0077 |
| Corn steep liquor ($X_2$) | 7651.63 | 1 | 7651.63 | 4.13 | 0.0630 |
| Hysoy ($X_3$) | 7455.66 | 1 | 7455.66 | 4.03 | 0.0661 |
| $X_1X_2$ | 818.10 | 1 | 818.10 | 0.44 | 0.5179 |
| $X_2X_3$ | 691.18 | 1 | 691.18 | 0.37 | 0.5518 |
| $X_1X_3$ | 1848.32 | 1 | 1848.32 | 1.00 | 0.3360 |
| $X_1^2$ | 4514.57 | 1 | 4514.57 | 2.44 | 0.1425 |
| $X_2^2$ | 10433.55 | 1 | 10433.55 | 5.63 | 0.0337 |
| $X_3^2$ | 7597.59 | 1 | 7597.59 | 4.10 | 0.0639 |
| Residual | 24076.49 | 13 | 1852.04 | | |
| Lack of Fit | 19917.32 | 8 | 2489.67 | 2.99 | 0.1213 |
| Pure Error | 4159.17 | 5 | 831.83 | | |
| Corrected total | 66937.16 | 22 | | | |

The model predicted NucB production of 480 U/ml under optimum levels of the three factors ($X_1$ – yeast extract of 87g/L, $X_2$ – corn steep liquor of 36 g/L and $X_3$ – hysoy of 45 g/L). The validation experiment performed using these optimum conditions in shake flask cultivation resulted in 471 U/ml NucB activity which validates the accuracy of the model. Additional experiments on nuclease activity outside the coded levels of the three significant variables were performed. Increasing the levels up to 2.63 (in terms of coded values of the nutrient factors) decreased nuclease activity. When the results of these additional experiments were incorporated into the dataset used for model development, the contour plots illustrated the interactive effects of the independent variables on the NucB production (Figure 3.4 a-c).

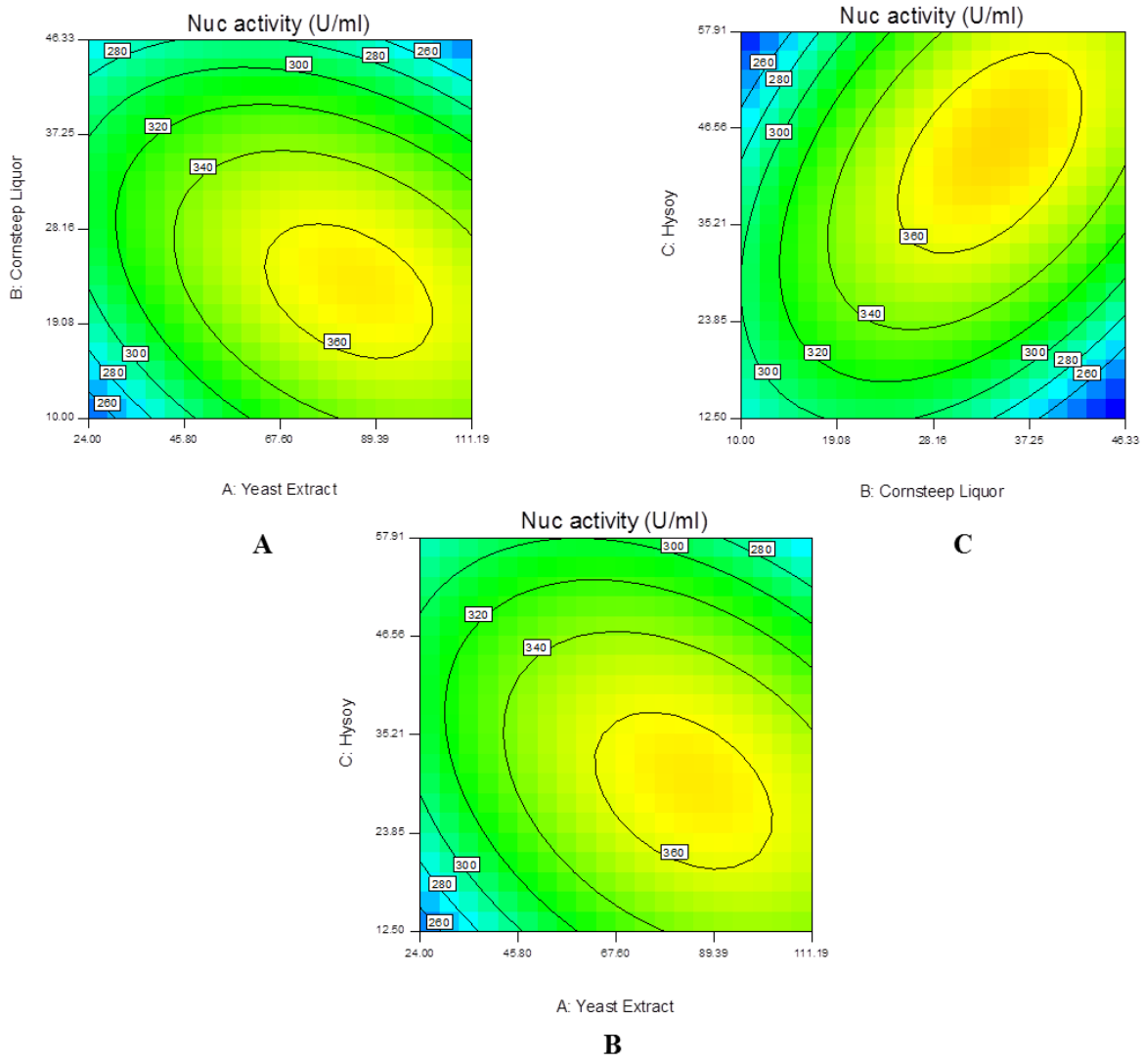



**Figure 3.4 Contour plots for the optimisation of NucB production in *B. licheniformis* EI-34-6: The interactive effects of two significant variables are shown, expressed as concentrations (g/L) in the medium A: yeast extract vs corn steep liquor with hysoy at 25 g/L B: yeast extract vs hysoy with corn steep liquor at 20 g/L C: corn steep liquor vs hysoy with yeast extract at 48 g/L.**

RSM using CCD designed experiments enabled the optimisation of the NucB production in the presence of animal free media components such as yeast extract, hysoy and corn steep liquor (CSL). In commercial production, complex nitrogen sources such as yeast extract and soy are often used as sources of proteins, vitamins and cofactors for growth and development of microbes, while CSL is considered an excellent source of free amino acids and micro nutrients that enhance the spore count (Table 3.3). In this study, the optimal combination of these media components was demonstrated to increase NucB production with statistical significance. These results correlate with earlier reports on derivatives of soy, corn products and yeast extract improving growth, sporulation and other metabolite production in various *Bacillus*

species (Table 3.3). Also, soy and corn steep products were used as inexpensive sources for production of other bioproducts (Maddipati *et al.*, 2011; Liu *et al.*, 2012; Saxena and Tanner, 2012). In this work, the optimum concentration of yeast extract (87 g/L), hysoy (45 g/L) and corn steep liquor (36 g/L) in the production medium resulted in a 10-fold (from 47 U/ml to 471 U/ml) increase in the NucB production at shake flask levels that will facilitate the scale up and commercialisation of the enzyme.

### *3.3.3 Effect of phosphate concentration on Mn induced NucB production.*

This study focused on applying the understanding of the physiological behaviour of *B. licheniformis* during phosphate addition, which is also important for industrial scale NucB production. In a biofilm environment, when cells lyse they release DNA into the extracellular space and cells become inorganic phosphate (Pi) limited. As Pi is one of the products of the degradation of nucleic acids, cells tend to secrete extracellular nucleases to recover lost phosphate (Mulcahy *et al.*, 2010). NucB production was found to commence in the late exponential phase, when the phosphate concentration decreased from 2.3 mM to ~0.3 mM and nuclease activity reached maximum at 48 h when biomass level decreased and the phosphate level was not detectable (Figure 3.5a). Interestingly, this investigation confirmed that phosphate levels were depleted when there was maximum NucB production and this corresponded to cells reaching the death phase. Also, experiments with increasing concentrations of phosphate (0.5 up to 6 mM) addition after 24 h post cultivation showed NucB synthesis gradually decreased (nuclease activity checked after 48 h) with increasing concentration of residual phosphate (Figure 3.5b). Similar observations were reported for studies on the biosynthesis of nuclease by *Proteus mirabilis* where phosphate addition had end-product inhibition effects (Salikhova *et al.*, 2000). Hence, in this experiment NucB production appears to be inhibited by the presence phosphate in the medium during cultivation.

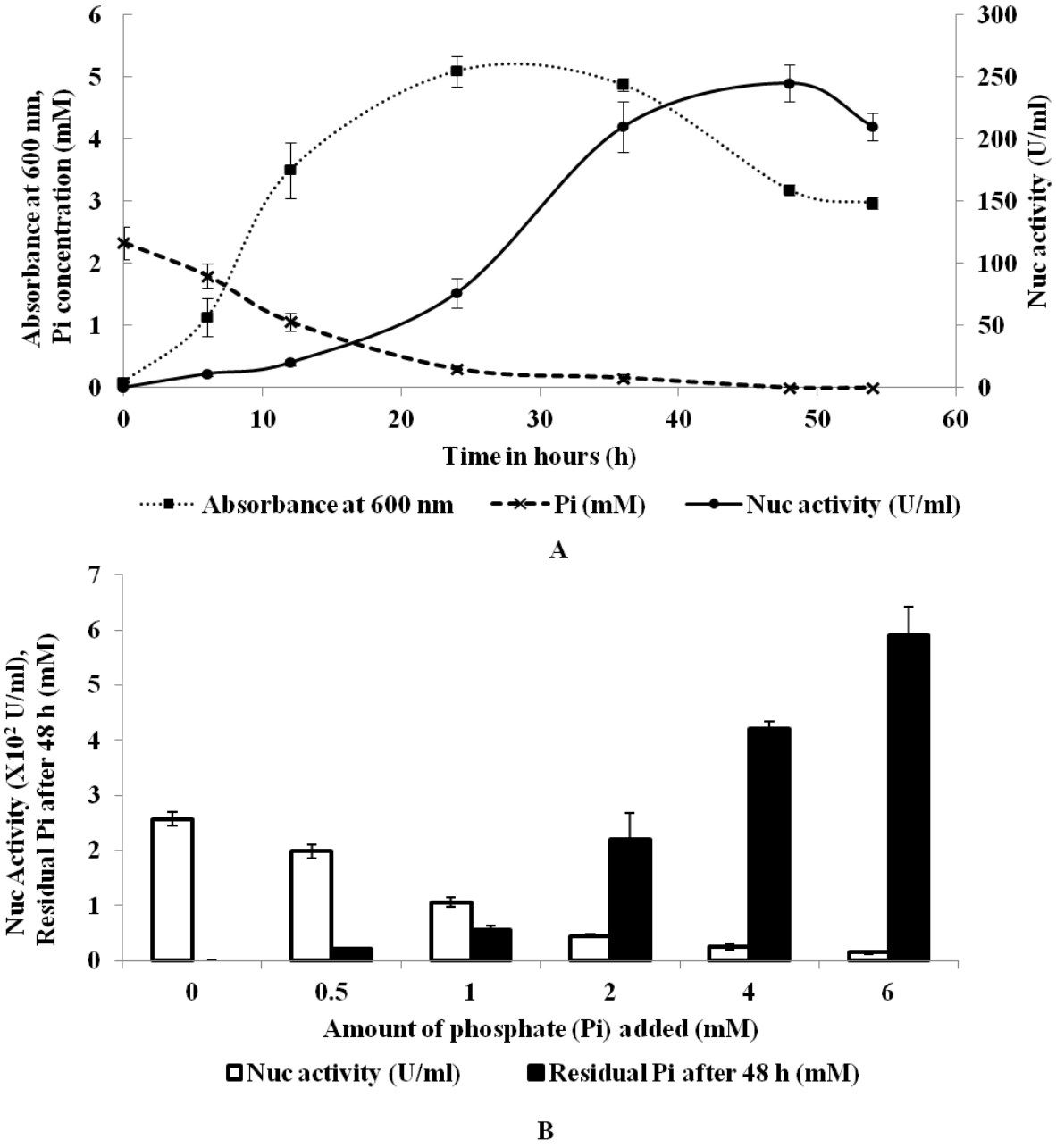



**Figure 3.5 The effect of phosphate addition on NucB production in LB medium with 100 µM $MnSo_4.H_2O$ A. Time trajectory of the *B. licheniformis* EI-34-6 growth, NucB production and phosphate concentration B. Addition of various concentrations of phosphate to 24 h post inoculation culture growing in LB medium with manganese.**

Therefore, we speculate that for large scale cultivation it is important to achieve high cell density using the optimum production medium and to reach phosphate depletion state for improving NucB production. This is one of the few studies to have applied an understanding of growth physiology to enhancement of enzyme synthesis and secretion.

## 3.4 SUMMARY

Subsequently, in this chapter production of an extracellular nuclease from *Bacillus licheniformis* EI-34-6 was achieved. Use of manganese as a secretory stimulus increased NucB production 5-fold. Statistical design of experiments identified yeast extract, hysoy and corn steep liquor as key variables for the production NucB, and their concentration optimisation gave 10-fold increased NucB secretion. Thus, NucB production was enhanced using unique physiology driven optimisation approach. Hence, the relationship between cell physiology and optimisation of process parameters is pivotal and that understanding the relationship between them could improve microbial bioprocesses and enzyme production efforts in a broad range of target proteins.

# Chapter 4 Characterisation of a recombinant biofilm dispersing endonuclease, NucB, produced by *Bacillus subtilis*

## 4.1 INTRODUCTION

Bacteria can actively secrete endogenous nucleases to escape from the biofilm in which they naturally grow. In previous work, we identified NucB, an extracellular nuclease produced by a marine strain of *Bacillus licheniformis,* which cleaves eDNA and disperses Gram positive and Gram negative biofilms (Nijland *et al.,* 2010). This enzyme was used to disperse natural biofilms on tracheoesophageal speech valves (Shakir *et al.,* 2012) and on biofilms produced by bacteria associated with chronic rhinosinusitis (Shields *et al.,* 2013). Hence, NucB, a microbial nuclease has potential as a biofilm dispersal compound. However, large quantities of this enzyme are needed to understand its biological role, and to allow its application in biofilm related medical and industrial problems.

NucB was initially produced in the wild type marine strain of *Bacillus licheniformis* EI-34-6 and a 10-fold increase in production of 10 mg/L was achieved (Rajarajan *et al.,* 2012). In order to further increase production, we heterologously expressed recombinant NucB in a subtilin regulated gene expression (SURE) host *Bacillus subtilis* NZ8900 (Nijland *et al.,* 2010). The advantage of using *Bacillus subtilis,* which is generally regarded as safe organism as host had been presented in chapter 1 in section 1.3.1. In addition, we used high cell density culture (HCDC) which enables increased productivity of desired proteins (Chen, 2012). The results indicate the feasibility of rapid purification of stable NucB at high concentrations. In this chapter, far UV circular dichroism (CD) spectroscopy, a method which is used to determine the amount of secondary structure in proteins in solution, and differential scanning calorimetry (DSC), a method used to understand the thermal stability of protein structure in solution, were used. Although, the structural information of protein was analysed using expensive X-ray diffraction (XRD) pattern in other studies, the above mentioned biophysical techniques have the advantages that they can be applied to molecules in solution. Additionally, it do not require crystallisation and XRD, hence cost effective techniques with limited resources. This is the first study to report high cell density culture of the *Bacillus* SURE expression system for heterologous protein production. Subsequently,

this is the first assessment of the biophysical and structural properties of the purified recombinant NucB.

## 4.2 MATERIALS AND METHODS

### *4.2.1 Microorganism and growth medium*

The *nucB* gene of the marine isolate *Bacillus licheniformis* EI-34-6 was previously cloned in the laboratory into a SURE expression vector pNZ8901 (Nijland *et al.*, 2010) and transformed into the SURE expression host *Bacillus subtilis* NZ8900 using the natural competence of the host strain and selection for chloramphenicol resistance (100mg/L) (Nijland *et al.*, 2010). Cells from a glycerol stock were streaked onto a Luria-Bertani (LB) plate containing 100 mg/L kanamycin and 100 mg/L chloramphenicol and incubated overnight at 37°C to obtain a pure single colony.

### *4.2.2 Medium pre-optimisation and growth conditions*

Initially, a medium pre-optimisation study was carried out in 500ml shake flasks and at 5L bioreactors. Two different basal media (pH 7.0): LB medium (Rajarajan *et al.*, 2012) and Hy-Soy™ medium modified from previous study (Cooke *et al.*, 2004) consisted of (g/L): Hy-Soy™ (Sheffield, UK), 25; yeast extract, 48; $NH_4Cl$, 1; $MgSO_4$, 0.25; $KH_2PO_4$, 3 and $Na_2HPO_4$, 6 were used. In all cultivation experiments, the media was supplemented with 100 mg/L kanamycin and 100 mg/L chloramphenicol. A single colony picked from a plate (prepared as described in section 4.3.1) was inoculated into 100ml LB/Hy-Soy™ medium in Erlenmeyer flasks (500ml). The inoculated seed flasks were incubated for 12h at 37°C and 200rpm on a rotary shaker. For the subsequent experiments, production flasks were inoculated with seed culture to a final optical density at 600nm ($OD_{600}$) of 0.1 and cultivated under the same growth conditions. When growth was in early exponential phase, the culture was induced with 1% cell free supernatant of *B. subtilis* ATCC 6633 as a natural source of subtilin (Bongers *et al.*, 2005). Cell growth and NucB production was further monitored until late exponential phase.

### *4.2.3 Central composite design (CCD) and statistical analysis*

After confirming the effect of the Hy-Soy™ medium, the levels of the media components were selected for study using a central composite design (CCD). The design matrix consisted of five coded levels (-2, -1, 0, +1, +2) and a set of 54 experimental runs, including replicates of the medial points. The range of the variables with coded and un-coded values were chosen based on low and high levels of 50% decrease and increase of levels in the production medium shown in Table 4. 1. The statistical software package Design Expert 8.0 (Stat-Ease Inc., Minneapolis, USA) was used to analyse the experimental results. Dry cell weight of *B. subtilis* in g/L was used as the response variable for the statistical analysis. An analysis of variance (ANOVA) was performed on experimental data to validate and evaluate the significance of the model.

**Table 4.1 Actual and coded factors for 6 independent variables used in central composite design optimisation**

| | Coded and actual levels | | | | |
|---|---|---|---|---|---|
| Variables (g/L) | -2 | -1 | 0 | +1 | +2 |
| Hysoy | 4.58 | 12.5 | 25.0 | 37.5 | 55.0 |
| Yeast extract | 8.78 | 24.0 | 48.0 | 72.0 | 105 |
| $KH_2PO_4$ | 0.55 | 1.50 | 3.00 | 4.50 | 6.55 |
| $Na_2HPO_4$ | 1.10 | 3.00 | 6.00 | 9.00 | 13.0 |
| $NH_4Cl$ | 0.18 | 0.50 | 1.00 | 1.50 | 2.20 |
| $MgSO_4$ | 0.05 | 0.13 | 0.25 | 0.38 | 0.55 |

### *4.2.4 Batch cultivation studies and effect of dissolved oxygen*

Batch cultivation was carried out using a 5L bioreactor (New Brunswick Scientific, UK). The bioreactor, containing 2.9 L of sterilised medium, was inoculated with 50ml of a seed culture of $OD_{600}$ 0.15. Cultivation temperature was controlled at 37°C, pH at 7.00 ± 0.05 and foaming was controlled by addition of 4% polypropylene glycol. The dissolved oxygen tension (DOT) level was controlled at the required values (0%, 20% and 30%) of air saturation by the manipulation of agitation between 200 to 1500 rpm and oxygen was enriched through mass flow control when required. Batch cultivation at

each DOT level was carried out in duplicate. Expression of NucB was induced in mid-exponential phase, sampled regularly at hourly intervals and harvested in the late exponential phase. For further purification of NucB protein, culture supernatants (2.7 L) were passed to the University protein purification facility run by Prof. Alastair Hawkins, who subsequently provided purified protein (see section 4.2.6)

### *4.2.5 Determination of growth and protein concentration*

Culture medium samples were withdrawn at the sample time intervals, in duplicate and centrifuged at 13000rpm for 10min. The cell pellets were re-suspended in 0.9 % (w/v) NaCl and the cell suspensions were then filtered through pre-weighed dry membrane filter and then dried at 80°C to constant weight to determine dry cell weight (DCW) in g/L. The expression levels of NucB were determined using bovine serum albumin (BSA) as standard protein by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% NUPAGE gel (Invitrogen, UK). Gels were stained with NUPAGE colloidal stain and captured as image. Standard BSA protein band intensity and NucB protein band intensity was quantified from gel by densitometry using Image J (Schneider *et al.*, 2012). NucB concentration was determined using calibration curve was generated using BSA protein concentration.

### *4.2.6 Source of purified NucB*

NucB at >95% purity was a gift from Professor Alastair R. Hawkins. At the time of this thesis submission, the details of NucB purification method are in the early stages of a patent filing by Prof. Hawkins and consequently are confidential until the patent application is published.

### *4.2.7 Biochemical characterisation of NucB*

**Optimisation of temperature, pH and metal ions**

The optimum temperature and pH of the enzyme were determined by measuring the nuclease activity at various temperatures (0-60°C) at pH 7.0 and pH (3-10) at 37°C and with various metal ions $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Hg^{2+}$, $Co^{2+}$ at concentration range 0-10 mM

using 50 mM Tris-HCl buffer with salmon sperm DNA as substrate as described earlier (Rajarajan *et al.*, 2012). Nuclease activity was measured by monitoring an increase in optical density at 260 nm. One unit of nuclease activity is defined as the amount of enzyme that causes an increase of 1.0 OD at 260 nm after 30 min at 37 °C. Assays were carried out in triplicate.

**Effect of inhibitors**

The effect of inhibitors on NucB was investigated by carrying out nuclease assays with the chelating agent EDTA and Iron (III) chloride.

**Determination of specificity and mode of action**

To determine the specificity of NucB, the nuclease assay was performed with double stranded DNA or RNA as substrates under standard assay conditions.

**Determination of endo-nucleolytic activity**

To determine the mode of action, plasmid DNA was used as a substrate under standard conditions and analysed in 0.8% agarose gel.

### *4.2.8 Biophysical characterisation of NucB*

**Differential scanning calorimetry (DSC)**

The methodology and rationale for differential scanning calorimetry (DSC) is contained within (Cooper *et al.*, 2000). Protein thermal transition characteristics in solution were determined by DSC using a Micro-Cal VP-DSC (cell volume 0.52 mL). Samples were scanned over a range from 25°C–80°C, with a scan rate of 90°C $h^{-1}$, and a filtering period of 16 sec with protein at 30 µM and 50 mM Tris-HCl buffer in the reference cell. Solutions were gently degassed prior to loading. Thermograms were corrected for instrumental baseline, using control buffer which was scanned under identical conditions, and analysed using standard Micro-Cal ORIGIN software. Protein concentrations were measured spectrophotometrically using the molar absorption

coefficient calculated from the amino acid sequence by the vector NTI suite of programs.

**Circular dichroism (CD) spectroscopy**

The methodology and rationale for Circular dichroism CD spectroscopy is contained within (Kelly *et al.*, 2005). CD spectra of the NucB protein (0.39 mg/ml) in the far-UV wavelength range between 250 to 190 nm were recorded at 20°C on a Jasco model J-810 spectropolarimeter (JASCO Inc., Japan). Each spectrum was an average of five continuous scans. Scans were recorded at standard sensitivity with a band width of 2 nm, scanning speed of 50 nm $min^{-1}$. Calculations and data analysis were carried out using Jasco Spectra analysis software. The acquired spectra were corrected by subtracting reference blank runs as appropriate protein free buffer solutions. The CD spectrum obtained was analysed using Dichro Web services (Whitmore and Wallace, 2004).

### *4.2.9 Secondary and Tertiary structure prediction of NucB using bioinformatics tools*

Protein secondary structure composition and prediction were performed using bioinformatics tools available online: the Jpred 3 server (Cole *et al.*, 2008) and the tertiary structure model obtained using QUARK, *ab initio* program for template-free proteins (Xu and Zhang, 2012).

## 4.3 RESULTS AND DISCUSSION

### *4.3.1 High cell density cultivation of B. subtilis and enhanced production of NucB*

Growth of *B. subtilis* and NucB production using two different basal media was studied. Initially in shake flasks, higher final cell growth and increased NucB concentration was obtained in cultivations using Hy-Soy™ medium than those using LB medium. The time trajectory for batch cultivation showed exponential phase until 10h after which growth reached stationary phase. For recombinant protein expression based on an inducer, it is usually optimal to add the inducer when an intermediate cell density was obtained (Cheng *et al.*, 2011). The cells were therefore subtilin induced at early-

exponential phase after which NucB secretion occurred and increased concomitantly with growth, suggesting that the process is growth associated (data not shown). Fig. 4.1 presents the SDS-PAGE gel showing the tightly controlled NucB production by subtilin induction. Lane 2 shows the cell culture supernatant before induction where NucB was not produced and lanes 3 to 9 contained samples after subtilin induction and showed increasing NucB yield until harvest time. This study showed controlled *nucB* gene expression by the SURE system which resulted in moderate cell density before induction and enhanced growth and NucB production after induction. This observation demonstrated that the SURE system was very efficient as compared earlier with other induction based *Bacillus* expression systems in terms of the amount of expressed recombinant products (Vavrovai *et al.*, 2010).

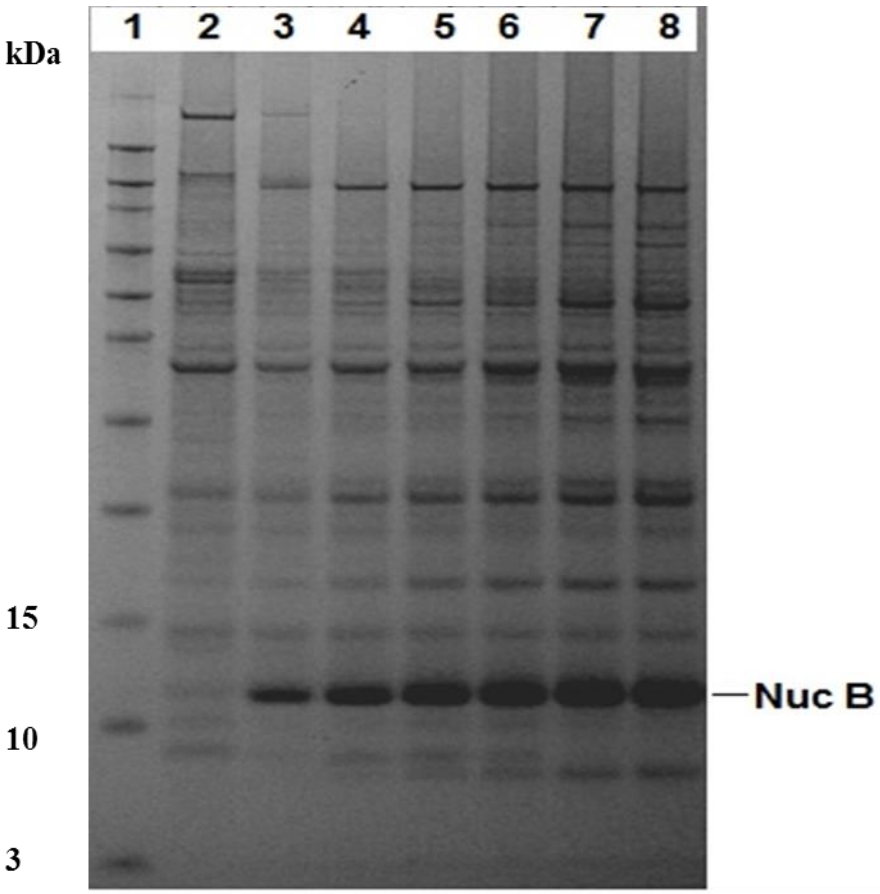



**Figure 4.1 SDS-PAGE gel image of crude supernatant of *B. subtilis* NZ8900. Lane 1 – Novagen pre-stained protein ladder ranging from 3 kDa to 210 kDa, Lane 2 – un-induced culture supernatant, Lane 3 to 8 – induced culture supernatant at 5.2, 6.2, 7.5, 8.2, 9.2, 10.2 h, NucB is pointed at approximately 12 kDa.**

Previous studies reported that the secretion level of recombinant enzymes such as serine protease and nattokinase were increased in the presence of $Na^+$, $K^+$, $Mg^{2+}$ or $Ca^{2+}$ in the medium (Chen *et al.*, 2007a). Also, soybean hydrolysate, peptone and yeast extract are most commonly used organic sources for industrial scale production of phytase and nattokinase (Vuolanto *et al.*, 2001; Chen *et al.*, 2007a). These studies also support the fact that presence of the most commonly available carbon sources, such as glucose, has a negative effect on enzyme production (Chen *et al.*, 2007a). The reason for this is that during cultivation acidic by-products such as acetates are produced that have a strong inhibitory effect on cell growth and also genetic instability (Vuolanto *et al.*, 2001). In this work, these facts were considered and $KH_2PO_4$, $Na_2HPO_4$, $MgSO_4$, Hy-Soy™ and yeast extract were included in the production medium and no additional sugars was present which significantly increased the growth of *B. subtilis* as well as leading to improved NucB production. Since NucB is growth associated, a batch cultivation leading to high cell density culture will directly increase the NucB production. Similarly, high cell density associated recombinant protein production was reported in other strains of *B. subtilis* (Park *et al.*, 1992; Huang *et al.*, 2004; Cho *et al.*, 2010) and *E. coli* (Choi *et al.*, 2006). The six components of the Hy-Soy™ medium were therefore considered critical for medium formulation and their concentration in the medium was optimised using CCD to increase the cell density of *B. subtilis* during cultivation which would then enhance NucB production.

There were 54 experimental trials carried out and dry cell weight (DCW) was taken as the measurement of cell density of *B. subtilis* and used as the response variable. This is presented in Table 4.2, along with predicted responses. Based on the analysis of variance for the responses a maximum dry cell weight was predicted as 11 g/L under optimum medium concentrations. The value of DCW obtained from cultivations carried out under these conditions was 11 ± 1 g/L confirming the model fit. This optimum production medium (OPM) was used to achieve maximum *B. subtilis* cell growth and after subtilin induction cells produced a maximum of 40 mg/L of NucB after 10 h cultivation in shake flasks. The presence of Hy-Soy™ and yeast extract also increased wild type NucB production (Rajarajan *et al.*, 2012). However, in the current study in recombinant strain the cultivation period was shorter and the productivity increased to 15-fold compared to basal media cultivation in shake flask using OPM.

**Table 4.2 Central composite design matrix and *B. subtilis* dry cell weight (DCW) as actual and predicted responses**

| Run | Hysoy | Yeast Extract | $KH_2PO_4$ | $Na_2PO_4$ | $NH_4Cl$ | $MgSO_4$ | DCW (g/L) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Actual | Predicted |
| 1 | 0 | 0 | 0 | 0 | +2 | 0 | 4.2 | 4.3 |
| 2 | +2 | 0 | 0 | 0 | 0 | 0 | 5.7 | 5.4 |
| 3 | 0 | 0 | +2 | 0 | 0 | 0 | 4.2 | 3.9 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 3.7 | 4.0 |
| 5 | 0 | 0 | 0 | 0 | -2 | 0 | 3.8 | 4.1 |
| 6 | 0 | 0 | -2 | 0 | 0 | 0 | 3.4 | 3.9 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 3.4 | 4.0 |
| 8 | 0 | 0 | 0 | 0 | 0 | -2 | 4.2 | 4.1 |
| 9 | 0 | 0 | 0 | -2 | 0 | 0 | 3.0 | 3.2 |
| 10 | -2 | 0 | 0 | 0 | 0 | 0 | 2.5 | 3.1 |
| 11 | 0 | -2 | 0 | 0 | 0 | 0 | 2.2 | 1.8 |
| 12 | 0 | +2 | 0 | 0 | 0 | 0 | 4.8 | 5.5 |
| 13 | 0 | 0 | 0 | 0 | 0 | +2 | 4.1 | 4.5 |
| 14 | 0 | 0 | 0 | +2 | 0 | 0 | 5.1 | 5.1 |
| 15 | 1 | 1 | -1 | 1 | 1 | -1 | 5.8 | 5.6 |
| 16 | -1 | 1 | -1 | -1 | -1 | 1 | 4.4 | 3.7 |
| 17 | -1 | -1 | -1 | -1 | -1 | -1 | 3.3 | 2.6 |
| 18 | -1 | 1 | 1 | 1 | -1 | 1 | 4.9 | 4.6 |
| 19 | 1 | -1 | -1 | 1 | 1 | 1 | 5.3 | 4.6 |
| 20 | -1 | 1 | -1 | 1 | 1 | 1 | 5.8 | 5.5 |
| 21 | -1 | 1 | 1 | -1 | 1 | 1 | 4.3 | 4.1 |
| 22 | 0 | 0 | 0 | 0 | 0 | 0 | 4.1 | 4.0 |
| 23 | 1 | 1 | 1 | 1 | -1 | -1 | 6.0 | 5.9 |
| 24 | 1 | -1 | 1 | -1 | 1 | 1 | 3.4 | 3.9 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 4.0 | 4.0 |
| 26 | 1 | 1 | -1 | -1 | -1 | -1 | 3.8 | 3.9 |
| 27 | 1 | 1 | 1 | -1 | 1 | -1 | 4.9 | 4.4 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 | 5.7 | 4.0 |
| 29 | 0 | 0 | 0 | 0 | 0 | 0 | 3.8 | 4.0 |
| 30 | -1 | -1 | 1 | -1 | 1 | -1 | 3.0 | 2.7 |
| 31 | -1 | -1 | -1 | 1 | 1 | -1 | 2.5 | 2.9 |
| 32 | 1 | -1 | -1 | -1 | -1 | 1 | 3.7 | 3.9 |
| 33 | 1 | -1 | 1 | 1 | -1 | 1 | 4.5 | 4.2 |
| 34 | -1 | -1 | 1 | 1 | -1 | -1 | 2.4 | 2.5 |
| 35 | 1 | -1 | -1 | 1 | -1 | -1 | 4.0 | 4.0 |
| 36 | -1 | 1 | -1 | 1 | -1 | -1 | 5.7 | 5.0 |
| 37 | -1 | -1 | -1 | -1 | 1 | 1 | 3.0 | 2.9 |
| 38 | -1 | -1 | 1 | -1 | -1 | 1 | 2.3 | 2.3 |
| 39 | 1 | 1 | 1 | 1 | 1 | 1 | 5.7 | 6.2 |
| 40 | -1 | 1 | -1 | -1 | 1 | -1 | 3.8 | 3.9 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 41 | 0 | 0 | 0 | 0 | 0 | 0 | 4.0 | 4.0 |
| 42 | 1 | 1 | -1 | -1 | 1 | 1 | 5.0 | 4.6 |
| 43 | 0 | 0 | 0 | 0 | 0 | 0 | 3.8 | 4.0 |
| 44 | 1 | -1 | 1 | -1 | -1 | -1 | 3.6 | 3.7 |
| 45 | -1 | -1 | 1 | 1 | 1 | 1 | 2.8 | 2.5 |
| 46 | -1 | 1 | 1 | 1 | 1 | -1 | 5.7 | 5.3 |
| 47 | 0 | 0 | 0 | 0 | 0 | 0 | 4.9 | 4.0 |
| 48 | 0 | 0 | 0 | 0 | 0 | 0 | 3.5 | 4.0 |
| 49 | -1 | -1 | -1 | 1 | -1 | 1 | 2.5 | 2.8 |
| 50 | 1 | 1 | -1 | 1 | -1 | 1 | 6.0 | 6.1 |
| 51 | 1 | 1 | 1 | -1 | -1 | 1 | 5.2 | 4.7 |
| 52 | 1 | -1 | -1 | -1 | 1 | -1 | 3.0 | 3.1 |
| 53 | -1 | 1 | 1 | -1 | -1 | -1 | 3.6 | 4.1 |
| 54 | 1 | -1 | 1 | 1 | 1 | -1 | 3.2 | 3.7 |

To determine whether the process optimisation could be scaled up, the batch cultivation was performed using the OPM in a 5 L bioreactor. To investigate the effect of the dissolved oxygen level on *B. subtilis* growth and NucB production, 5 L batch cultivations with dissolved oxygen concentration 0 %, 20 % and 30 % of air saturation were carried out. The comparison of the performance and kinetic parameter values of cell growth and NucB production by *B. subtilis* NZ8900 at different dissolved oxygen tension levels is shown in Table 4.3. The maximum biomass concentration ($X_{max}$) increased almost 1.25 times with increasing DOT level from 0 to 20 % saturation and the $X_{max}$ increased further 1.28 times with increase of DOT level from 20 to 30 % saturation. In previous studies, DO greater than 20 % did not affect growth rate of *B. subtilis* but it affected the keratinase enzyme production rate (Wang and Shih, 1999). In contrast, data obtained in this chapter showed a substantial increase in NucB production, about 1.45 times higher, was obtained by increasing DOT level from 0 to 20 % saturation. Also, NucB production was 1.5 times higher with increasing DOT from 20 to 30% saturation.

**Table 4.3 Comparison of performance and kinetic parameter values of cell growth and NucB production in batch cultivation of *B. subtilis* NZ900 in optimum production medium at different dissolved oxygen tension (DOT) levels in a 5 L bioreactor**

| | DO level* | | |
|---|---|---|---|
| Kinetic parameter values | 0 % | 20 % | 30 % |
| Maximum biomass concentration, $X_{max}$ ($g\ L^{-1}$) | 20.2 ± 0.4 | 25.3 ± 1.2 | 32 ± 1.5 |
| Maximum specific growth rate, $\mu_{max}$ ($hr^{-1}$) | 0.21 | 0.37 | 0.42 |
| Maximum NucB concentration, $P_{max}$ ($mg\ L^{-1}$) | 95.7 ± 1.5 | 138.7 ± 3.3 | 210.3 ± 2.6 |
| NucB specific production rate (mg /g Biomass.hr) | 1 | 2.1 | 2.7 |

Mean value ± standard error (n = 3), ANOVA tests for each dissolved oxygen level showed p-value < 0.001 for both biomass and NucB concentration, hence results are statistically significant

Dissolved oxygen limitation has been extensively reported in high cell density cultivations of *E. coli* as affecting the cell growth and recombinant product yield (Soini *et al.*, 2008). Similarly, in this study for efficient growth and NucB production in *B. subtilis* there is a requirement for aerobic conditions and maintaining 30% DOT level gave nominal highest yield. There were difficulties in process control due to foam formation during further increase in DOT levels. Also in economical perspective, controlling DO at 30% air saturation was ideal and robust for large scale production process. Figure 4.2 shows the sequence of scale up involved in the current study. When LB basal medium was changed to Hysoy™ basal medium, recombinant *B. subtilis* expressed NucB from 3 mg/L to 14 mg/L, which is a 5-fold increase in production. In literature, fed-batch cultivation approach was used to achieve high cell density of recombinant *B. subtilis* to obtain 32 g/L DCW (Vuolanto *et al.*, 2001). Conversely, in this study, process engineering using statistical design of experiments enabled to achieve 32 g/L DCW by high cell density batch cultivation. This is a 20-fold overall increase in cell density. This is therefore one of the few study showing high cell density culture achievable by *B. subtilis* in a batch cultivation approach. Also this enabled a 68-fold (210 mg/L) enhanced NucB production

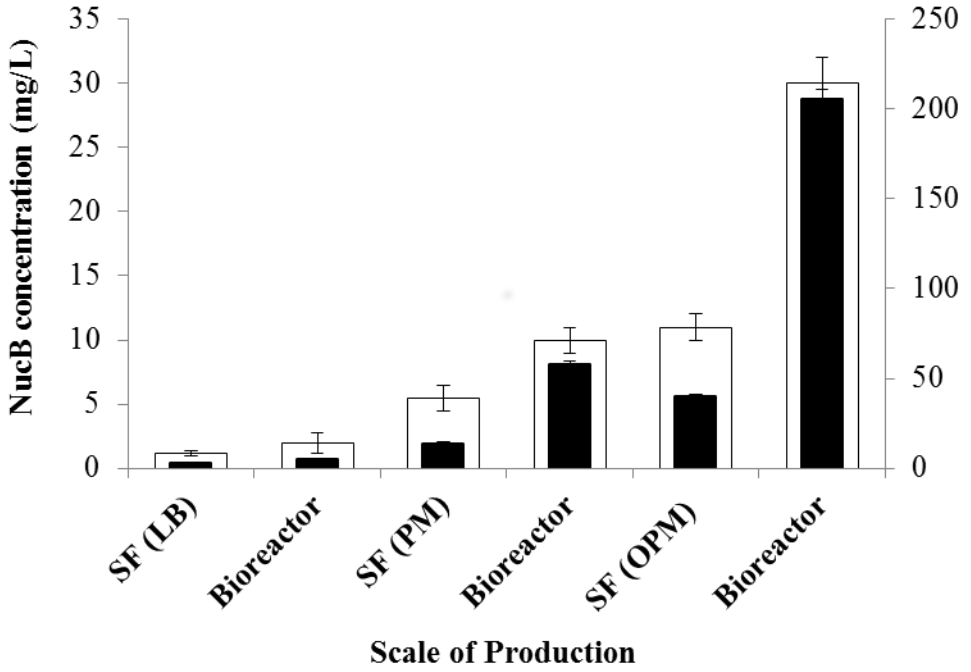



**Figure 4.2 Enhancement of growth (white bars) and NucB production (black bars) by *B. subtilis* from non-optimised to optimised production medium using shake flasks and 5 L bench top bioreactor. Cultivations were carried out under conditions described in section 4.3.2 and the final value at 10h (harvest time). LB represents Luria Bertani medium, PM represents HySoy™ medium, OPM represent optimum production medium**

***4.3.2***

### *4.3.3 Analysis of purified NucB*

The optimum production medium was used for the maximum production and purification of NucB. Increasing volumes of purified NucB were subject to analysis by SDS PAGE to look for the presence of any other contaminating proteins (Figure 4.3).

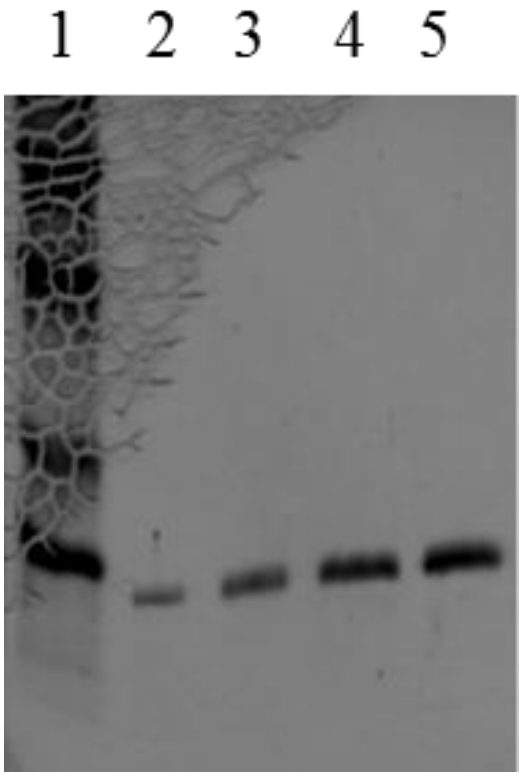



**Figure 4.3 Analysis of the purified NucB by SDS-PAGE. The protein samples were electrophoresed on a 15% polyacrylamide gel and visualised by coomassie blue staining. Lane 1 – molecular markers 43, 29, 18 and 12.4 kDa. Lanes 2 to 5 NucB at increasing loading volumes of 1.5, 3, 4.5 and 6 µl. The specific activity of NucB obtained was 15000 U/mg protein (A.R.Hawkins, unpublished data)**

The NucB purified was greater than 95% purity with a specific activity of 15000 U/mg of protein.

### *4.3.4 Biochemical and biophysical characterisation of recombinant NucB*

Characterisation was carried out to assess the product attributes such as purity, activity, specificity, mode of action, aggregation, secondary and tertiary structures, thermal stability and stability during storage. The biochemical properties of NucB are summarised in Table 4.4. The optimum temperature for the degradation of nucleic acids by NucB is 37 °C. However, the enzyme is effective over a temperature range of 4-60 °C. The temperature storage stability of purified recombinant NucB showed no loss of activity when stored at -80°C for more than 6 months. However, there was a slight loss of activity, as expected, during the transition between freezing and thawing. The effect of storage at -20°C and 4°C showed it retains greater than 75% activity examined every 2 months up to a year (data not shown).

**Table 4.4 Properties of nuclease NucB**

| Property | Value |
|---|---|
| Mr | 12 kDa |
| Optimum temperature | 37°C |
| Optimum pH | 8 |
| Metal ion requirement | $Mn^{2+}$ (5 mM concentration) |
| Inhibitors | $Fe^{2+}$ and EDTA |
| Substrate specificity | dsDNA>> RNA |
| Mode of action | Endonuclease |

NucB show optimum activity at pH 8.0 and is effective over a temperature range of 6 to 9. These results show NucB is active across a similar range of temperature as that of other microbial extracellular nuclease (Healy *et al.*, 1999). NucB exhibited obligate requirement for divalent metal ion. It showed optimum nuclease activity with 5 mM $Mn^{2+}$ in 50 mM Tris-HCl buffer (pH 8.0) and 75% activity with $Mg^{2+}$ whereas 40% activity with $Ca^{2+}$. The manganese ion requirement for NucB activity is similar to the endonuclease, Stn β, produced from *Streptomyces thermonitrificians* (Patil *et al.*, 2005), deoxyribonuclease, Rsn, from fungi *Rhizopus stolonifer* with double strand DNA substrates (Rangarajan and Shankar, 1999), Nuc C1 from another fungi *Cunninghamella echinulata* (Ho *et al.*, 2001) and endonuclease, NucA from filamentous cyanobacteria *Anabena* sp. (Rangarajan and Shankar, 2001). However, it is different to the thermostable nuclease, NucH, from *Staphylococcus hyicus* (Chesneau and Solh, 1994), which showed absolute requirement for $Ca^{2+}$, and endonuclease from *Serratia marscens*, which is active only with $Mg^{2+}$ (Benedik and Strych, 2006).

Divalent cation $Fe^{2+}$ inhibited the enzyme at concentration range of 1-3 mM and also, the chelating agent EDTA inhibited NucB activity at concentrations as low as 0.5 mM which is similar to observations on Nuc C1 and DNase, SaD 1 from *Streptomyces aureofaciens* (Yang, 2011). NucB has specificity towards double stranded DNA as it showed high activity on DNA and only up to 40 % activity on RNA. It was able to readily degrade the plasmid DNA, pUC18 which confirms that NucB is an endonuclease by mode of action. This is also been reported on native NucB from *B. subtilis* (Vansinderen *et al.*, 1995) and is similar in characteristics to other reported microbial nucleases (Rangarajan and Shankar, 2001).

Figure 4.4 shows typical DSC thermograms, and the enthalpy of unfolding is summarised in Table 4.5. Inspection of Figure 4.4 and Table 4.5 shows that the midpoint for NucB unfolding (Tm) was 57.5°C and that the protein was completely unfolded at 80°C. Table 4.5 shows that approximately 55% of NucB molecules unfolded at 80°C were able to refold when passively cooled to 25°C. Previously, reversible folding has been reported for NucH, a thermonuclease which retained nuclease activity after heating to 100°C (Chesneau and Solh, 1994). Another study of thermonuclease from *Staphylococcus aureus* demonstrated reversible thermal refolding using DSC (Tanaka *et al.*, 1993). The DSC scan of NucB is therefore comparable to this previous observation. However, NucB being a small, mesophilic endonuclease and showing reversible unfolding during thermal denaturation is an unusual observation.

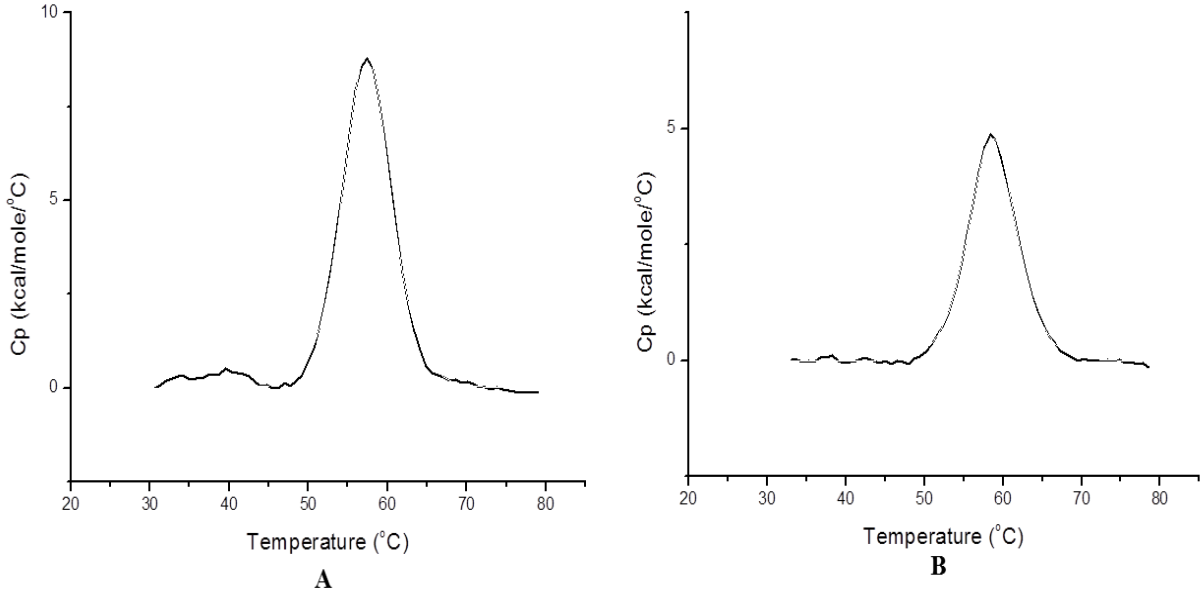



**Figure 4.4 DSC thermograms of the recombinant NucB (0.39 mg/ml) at 90°C per hour in pH 8.0 50 mM Tris-HCl buffer, 1 mM DTT. After subtraction of a buffer baseline the data were analysed by fitting to the non-two state model contained within the MicroCal Origin software package. Panel A = DSC thermogram showing the first unfolding; Panel B = DSC thermogram showing the unfolding of the refolded protein after passive cooling to 25°C.**

**Table 4.5 DSC thermograms, and enthalpies of unfolding for first and refolded protein**

| Parameters | Tm (°C) | ΔH (KJ/mol) |
|---|---|---|
| First unfolding | 57.5 ± 0.1 | 62.5± 4.4 |
| Unfolding of refolded protein | 58.5 ± 0.1 | 32.7 ± 3.0 |

Study also examined the secondary structure of NucB by CD spectroscopy at standard buffer conditions. The analysis of the CD spectrum of the purified NucB showed presence of mixed alpha helical and beta sheet conformation (Figure 4.5). The data in figure 4.5 were further analysed by the CDSSTR analysis programme in conjunction with reference set 4 contained within the DichroWeb service. The results of the analysis are shown in Figure 4.6.

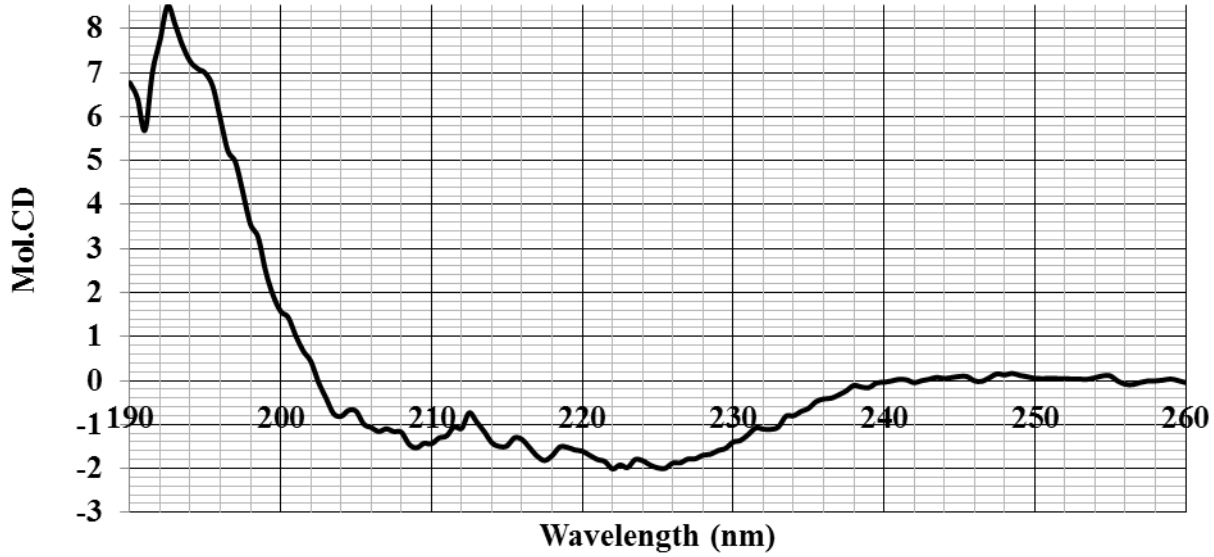



**Figure 4.5 Secondary structure analysis of NucB by CD spectroscopy.**

The DichroWeb analysis gave an NRMSD (normalised root mean square deviation) of 0.011, giving confidence that the experimental and reconstructed data have a good fit. NMRSD values range from 0 (perfect fit) to 1 (no fit whatsoever). In practise, NMRSD values above 0.1 are usually taken to indicate that the fit is unlikely to be correct. Hence, the data obtained here by CD analysis is a good fit. The DichroWeb analysis predicts 32% α-helix, 24% β-sheets and 44% coil or disordered structure. This observation is in contrast with *Anabena* sp. nuclease NucA and *Serratia* nuclease which showed lower α-helical and β-sheet content (Meiss *et al.*, 1998).

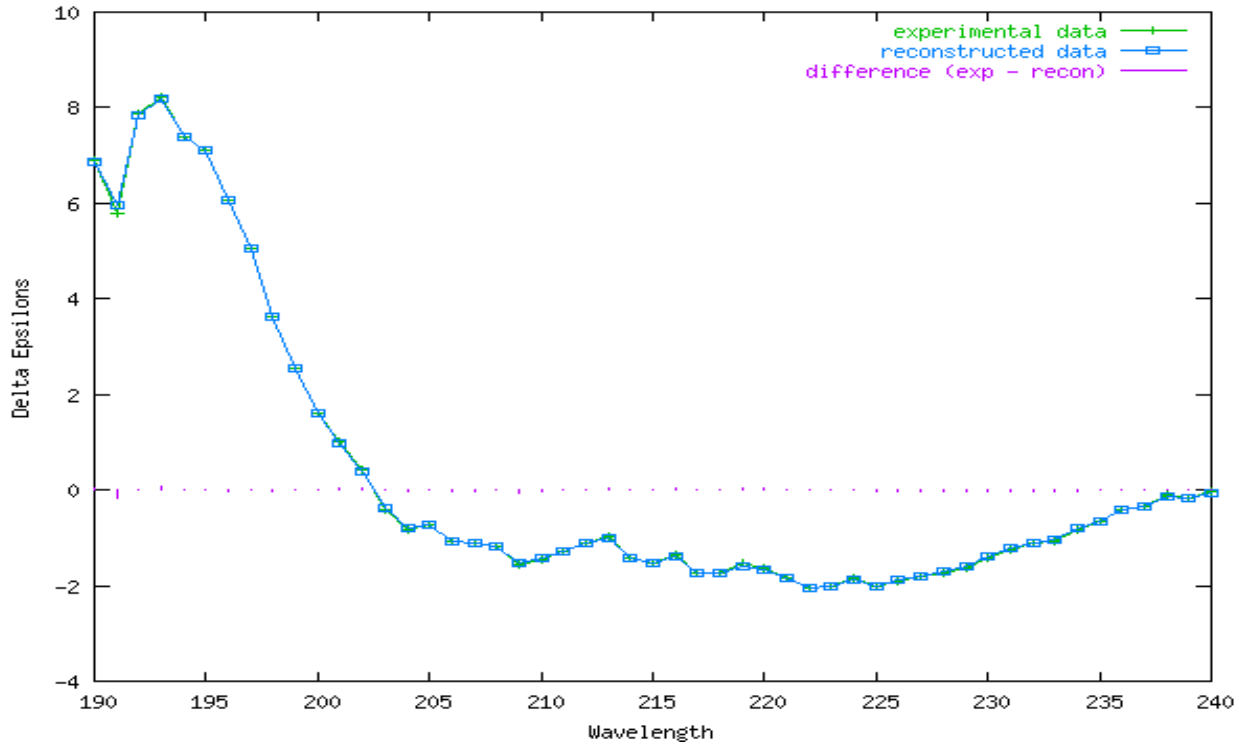



**Figure 4.6 Far-UV CD spectra generated by DichroWeb showing the secondary structure content of recombinant NucB. The spectrum in green is the experimental data and spectrum in blue is the reconstructed data**

### *4.3.5 NucB structure prediction using bioinformatics tools*

We have used bioinformatics tools and web-services for the prediction of the NucB structure. The secondary structure evaluation by Jpred 3 retrieved similar sequences from Uniprot database which included NucB, a sporulation specific extracellular nuclease of other *Bacillus* species and NucA, DNA entry/competence specific nucleases and aligned the multiple sequences using CLUSTAL. Jpred 3 then provided Jalview of three state predictions (α-helix, β-strand and coil) shown in Fig 4.5. The confidence score for prediction ranges from 0-9 and the score of buried to exposed residues range from 0-9. The coloured amino acid sequences are the most conserved region and suggested to be the active site. Previous study on characterising endo-exonuclease from *Armillaria mellea* showed 42% identity to NucB sequence also denoted the active site present within the 38 residues covering DRDE conserved domain (Healy *et al.*, 1999).

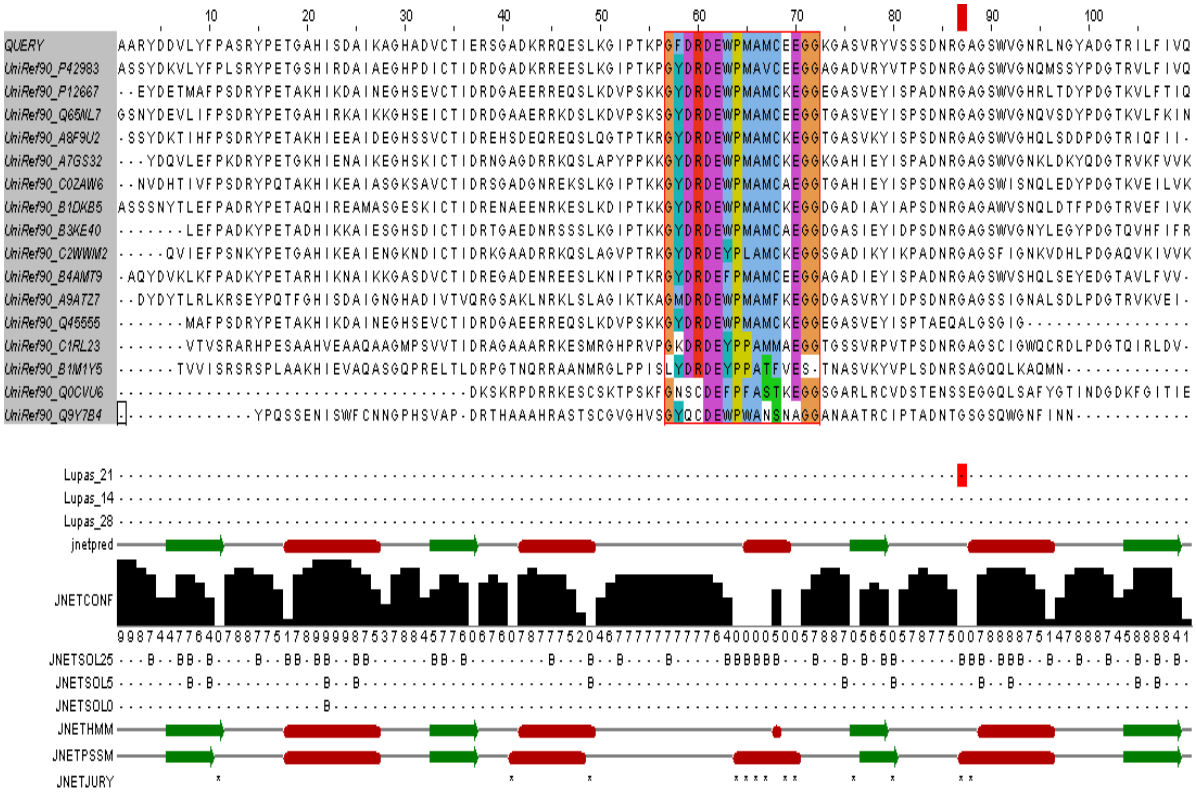



**Figure 4.7 NucB protein sequence annotated with the secondary structure predicted by Jpred 3 web-services**

Spectrum generated using Far-UV CD presented in Fig 4.4 compared with the structural composition calculated from Jpred 3 predictions showed number of α-helix and β-strands, coils and disordered residues are in good agreement (Table 4.5).

**Table 4.6 Comparison of calculated secondary structure as percentage fractions using predicted method and experimental method**

| Secondary structures | | Jpred 3 prediction (%) | CD spectrum analysis (%) |
|---|---|---|---|
| α- helix | H | 32 | 32 |
| β- strands | E | 21 | 24 |
| Coils and Disordered | C | 47 | 44 |

Some of well-known sugar non-specific nucleases such as; *Serratia* nuclease is a dimer, with 2 equal monomer units that has a central β-sheet formed by six-antiparallel β-strands flanked by four short α-helices. NucA of *Anabena* sp., and periplasmic nuclease of *Vibrio vulnificus* contains a ββα-metal motif. Also, EndA nuclease of *Streptococcus*

*pneumoniae* and prophage-encoded extracellular DNase from *Streptococcus pyogenes* revealed DRGH conserved domains that contain ββα-metal finger motif (Korczynska *et al.*, 2012). Another sugar non-specific nuclease is staphylococcal nuclease which contains two anti-parallel β-sheets and three α-helices (Yang 2011). The results infer literature reported nuclease structures are different from predicted NucB structure. Therefore, NucB suggested to be considered as a separate class of nucleolytic enzyme compared to sugar non-specific nucleases that showed secondary structure composition and active site around DRGH conserved domain.

Three dimensional structural models developed using QUARK *ab-initio* web-service is shown in Fig.4.6 A. Model with the predicted binding site shown in Fig 4.6 B and includes residues 35, 36, 37, 38, 42, 44, 45, 48. The predicted PDB model was validated by searching in the DALI server to map against biologically similar structural proteins. The results showed NucB 3-D model matching to DNA binding protein (PDB: 3VIB) of *Neisseria gonorrhoea* with superimposed binding domain (Fig. 4.6 C).

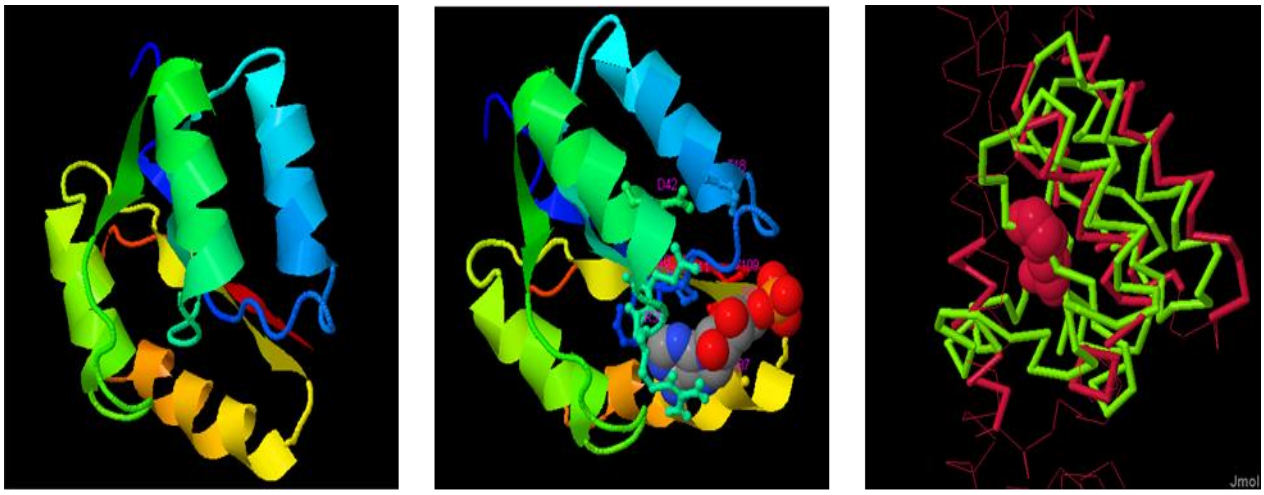



**Figure 4.8 The three-dimensional models of NucB constructed using QUARK *ab-initio* program A. predicted tertiary structure B. predicted binding site and C. structural alignment to a DNA binding protein**

NucB showed structural similarity to another DNA binding protein which is a GIY-YIG N-terminal endonuclease domain of uvrc from *Bacillus caldotenax* (Truglio *et al.*, 2005)

and an alpha/beta-fold characteristic domain of nucleotide utilising enzyme of *E. coli* (Chao *et al.*, 2008).

## 4.4 SUMMARY

In this chapter, the optimum culture conditions for high cell density cultivation of recombinant *B. subtilis* enhanced NucB production 68-fold. NucB produced by this method was able to be purified and had specific activity of 15000 U/mg of protein. Biochemical characterisation demonstrated NucB works optimum at 37°C, pH 8.0 in the presence of 5mM $Mn^{2+}$ and it is an endonuclease which breaks DNA at greater magnitude than RNA. Biophysical characterisation by DSC displayed NucB as thermally stable enzyme which can reversibly unfold retaining 55% of its activity. CD spectroscopy showed NucB contains 32 % α-helical and 24 % β-sheets which are in agreement with the secondary structure predictions. Bioinformatics tools enabled tertiary structure prediction of NucB and conformation was in alignment to reported nucleases structures. Hence, understanding the characteristics of NucB may be useful for potential biotechnological applications of NucB as an anti-biofilm enzyme.

# Chapter 5 Optimisation of extracellular deoxyribonuclease production by diverse bacteria

## 5.1 INTRODUCTION

To expand on the range of extracellular nucleases that could be applicable for degrading the eDNA in the biofilm matrix, this chapter accelerate the production optimisation of extracellular nucleases from diverse bacteria which may be active and stable over a wide range of temperatures. Given the potential use of nucleases in detergents and cleaning solutions (see Chapter 6), the industrial extracellular nuclease enzyme production is likely to be intensified considerably in the near future. Although enzymes are commonly produced by plants, animals and microbes, microbial enzymes are easily accessed and could be produced naturally in a less expensive way using pilot scale cultivation (Gupta *et al.*, 2002).

Optimising the growth media and other physico-chemical factors will determine the viability of industrial processes for enzymatic production (Thiry and Cingolani, 2002). There are more than 3000 enzymes known today and only few are industrially exploited. However, they are mainly extracellular enzymes that degrade naturally occurring polymers starch, pectin, protein, and cellulose (Sanchez-Porro *et al.*, 2003). This implies extracellular nucleases for biotechnological applications are still unexplored. Hence, this requires development of processes for producing microbial extracellular nucleases that can be scaled-up for commercial applications.

The ability to produce extracellular enzymes is variable between species and even within the strains of same species (Zhou *et al.*, 2009). Hence, it's important to select the strains for commercial production of specific enzymes which have the capacity for producing the desired enzymes in highest amounts. Temperature is one specific growth condition which influences the enzyme production capability for microbes. This readily alters certain properties such as activity and stability of enzymes produced (Ferrer *et al.*, 2007). Thermophiles are extremophiles which produce thermostable enzymes that are more versatile, and have high activity and storage stability which extends their shelf life at elevated temperatures (Devi *et al.*, 2010). In industrial settings, where biofouling and bacterial contamination is a major issue, the working conditions for removal of these

biofilms require highly active and thermostable enzymes. Hence it is necessary to identify the microorganisms which can produce nucleases at extreme conditions which can be used in wider array of industrial processes.

In Chapter 2, it was demonstrated that extracellular nucleases can be produced by diverse bacteria and at different temperature conditions showing DNase activity at their growth temperature. In this chapter, amongst identified good nuclease producers from chapter 2, three diverse bacteria *Bacillus* sp. NR-AV-5, *Bacillus* sp. NR-T-2 and *Streptomyces* sp. NR-Sr-1 were studied for enhanced production of nuclease. Based on the growth characteristics of the isolates, the culture conditions for enzyme production using statistical design of experiments were optimised in shake flasks scale.

## 5.2 MATERIALS AND METHODS

### *5.1.1 Morphological characterisation of the bacterial isolates*

Three DNase producing bacterial isolates were subjected to morphological characterisation using microscopic studies. Colony size, shape, colour, appearance, cell motility and sporulation ability of the isolates was observed and recorded. Gram staining was carried out using standard Gram staining kit (Sigma, UK). Isolates were grown in nutrient agar (Oxoid, UK) plates. Salt tolerance experiments were performed on nutrient agar with NaCl in various concentrations (0, 2, 4, 6, 8, 10, 12, 14 and 16% (w/v)) and optimum temperature for growth was determined by culturing isolates at various temperature (between 20, 25, 30, 35, 40, 45, and 50 °C).

### *5.1.2 Bacterial strains and culture condition*

The strains which were identified as good nuclease producers in chapter 2, section 2.3.2 have been used in this study. Strains *Bacillus* sp.NR-AV-5 and *Bacillus* sp. NR-T-2 were maintained on nutrient agar plates grown at 37 and 45°C respectively. *Streptomyces* NR-Sr-1 was maintained on Difco™ ISP medium 4 (BD, UK) agar plate grown at 30°C. Seed cultures were prepared by transferring five single colonies from agar plates into 500ml shake flask containing 100ml of nutrient broth for *Bacillus* strains and nutrient broth with 1% (w/v) glucose for *Streptomcyes* strain. The strains

were incubated at their optimum growth temperature on rotary shaker at 200 rpm for 24-36h. Growth was determined by measuring the optical density at 600 nm ($OD_{600}$). For optimisation experiments, strains were cultured in shake flasks at an initial cell density of 0.1 at $OD_{600}$, in the indicated medium at pH 7.0. Cultures were maintained at same growth conditions for 30-72 h. The cell pellets were removed by centrifugation at 13000 g for 10 min. Supernatants was harvested as crude nucleases. All experiments were performed in triplicate and the standard deviation was within 5%.

### *5.1.3 Assay of DNase activity*

DNase activity was determined as described in Rajarajan et al. (Rajarajan *et al.*, 2012). For each strain, the reactions were performed at their respective optimum growth temperature. One unit of nuclease activity is defined as the amount of enzyme that causes an increase of 1.0 OD at 260 nm after 30 min at respective growth temperature. Assays were carried out in triplicate.

### *5.1.4 Plackett-Burman (P-B) optimisation for DNases production*

For *Bacillus* species nuclease production, eleven components including carbon, nitrogen and salts were selected based on the literature survey performed on *Bacillus* production optimisation as described in Chapter 3 and for *Streptomyces* in addition to carbon, nitrogen and salts, agitation and harvest time were taken as two other factors for P-B experimental design based on information from literature. Table 5.1, 5.2 and 5.3 illustrates the factors under investigation as well as levels of each factor used in the experimental design for *Bacillus* sp.NR-AV-5, *Bacillus* sp. NR-T-2 and *Streptomyces* sp.NR-Sr-1 respectively. Nuclease activity was measured as response. The design was developed by Minitab 16 statistical software (Minitab, Inc., State college, PA, USA). In the present study 11 variables were evaluated in 12 experimental trials (middle points were included as additional trials in some experiments). Regression analysis determined the factors that had a significant ($p < 0.05$) effect on the nuclease yield, and these factors were evaluated in further optimisation experiments.

**Table 5.1 Placket-Burman experimental plan for choosing significant factors of DNase production optimisation by *Bacillus* sp.NR-AV-5.**

| Factors | Components | Units | Low (-) | High (+) |
|---|---|---|---|---|
| A | Hysoy | g/L | 12.5 | 37.5 |
| B | Yeast extract | g/L | 24 | 72 |
| C | $KH_2PO_4$ | g/L | 1.5 | 4.5 |
| D | $Na_2HPO_4$ | g/L | 3 | 9 |
| E | $NH_4Cl$ | g/L | 0.5 | 1.5 |
| F | $MgSO_4$ | g/L | 0.13 | 0.38 |
| G | Corn steep liquor | g/L | 2 | 20 |
| H | Sucrose | g/L | 1 | 10 |
| I | L-Glutamic acid | g/L | 2.5 | 20 |
| J | Glucose | g/L | 1 | 10 |
| K | Glycerol | g/L | 1 | 10 |

**Table 5.2 Placket-Burman experimental plan for choosing significant factors of DNase production optimisation by *Bacillus* sp.NR-T-2.**

| Factors | Components | Units | Low (-) | High (+) |
|---|---|---|---|---|
| A | Hysoy | g/L | 3.8 | 38 |
| B | Yeast extract | g/L | 4.8 | 48 |
| C | Corn steel liquor | g/L | 2 | 20 |
| D | $NH_4Cl$ | g/L | 0.5 | 10 |
| E | $MgSO_4$ | g/L | 0.1 | 0.4 |
| F | $(NH4)_2SO_4$ | g/L | 0.5 | 10 |
| G | $KH_2PO_4$ | g/L | 1.5 | 4.5 |
| H | Glycerol | g/L | 2 | 20 |
| I | Glucose | g/L | 2 | 20 |
| J | Sucrose | g/L | 2 | 20 |
| K | $Na_2SO_4$ | g/L | 0.1 | 0.5 |

**Table 5.3 Placket-Burman experimental plan for choosing significant factors of DNase production optimisation by *Streptomyces* sp.NR-Sr-1.**

| Factors | Components | Units | Low (-) | High (+) |
|---|---|---|---|---|
| A | Agitation | rpm | 100 | 200 |
| B | Harvest time | h | 30 | 72 |
| C | Yeast extract | g/L | 2.5 | 25 |
| D | Hysoy | g/L | 2.5 | 25 |
| E | Tryptone | g/L | 2.5 | 25 |
| F | Glycerol | g/L | 1 | 10 |
| G | Glucose | g/L | 1 | 10 |
| H | $NaH_2PO_4$ | g/L | 0.9 | 9 |

| | | | | |
|---|---|---|---|---|
| I | $KH_2PO_4$ | g/L | 0.5 | 5 |
| J | $MgSO_4$ | g/L | 0.03 | 0.3 |
| K | $NH_4Cl$ | g/L | 0.2 | 2 |

### *5.1.5 Response surface methodology (RSM) for DNases production*

The significant variables from P-B design analysis for DNase production were selected and the ranges were decided based on levels used in the literature as described in Chapter 3. RSM (Khuri & Mukhopadhyay, 2010) using central composite design (CCD) was used to optimise the key factor levels to maximise DNase production. The factors and their coded and uncoded values at five levels are listed in Table 5.4 and 5.6 for *Bacillus* sp. NR-AV-5 and *Streptomcyes* NR-Sr-1 respectively for 3 significant variables and in Table 5.5 for *Bacillus* sp. NR-T-2 for 2 significant variables.

**Table 5.4 Factors and the concentration levels used for CCD optimisation study of *Bacillus* sp.NR-AV-5**

| | Concentration (g/L)/levels | | | | |
|---|---|---|---|---|---|
| **Factors** | **-1.63** | **-1** | **0** | **1** | **1.63** |
| **Yeast extract (A)** | 7.6 | 24.0 | 48.0 | 72.0 | 88.4 |
| **Corn steep liquor (B)** | 3.1 | 10.0 | 20.0 | 30.0 | 36.8 |
| **Hysoy (C)** | 4.0 | 12.5 | 25.0 | 37.5 | 46.0 |

**Table 5.5 Factors and the concentration levels used for CCD optimisation study of *Bacillus* sp. NR-T-2**

| | Concentration (g/L)/levels | | | | |
|---|---|---|---|---|---|
| **Factors** | **-1.41** | **-1** | **0** | **1** | **1.41** |
| **Yeast extract (A)** | 14.1 | 24.0 | 48.0 | 72.0 | 81.9 |
| **Hysoy (B)** | 3.8 | 10.0 | 25.0 | 40.0 | 46.2 |

**Table 5.6 Factors and the concentration levels used for CCD optimisation study of *Streptomyces* sp.NR-Sr-1**

| | Concentration (g/L)/levels | | | | |
|---|---|---|---|---|---|
| **Factors** | **-1.63** | **-1** | **0** | **1** | **1.63** |
| **Hysoy (A)** | 0.5 | 8 | 19 | 30 | 37.5 |
| **Glucose (B)** | 1.0 | 2 | 3.5 | 5 | 6 |
| **Yeast extract (C)** | 0.5 | 8 | 19 | 30 | 37.5 |

A total of 20 experimental runs were required to estimate the DNase activity in NR-AV-5 and NR-Sr-1 whereas 14 experimental runs were required to estimate the DNase activity in NR-T-2. Second order polynomial models were fitted to the data and the coefficients were calculated using the Minitab 16 statistical software package (Minitab, Inc., State college, PA, USA) including regression analysis. Validation experiments were performed with optimum factor concentrations of the predicted model to determine the model fit. For each variable, the quadratic model predictions were represented as contour plots to visualise the main and interactive effects.

## 5.3 RESULTS AND DISCUSSIONS

### *5.1.6 Morphological and growth characterisation*

The isolates were morphologically characterised as listed in Table 5.7 and the growth characteristics of the strains showed varied requirements for growth temperature and salt tolerance ability.

**Table 5.7 Morphological and growth characterisation of diverse bacteria used in this study**

| Characteristics | NR-Sr-1 | NR-AV-5 | NR-T-2 |
|---|---|---|---|
| **Colony** | | | |
| Colour | brown | frost | pale white |
| Appearance | rough and powdery | shiny and dry | rough and adherent |
| Shape | circle | undulate | wrinkled |
| Size (mm) | 5-7 | 5-7 | 5-7 |
| **Cell** | | | |
| Gram's stain | + | + | + |
| Motility | non-motile | motile | motile |
| Spore formation | yes | yes | yes |
| **Growth** | | | |
| Temperature range (°C) | 25 to 35 | 20 to 45 | 30 to 50 |
| Optimum temperature (°C) | 30 | 37 | 45 |
| Salt tolerance ( % NaCl) | 0 to 6 | 0 to 8 | 2 to 16 |
| Optimum salt (%) | 2 | 2 to 4 | 2 to 6 |
| Growth period (h) | 48 | 48 | 48 |

The morphological observation of colony and cell characteristics of all three bacteria studied here agreed generally to the reported literature. Growth characteristic showed *Streptomyces* sp. NR-Sr-1 and *Bacillus* sp. NR-AV-5 were mesophilic with optimum

growth temperature at 30 and 37°C respectively which also agrees to previous studies of other *Streptomyces* and *Bacillus* spp., (Patil *et al.*, 2005; Zhou *et al.*, 2010). *Bacillus* sp. NR-T-2 was a moderate thermophile with growth optimum at 45°C, similar observation has been observed in an earlier study of *Bacillus licheniformis* which was able to produce thermostable esterase (Alvarez-Macarie *et al.*, 1999). This suggests nuclease produced by NR-T-2 will have greater thermostability. The salt tolerance study had pronounced effect on growth of all three bacteria which showed these bacteria can grow from 2-16% NaCl. Previously, strain *Streptomyces clavuligerus* which showed optimum growth at 5% NaCl had the ability to produce alkaline proteases (Thumar and Singh, 2007). However, in present study NR-Sr-1 had only optimum growth at 2% NaCl and this may not produce enzyme with alkaline properties. The level of salt tolerance reported previously for *Bacillus licheniformis* and *Bacillus amyloliquefaciens* varied from 0-10% (Palmisano *et al.*, 2001) which is slightly lower range compared to the data presented here. Also, NR-AV-5 requires 2-4 % optimum salt and NR-T-2 requires 2-6 % salt for optimum growth suggesting they are moderately alkaliphilic strains. Hence, these results propose the isolates chosen for this nuclease production study are distinct and may be advantageous in producing diverse nucleases.

### *5.1.7 P-B design to identify the key components for nuclease production*

The critical components were chosen as described in section 5.2.4 and P-B design was applied to identify the significant components. Components for DNase production by P-B experiments with high and low levels and DNase activity responses are shown in Table 5.8, 5.9 and 5.10. The DNase activity produced by NR-AV-5 range from 63-378 U/ml, produced by NR-T-2 range from 76-393 U/ml and produced by NR-Sr-1 range from 30-451 U/ml.

**Table 5.8 P-B experimental design matrix for nuclease production in *Bacillus* sp. NR-AV-5**

| Run order | A | B | C | D | E | F | G | H | I | J | K | Nuc activity (U/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | - | + | + | - | + | - | - | - | + | + | + | 232 |
| **2** | + | + | - | + | + | - | + | - | - | - | + | 349 |
| **3** | + | - | + | + | - | + | - | - | - | + | + | 243 |
| **4** | - | - | + | + | + | - | + | + | - | + | - | 244 |
| **5** | - | - | - | + | + | + | - | + | + | - | + | 81 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **6** | + | + | + | - | + | + | - | + | - | - | - | 343 |
| **7** | + | - | + | - | - | - | + | + | + | - | + | 346 |
| **8** | - | - | - | - | - | - | - | - | - | - | - | 63 |
| **9** | - | + | + | + | - | + | + | - | + | - | - | 311 |
| **10** | + | + | - | + | - | - | - | + | + | + | - | 378 |
| **11** | + | - | - | - | + | + | + | - | + | + | - | 356 |
| **12** | - | + | - | - | - | + | + | + | - | + | + | 337 |

**Table 5.9 P-B experimental design matrix for nuclease production in Bacillus sp. NR-T-2**

| **Run order** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **Nuc activity (U/ml)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | + | + | - | + | + | - | + | - | - | - | + | 393 |
| **2** | + | - | + | - | - | - | + | + | + | - | + | 237 |
| **3** | - | - | - | - | - | - | - | - | - | - | - | 149 |
| **4** | - | + | - | - | - | + | + | + | - | + | + | 232 |
| **5** | + | + | - | + | - | - | - | + | + | + | - | 376 |
| **6** | + | + | + | - | + | + | - | + | - | - | - | 368 |
| **7** | + | - | + | + | - | + | - | - | - | + | + | 205 |
| **8** | - | + | + | - | + | - | - | - | + | + | + | 309 |
| **9** | - | + | + | + | - | + | + | - | + | - | - | 347 |
| **10** | - | - | - | + | + | + | - | + | + | - | + | 124 |
| **11** | + | - | - | - | + | + | + | - | + | + | - | 149 |
| **12** | - | - | + | + | + | - | + | + | - | + | - | 76 |

**Table 5.10 P-B experimental design matrix for nuclease production in Streptomyces sp. NR-Sr-1**

| **Run order** | **A** | **B** | **C** | **D** | **E** | **D** | **G** | **H** | **H** | **J** | **K** | **Nuc activity (U/ml)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | + | + | - | + | + | - | + | - | - | - | + | 325 |
| **2** | - | + | - | - | - | + | + | + | - | + | + | 180 |
| **3** | + | - | + | + | - | + | - | - | - | + | + | 299 |
| **4** | - | - | - | - | - | - | - | - | - | - | - | 30 |
| **5** | - | + | + | + | - | + | + | - | + | - | - | 417 |
| **6** | + | - | + | - | - | - | + | + | + | - | + | 224 |
| **7** | - | - | - | + | + | + | - | + | + | - | + | 187 |
| **8** | + | - | - | - | + | + | + | - | + | + | - | 166 |
| **9** | + | + | - | + | - | - | - | + | + | + | - | 229 |
| **10** | - | + | + | - | + | - | - | - | + | + | + | 144 |
| **11** | + | + | + | - | + | + | - | + | - | - | - | 141 |
| **12** | - | - | + | + | + | - | + | + | - | + | - | 451 |

Any factor with $p < 0.05$ was considered to have a significant influence on the response at 95% confidence level. In the regression analysis for NR-AV-5, hysoy (p = 0.006), yeast extract (p = 0.009) and corn steep liquor (p = 0.009) were identified as statistically significant. For NR-T-2, hysoy (p = 0.047) and yeast extract (p = 0.021) and for NR-Sr-1 hysoy (p = 0.015), glucose (p = 0.008) and yeast extract (p = 0.011) were indicated as statistically significant. The estimated positive and negative effects of the factors with their *p*-values are shown graphically in Figure 5.1, 5.2 and 5.3 for NR-AV-5, NR-T-2 and NR-Sr-1, respectively.

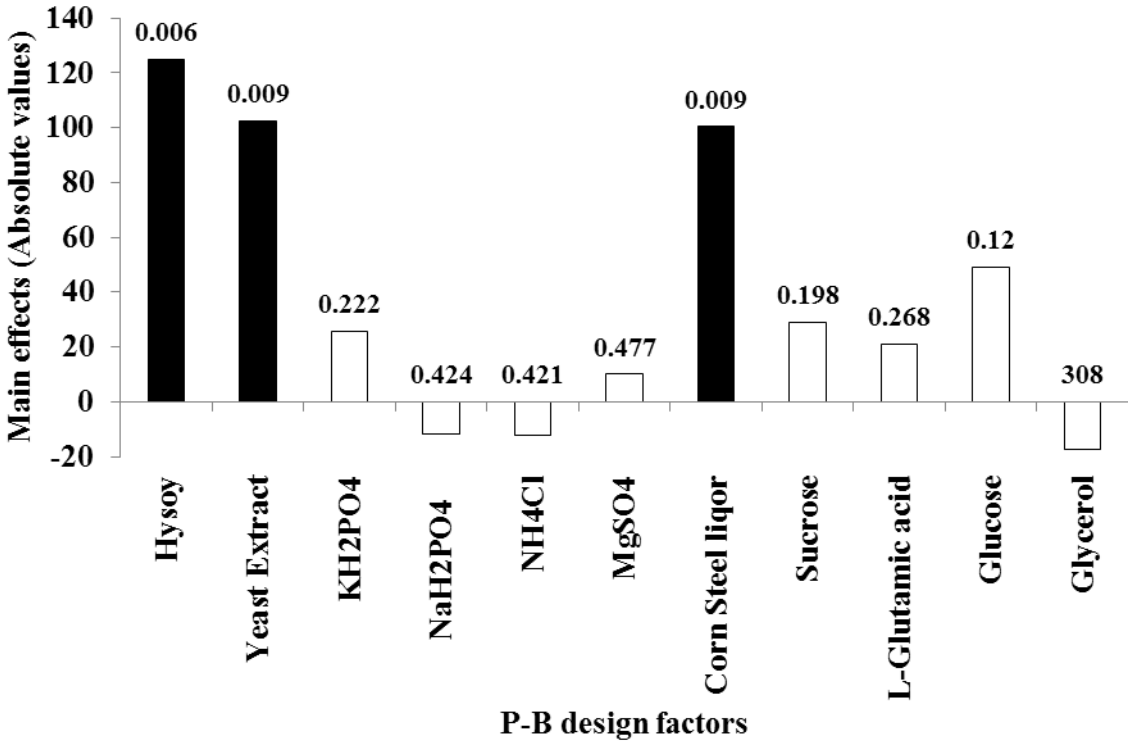



**Figure 5.1 The extent of positive and negative effects of the eleven factors on the Nuc activity by *Bacillus* sp. NR-AV-5 and the corresponding *p*-values (number above each bar) of the factors showing their significance (if p < 0.05) based on ANOVA using P-B design study. The black bars indicate the positively significant factors taken for next level optimisation.**

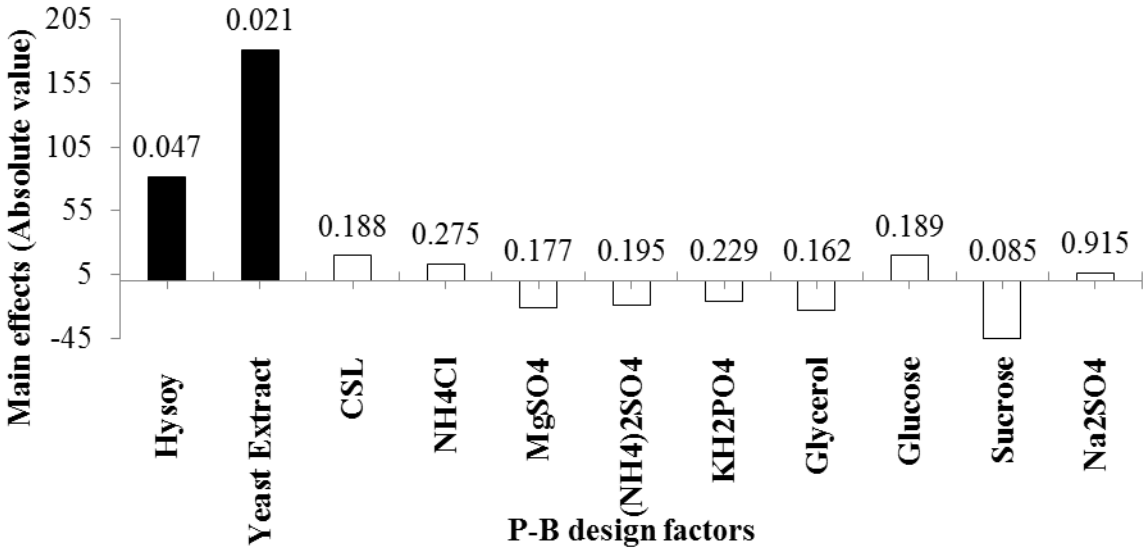



**Figure 5.2 The extent of positive and negative effects of the eleven factors on the Nuc activity by *Bacillus* sp. NR-T-2 and the corresponding *p*-values (number above each bar) of the factors showing their significance (if p < 0.05) based on ANOVA using P-B design study. The black bars indicate the positively significant factors taken for next level optimisation.**

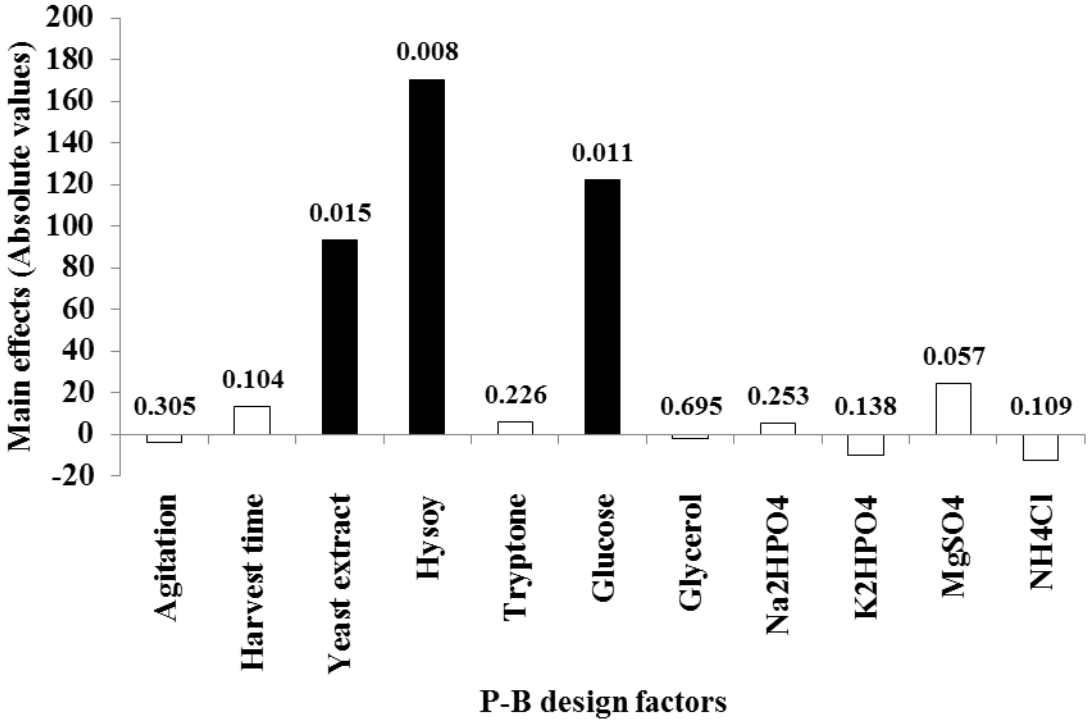



**Figure 5.3 The extent of positive and negative effects of the eleven factors on the Nuc activity by *Streptomyces* sp. NR-Sr-1 and the corresponding *p*-values (number above each bar) of the factors showing their significance (if p < 0.05) based on ANOVA using P-B design study. The black bars indicate the positively significant factors taken for next level optimisation.**

Additional components such as sucrose, which was previously reported to increase nuclease production in *Bacillus* (Zhou *et al.*, 2010) and $Na_2SO_4$ as well as L-glutamic acid which were reported to induce sporulation in *Bacillus* (Burnett *et al.*, 1986; de Vries *et al.*, 2005), were also included in the P-B design. Nevertheless, these factors did not have any significant effect on nuclease production. Factors which were significant for nuclease production by NR-AV-5 after 48 h harvest are similar to results obtained in Chapter 3 suggesting hysoy, yeast extract and corn steep liquor could be used in nuclease production of mesophilic *Bacillus* species and further optimisation studies.

For thermophilic strain NR-T-2 nuclease production after 48 h harvest showed hysoy and yeast extract as significant factors. Though corn steep liquor had a positive effect, it was not significant at 95% confidence level as the *p*-value was greater than 0.05. The results suggest that *Bacillus* sp. grown at temperature as high as 45°C increases the nuclease production in presence of hysoy and yeast extract. This result is in agreement with previous report on *Bacillus cereus* for RNase production optimisation which showed optimum enzyme production was achieved at 42°C (Zhou *et al.*, 2010).

For *Streptomyces* strain NR-Sr-1 grown at 30°C, glucose, yeast extract and hysoy had positive effects on nuclease production. The results of main effects of the factors also demonstrated that lower agitation (100 rpm) and 72 h harvest time had a positive influence on nuclease production. Previously, for *Streptomyces*, glucose, yeast extract and peptone was reported to enhance nuclease production efficiently at the end of exponential phase (Brnakova *et al.*, 2005). Hence, the results presented in this Chapter for *Streptomyces* are similar to reported literature however; hysoy replaced peptone as an economical substrate which was a significant variable for further optimisation.

### *5.1.8 Optimisation of significant factors using Response surface methodology*

#### ***Bacillus* sp.NR-AV-5**

Three media components which induced maximum nuclease production using P-B design were used in the RSM-based optimisation in NR-AV-5.20 experimental runs were performed using different concentrations of three variables using CCD. Experimentally measured nuclease activity values along with the model predicted

responses are presented in Table 5.11. The highest nuclease production was measure as 478 U/ml and the lowest production was measured as 163 U/ml.

**Table 5.11 CCD matrix for the experimental design with observed and predicted responses for medium optimisation for the nuclease production by *Bacillus* sp. NR-AV-5**

| Run Order | A | B | C | Nuc activity (U/ml) Observed | Predicted |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 2 | 326 | 377 |
| 2 | 0 | 2 | 0 | 302 | 358 |
| 3 | 0 | 0 | -2 | 263 | 292 |
| 4 | 1 | -1 | -1 | 308 | 265 |
| 5 | 0 | 0 | 0 | 423 | 454 |
| 6 | 0 | 0 | 0 | 478 | 454 |
| 7 | -1 | -1 | 1 | 356 | 317 |
| 8 | 1 | 1 | -1 | 413 | 395 |
| 9 | 0 | 0 | 0 | 445 | 454 |
| 10 | 1 | 1 | 1 | 416 | 350 |
| 11 | 2 | 0 | 0 | 326 | 381 |
| 12 | -1 | -1 | -1 | 163 | 171 |
| 13 | 0 | 0 | 0 | 475 | 454 |
| 14 | -2 | 0 | 0 | 255 | 279 |
| 15 | 0 | 0 | 0 | 434 | 454 |
| 16 | 0 | -2 | 0 | 220 | 244 |
| 17 | 0 | 0 | 0 | 480 | 454 |
| 18 | -1 | 1 | 1 | 337 | 323 |
| 19 | 1 | -1 | 1 | 381 | 367 |
| 20 | -1 | 1 | -1 | 367 | 324 |

From the responses analysed using Minitab statistical software package, a quadratic model was fitted to the measured nuclease activity data. Equation (5.1) represents the regression equation of the nuclease production from NR-AV-5 as a function of hysoy (A), yeast extract (B) and corn steep liquor (C) concentrations. All terms, even if they are in-significant, are included in this equation

$$\text{Nuc activity (U/ml)} = -496.95 + 19.1\,A + 13.9\,B + 28.9\,C - 0.3\,A^2 - 0.09\,B^2 - 0.4C^2 - 0.02AB - 0.09AC - 0.15BC \quad \text{Eq (5.1)}$$

Statistical testing of the model was performed by Fischer's statistical test for analysis of variance (ANOVA) and the results are shown in Table 5.12. The model F-value was

5.84 with a low probability value (P > F = 0.005) and the F-value for "lack of fit" was 6.77 with a high probability value (P > F = 0.82). The high model F-value and P > F below 0.05 indicate the model terms are significant. The "lack of fit" P > F greater than 0.05 implies it is non-significant and thus the model provides a good representation of the experimental data.

**Table 5.12 ANOVA for the selected quadratic model (Eq. 5.1) of CCD. Df represents degrees of freedom**

| Variables | Sum of squares | df | Mean square | *F*-value | *p*-value (Prob > *F*) |
|---|---|---|---|---|---|
| Model | 128228 | 9 | 14247.6 | 5.84 | 0.005* |
| Hysoy (*A*) | 12523 | 1 | 12522.8 | 5.13 | 0.047* |
| Yeast extract (*B*) | 15651 | 1 | 15650.9 | 6.41 | 0.030* |
| Corn steep liquor (*C*) | 8709 | 1 | 8709.5 | 3.57 | 0.088 |
| *AB* | 251 | 1 | 250.7 | 0.10 | 0.755 |
| *BC* | 10756 | 1 | 10756 | 4.41 | 0.062 |
| *AC* | 992 | 1 | 991.9 | 0.41 | 0.538 |
| $A^2$ | 17920 | 1 | 27408.2 | 11.23 | 0.007* |
| $B^2$ | 35881 | 1 | 41761.5 | 17.12 | 0.002* |
| $C^2$ | 25546 | 1 | 25545.6 | 10.47 | 0.009* |
| Residual | 24400 | 10 | 2440.0 | | |
| Lack of Fit | 21260 | 5 | 4251.9 | 6.77 | 0.82 |
| Pure Error | 3140 | 5 | 628.1 | | |
| Corrected total | 152628 | 19 | | | |

* $p < 0.05$ is significant

The 2D contour plots presented in Figure 5.2 are the graphical depiction of the model predictions. Three plots show the variation in the yield of nuclease, as a function of the varying concentrations of two variables at a time with the other variable being at its optimum level. These plots help to understand the interactions between two variables and locate the optimal levels (the darkest colour shading) of those variables.

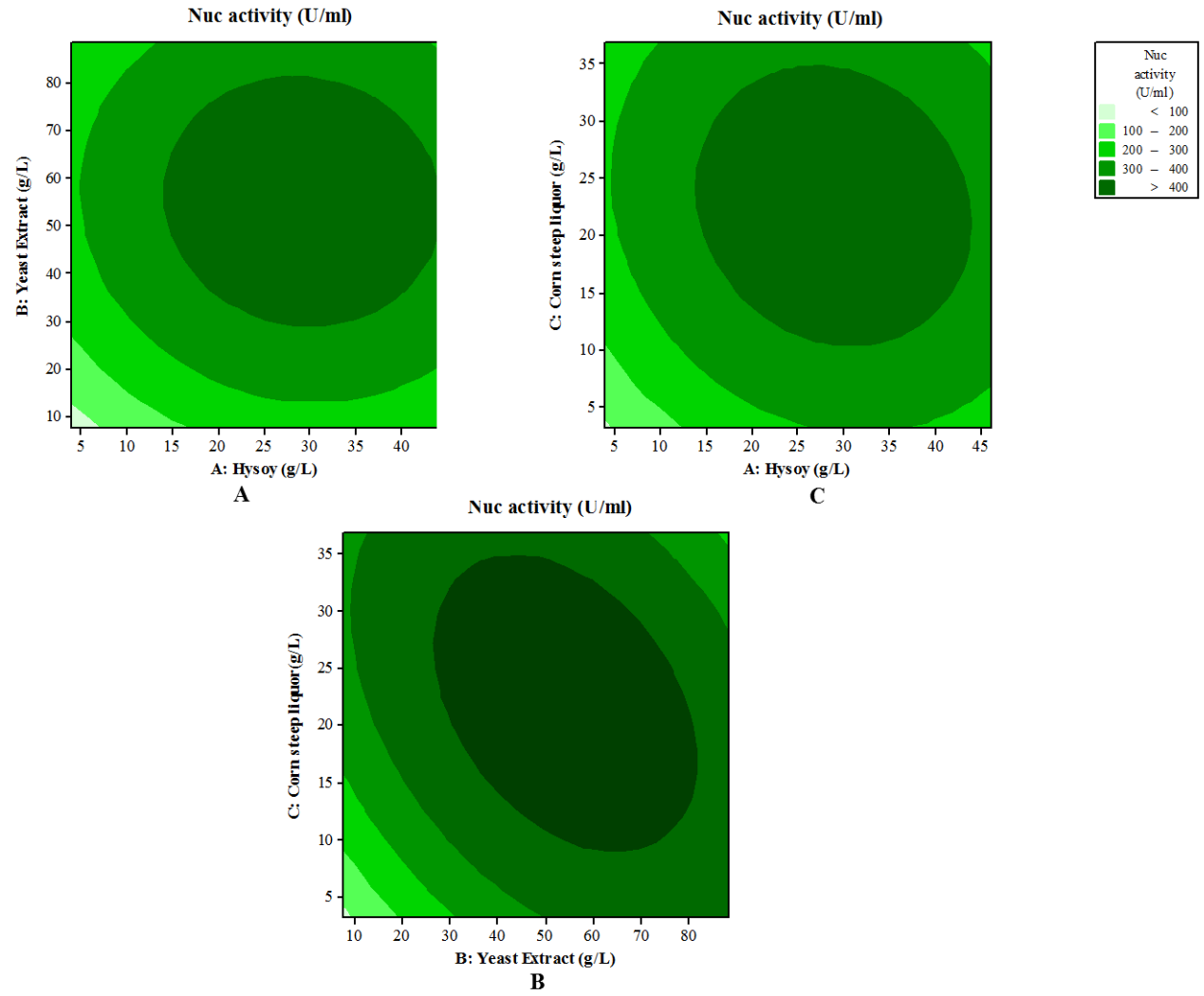



**Figure 5.4 Contour plots for the optimisation of NucB production in *Bacillus* sp. NR-AV-5: The interactive effects of two significant variables are shown, expressed as concentrations (g/L) in the medium A: Hysoy vs Yeast extract with Corn steep liquor at 20 g/L. B: Yeast extract vs Corn steep liquor with Hysoy at 25 g/L. C: Corn steep liquor vs Hysoy with Yeast extract at 48 g/L.**

As shown in Fig. 5.2, the maximum production of nuclease from Bacillus sp.NR-AV-5 was obtained when the concentrations of hysoy (Fig. 5.2 A, C), yeast extract (Fig. 5.2 A, B), and corn steep liquor (Fig 5.2 B, C) were 5–45 g/L, 10–80 g/L, and 5–35 g/L, respectively. The prediction of Minitab software package on maximum nuclease production was 464 U/ml at the optimum levels of hysoy (29 g/L), yeast extract (54 g/L) and corn steep liquor (22 g/L). The validation experiment performed using these optimum concentrations in shake flask cultivation resulted in 470 U/ml nuclease activity which validates the accuracy of the model. Initially in P-B design, NR-AV-5 produced minimum nuclease activity of 63 U/ml and after optimising the concentration levels of the three significant medium components the nuclease production increased 7.5 fold (470 U/ml).

***Bacillus* sp.NR-T-2**

Two media components which induced maximum nuclease production using P-B design were employed for RSM-based optimisation in NR-T-2. For the optimisation of the concentrations of components, 13 experimental runs were performed using different combinations of two variables using CCD. Nuclease activity that was the observed response of the design matrix along with the predicted responses is presented in Table 5.13. The highest nuclease production was recorded 474 U/ml and the lowest production was recorded as 246 U/ml.

**Table 5.13 CCD matrix for the experimental design with observed and predicted responses for medium optimisation of the nuclease production by *Bacillus* sp. NR-T-2**

| | | | Nuc activity (U/ml) | |
|---|---|---|---|---|
| RunOrder | A | B | Observed | Predicted |
| 1 | 1.4 | 0 | 320 | 344 |
| 2 | 0 | -1.4 | 318 | 327 |
| 3 | 0 | 1.4 | 404 | 409 |
| 4 | 0 | 0 | 454 | 458 |
| 5 | -1.4 | 0 | 254 | 245 |
| 6 | -1 | 1 | 337 | 343 |
| 7 | 0 | 0 | 472 | 458 |
| 8 | 0 | 0 | 474 | 458 |
| 9 | 1 | -1 | 375 | 355 |
| 10 | -1 | -1 | 246 | 249 |
| 11 | 1 | 1 | 395 | 377 |
| 12 | 0 | 0 | 447 | 458 |
| 13 | 0 | 0 | 441 | 458 |

The responses were analysed using Minitab statistical software package and the quadratic model obtained after the analysis of variance (eq. 5.2) shows the nuclease production from NR-T-2 as the function of variables hysoy (A) and yeast extract (B). All terms, even if they are in-significant, are included.

$$\text{Nuc activity (U/ml)} = -171.96 + 13.3\,A + 14.3B - 0.1\,A^2 - 0.2\,B^2 - 0.1AB \qquad \text{Eq (5.2)}$$

Statistical testing of the model was performed by Fischer's statistical test for analysis of variance (ANOVA) and the results are shown in Appendix B, Table B.1. The model F-value was 41.8 with a low probability value ($P > F < 0.0001$) and the F-value for "lack

of fit" was 2.26 with a high probability value (P > F = 0.224). The high model F-value and P > F below 0.05 indicate the model terms are significant. The "lack of fit" P > F greater than 0.05 implies it is non-significant and thus the model provides a good representation of the experimental data.

As shown in Figure 5.3, the maximum production of nuclease from *Bacillus* sp.NR-T-2 was obtained when the concentration of hysoy and yeast extract (Figure 5.2) was 5–45 g/L, and 10–80 g/L, respectively. The prediction of the model (eq. 5.2) indication that the maximum nuclease production was 478 U/ml at the optimum levels of hysoy (29 g/L) and yeast extract (53 g/L). The validation experiment performed using these optimum concentrations in shake flask cultivation resulted in 481 U/ml nuclease activity which validates the accuracy of the model.

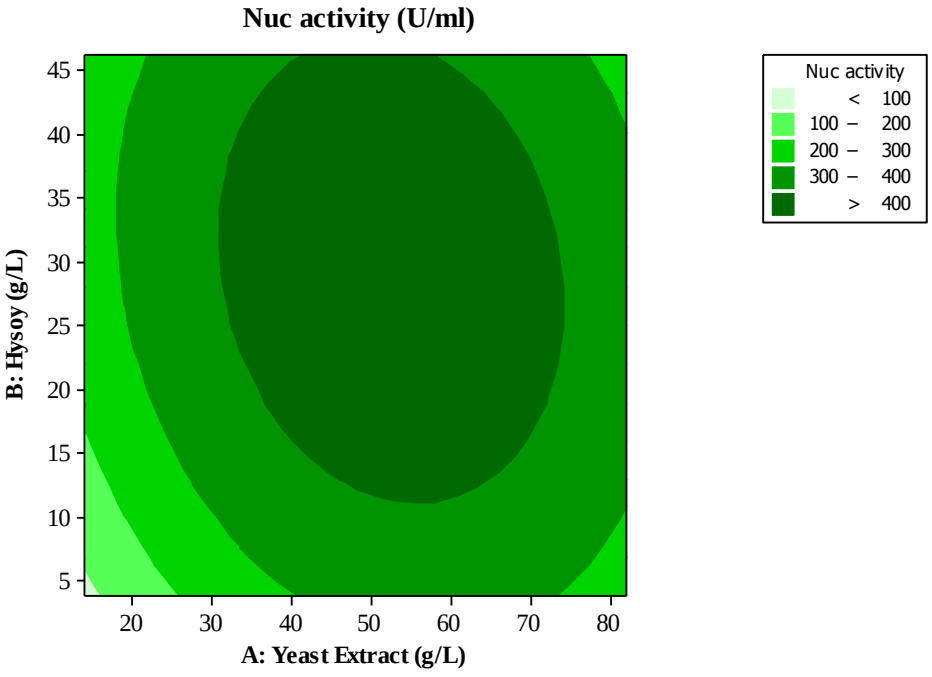



**Figure 5.5 Contour plots for the optimisation of Nuclease production in *Bacillus* sp. NR-T-2: The interactive effects of two significant variables are shown, Yeast extract vs Hysoy expressed as concentrations (g/L) in the medium.**

NR-T-2 produced minimal nuclease activity of 76 U/ml and after optimising the concentration levels of 2 significant medium components the nuclease production enhanced 6.3-fold.

There are no other literature reports available to date on deoxyribonuclease production optimisation of *Bacillus* to compare the results obtained in this Chapter for *Bacillus* species. Hence, comparing the data obtained in Chapter 3 on *Bacillus licheniformis* NucB production which showed maximum enzyme production of 471 U/ml, the *Bacillus* sp.NR-AV-5 yielded similar maximum productivity of 470 U/ml at 37°C after 48h, whereas the thermophilic *Bacillus* sp. NR-T-2 maximum nuclease productivity was 480 U/ml at 45°C after 48h. Hence, the data suggest that the production titers for extracellular deoxyribonuclease production are similar between the strains of *Bacillus* species. Nevertheless, ribonuclease production by *Bacillus firmus* optimised previously using statistical optimisation showed maximum RNase activity of 46.5 U/ml (Kumar *et al.*, 2010) and the nuclease optimisation results obtained in this Chapter and Chapter 3 is 10-fold higher than the previous report.

***Streptomyces* sp.NR-Sr-1**

Three media components, which induced maximum nuclease production using P-B design, were employed for RSM-based optimisation in NR-Sr-1. 20 experimental runs were performed using different combinations of three variables using CCD. Nuclease activity that was the observed response of the design matrix along with the predicted responses is presented in Table 5.14. The highest nuclease production was experimentally measured as 572 U/ml and the lowest production was recorded as 134 U/ml.

**Table 5.14 CCD matrix for the experimental design with observed and predicted responses for medium optimisation for the nuclease production by *Streptomcyes* sp. NR-Sr-1**

| | | | | Nuc activity (U/ml) | |
|---|---|---|---|---|---|
| Run Order | A | B | C | Observed | Predicted |
| 1 | 0 | 0 | 2 | 368 | 346 |
| 2 | 1 | 1 | -1 | 396 | 365 |
| 3 | 0 | 0 | 0 | 543 | 559 |
| 4 | -1 | -1 | 1 | 198 | 251 |
| 5 | 0 | 0 | 0 | 572 | 559 |
| 6 | -1 | -1 | -1 | 134 | 114 |
| 7 | 0 | 2 | 0 | 301 | 303 |
| 8 | 0 | 0 | 0 | 559 | 559 |
| 9 | -1 | 1 | -1 | 147 | 180 |

| | | | | | |
|---|---|---|---|---|---|
| 10 | 2 | 0 | 0 | 358 | 339 |
| 11 | 0 | 0 | 0 | 546 | 559 |
| 12 | 0 | 0 | 0 | 570 | 559 |
| 13 | 1 | -1 | -1 | 189 | 239 |
| 14 | 1 | -1 | 1 | 257 | 246 |
| 15 | 0 | 0 | 0 | 561 | 559 |
| 16 | -1 | 1 | 1 | 305 | 277 |
| 17 | 0 | 0 | -2 | 266 | 258 |
| 18 | 1 | 1 | 1 | 290 | 332 |
| 19 | 0 | -2 | 0 | 207 | 175 |
| 20 | -2 | 0 | 0 | 199 | 187 |

The responses were analysed using Minitab statistical software package and the quadratic model obtained after the analysis of variance (eq. 5.3) shows the nuclease production from NR-Sr-1 as the function of variables variables glucose (A), hysoy (B) and yeast extract (C). All terms, even if they are in-significant, are included.

$$\text{Nuc activity (U/ml)} = -929.9 + 38.9A + 372.2B + 38.2C - 0.9\,A^2 - 50.4\,B^2 - 0.8\,C^2 + 0.9\,AB - 0.3\,AC - 0.6\,BC \qquad \text{Eq (5.3)}$$

Statistical testing of the model was performed by Fischer's statistical test for analysis of variance (ANOVA) and the results are shown in Appendix B, Table B.2. The model F-value was 38.25 with a low probability value ($P > F < 0.0001$) and the F-value for "lack of fit" was 17.41 with a high probability value ($P > F = 0.4$). The high model F-value and $P > F$ below 0.05 indicate the model terms are significant. The "lack of fit" $P > F$ greater than 0.05 implies it is non-significant and thus the model provides a good representation of the experimental data.

As shown in Fig. 5.4, the maximum production of nuclease from *Streptomcyes* sp.NR-Sr-1 was obtained when the concentration of glucose (Fig. 5.4 A, C), hysoy (Fig. 5.4 A, B), and yeast extract (Fig 5.4 B, C) was 1–6 g/L, 5–35 g/L, and 5–35 g/L, respectively.

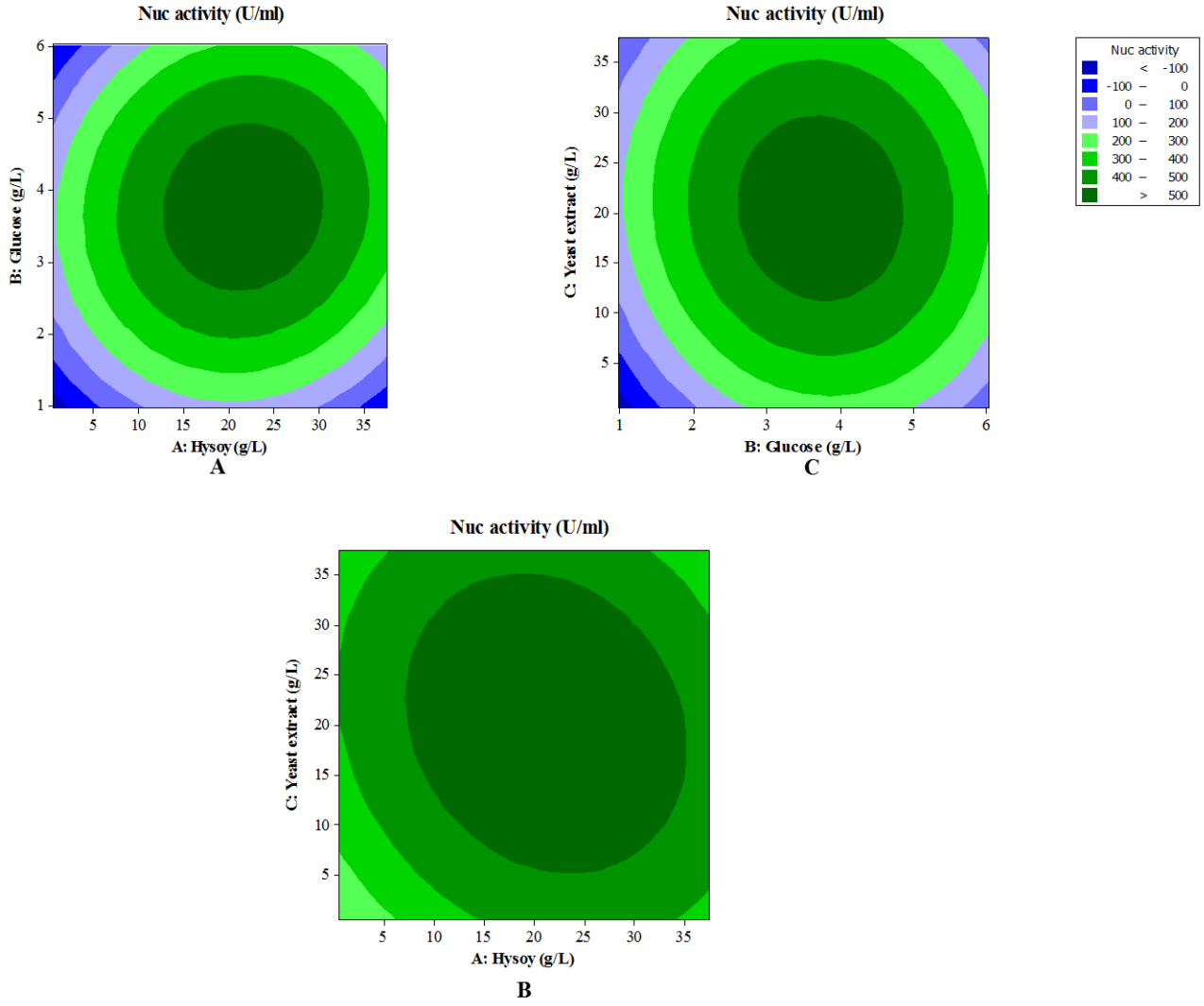



**Figure 5.6 Contour plots for the optimisation of Nuclease production in *Streptomyces* sp. NR-Sr-1: The interactive effects of two significant variables are shown, expressed as concentrations (g/L) in the medium A. Hysoy vs Glucose with Yeast extract at 20 g/L B. Yeast extract vs Hysoy with Glucose at 25 g/L C. Glucose vs Yeast extract with Hysoy at 48 g/L.**

The prediction of Minitab software package on maximum nuclease production was 565 U/ml at the optimum levels of glucose (4 g/L), hysoy (21 g/L) and yeast extract (20 g/L). The validation experiment performed using these optimum concentrations in shake flask cultivation resulted in 560 U/ml nuclease activity which validates the accuracy of the model.

In the P-B design study on NR-Sr-1, minimal nuclease level obtained was 30 U/ml and after optimising the concentration levels of 3 significant medium components the nuclease production enhanced 19-fold, also this was ~1.2-fold higher productivity compared to yields of nuclease from *Bacillus* strains reported in this chapter. In *Streptomyces thermonitrificans* the maximum nuclease levels obtained was 51 U/ml

after 40h at 45°C (Patil *et al.*, 2005) whereas, *Streptomyces* NR-Sr-1 produced maximum 570 U/ml after 72h at 30°C. This is about 11-fold increase to previous nuclease yield.

## 5.4 SUMMARY

In summary, in this chapter improved extracellular nucleases production was achieved by statistical optimisation in diverse bacteria grown at three different temperatures. Models fitted for enzymes productions by three diverse bacteria propose that the choice of medium components and cultivation parameters determined the desired nuclease enzyme production. The major medium constituents which were identified as significant were hysoy, yeast extract, corn steep liquor and glucose between all three diverse bacteria. In *Bacillus* sp.NR-AV-5 nuclease activity increased 7.5-fold after 48 h grown at 37°C, in *Streptomyces* sp.NR-Sr-1 nuclease production enhanced 19-fold after 72 h grown at 30°C and in *Bacillus* sp.NR-T-2 nuclease production boosted 6.3-fold after 48 h grown at 45°C. Hence, by selection of growth temperature, medium constituents and their optimum levels lead to the enhanced enzyme yield. These results obtained using statistical design of experiments indicate a promising methodology for process development of enzyme production which could support scale-up for commercial production of nucleases enzymes for biotechnological applications.

# Chapter 6 Application of microbial extracellular nucleases as potential anti-biofilm enzymes

## 6.1 INTRODUCTION

Extracellular matrix (ECM) is an adhesive layer which comprises of structural components of microbial biofilms. Monitoring and control of the biofilm accumulation formed by microbial species remains challenging task on any abiotic and biotic surfaces (Kim *et al.*, 2012). The use of chemical biocides, detergents and surfactants is the main strategy to control and prevent the formation of biofilms. Beyond this chemical treatment, mechanical removal has also been proposed (Chambliss-Bush, 2012). However, complete removal of biofilms by these methods is difficult due to the protection of the biofilms by the ECM. Therefore, there is a need for methods that are capable of removing biofilms by destroying the key structural component of ECM.

In Chapter 1, we reviewed recent studies which have shown most prokaryotic and eukaryotic biofilms contain extracellular DNA (eDNA) in their ECM as an important structural component. Studies have suggested that the secretion of eDNA has a purpose that is essential for microbial survival as biofilms. The purpose includes, sole nutrient source of carbon, nitrogen and phosphorous and horizontal transfer of genetic material that enables competence development (Zafra *et al.*, 2012). Also, eDNA form a structural network which facilitates strength and integrity to biofilms. Researchers have investigated the effect of eDNA degradation by DNase and the removal of a number of bacterial (Steinberger and Holden, 2005; Tetz *et al.*, 2009), yeast (Martins *et al.*, 2012) and fungal (Rajendran *et al.*, 2013) biofilms. Hence, DNase may have significant impact as an anti-biofilm agent for more widespread applications than anticipated earlier.

Deoxyribonucleases are important enzymes and are distributed widely in nature as investigated and concluded in Chapter 2. Previously, DNases from microorganisms were widely applied to remove nucleic acid contaminants to simplify the downstream processing of recombinant proteins and biopharmaceutical products, to reduce cell clumping and also in adenovirus purification (Benedik and Strych, 2006).

In this chapter, the effectiveness of an isolated extracellular deoxyribonuclease, NucB, to disperse single species biofilms is explored. The efficacy of NucB as anti-biofilm enzyme compared to other commercial nucleases in dispersing various microbial biofilms on surfaces such as plastic, glass and stainless steel is also investigated. It also shown that the crude supernatants of bacteria, the source of microbial extracellular nucleases were able to disperse the preformed biofilms.

## 6.2 MATERIALS AND METHODS

### *6.2.1 Enzymes and reagents*

NucB enzyme purified from a recombinant *Bacillus subtilis* strain was obtained from Prof. Alastair Hawkins and stock enzyme concentration was determined as 0.5 mg/ml using UV-Vis spectrophotometric method. Varidase enzyme mixture containing streptokinase and streptodornase was purchased from Amano Enzyme Inc. (Japan) and bovine DNase I (bDNase I) purchased from Sigma Aldrich (UK). Varidase and bDNase I total protein concentration was determined as 0.4 mg/ml and 4.6 mg/ml respectively using Lowry's method (1951). Standard buffer solution contained 50 mM Tris-HCl with 5 mM $MnSO_4.H_2O$, and 1 mM DTT at pH 8.0

### *6.2.2 Microbial strains and growth conditions*

The list of bacterial strains isolated and identified as described in Chapter 2 has been used in this study as model biofilms. The particular strains were chosen for their ability to form biofilms naturally to study dispersal. Also nuclease producers identified in Chapter 2 were used in this chapter as nuclease sources as presented in Table 6.1. All the strains were maintained at -80°C in 20% glycerol and recovered onto Luria Bertani agar (LBA) and for Baker's yeast onto media containing 1% yeast extract, 2% peptone, 5% glucose with 1.5% agar (YPGA). For inoculum preparation, an isolated colony was inoculated into LB broth (for bacteria) and YPG broth (for yeast) and incubated at 37°C, 160 rpm in an orbital shaker for 24h.

**Table 6.1 Panel of isolates used for biofilm formation and disruption assay and as nuclease sources.**

| Strain type for isolates | Denoted in this study | Biofilm source | Nuclease source |
|---|---|---|---|
| *S. cerevisiae* | Yeast | + | - |
| *Bacillus* sp. | NR-41 | + | - |
| *Pseudomonas* sp. | NR-P-1 | + | - |
| *Acinetobacter* sp. | NR-A-1 | + | - |
| *Ruegeria* sp. | NR-SA-1 | + | - |
| *Escherichia coli* | K-12 | + | - |
| *Bacillus* sp. | NR-T-2 | + | - |
| *Bacillus* sp. | NR-T-8 | + | - |
| *Bacillus licheniformis* | EI-34-6 | + | + |
| *Bacillus* sp. | NR-T-10 | + | + |
| *Bacillus* sp. | NR-AV-5 | + | + |
| *Bacillus* sp. | NR-P-19 | - | + |
| *Bacillus* sp. | NR-P-20 | - | + |
| *Bacillus* sp. | NR-P-21 | - | + |
| *Bacillus* sp. | NR-P-22 | - | + |
| *Bacillus* sp. | NR-P-23 | - | + |
| *Streptomyces* sp. | NR-Sr-1 | - | + |
| *Streptomyes* sp. | NR-Sg-2 | - | + |

### *6.2.3 Surfaces tested for biofilm formation and dispersal*

1. 96-well polystyrene plates (BD, UK)
2. Glass cover slips (BD, UK)
3. Stainless steel coupons (Grade 304, obtained from school of chemistry, Newcastle University).

### *6.2.4 Biofilm formation by bacterial isolates and dispersal by NucB*

Single s*trains were* inoculated in the wells of a 96-well microtiter plate (MTP) at $OD_{600}$ of 0.1 (200µl per well). Biofilms were formed over 24h time period at 37°C under static condition. Non-attached planktonic cells were removed by aspiration and washing with sterile phosphate buffered saline (PBS) solution. Working solution of NucB was prepared in standard buffer solution (described in section 6.2.2). To evaluate the effect of dispersal on preformed biofilms, 24h grown biofilms were treated with 225 µl of NucB (3µg/ml) at 37°C for 1 h. One hour incubation time was shown to sufficient for NucB activity on biofilms as shown in the previous studies (Nijland et al., 2010). The plates were washed three times thoroughly with PBS solution and air dried. The biofilms were stained with 225µl of 0.1% crystal violet (CV) solution by incubating at room temperature for 20 min. After washing, remaining crystal violet dye was solubilised in 225µl absolute ethanol and acetic acid mixture (ratio of 80:20). Then, 100µl of this solubilised mixture were transferred to a new polystyrene plate and the CV absorbance at 595nm was read in a microtiter plate reader (FLUOstar OPTIMA, BMG LABTECH). Analysis of the data was performed using Optima Mars analysis software and Microsoft Excel. Each assay was performed in triplicate.

### *6.2.5 Growth and visualisation of biofilms on glass coverslips*

The effect of NucB on biofilm disruption was visualised by light microscopy, phase contrast microscopy, confocal laser scanning microscopy (CLSM) and by scanning electron microscopy (SEM), using biofilms cultured on glass cover slips. Cells were diluted to 0.1 $OD_{600}$ into fresh media and 2 ml was added in a 24-well polystyrene microtiter plate (Becton Dickinson, UK) with sterile glass cover slips (13mm diameter) placed in each well. The plates were incubated static for 24 h at 37°C. The culture was harvested by aspiration after 24 h and 250 µl sterile PBS solutions were used to rinse each well twice. NucB working solutions were prepared with standard buffer (section 6.2.1). To the glass slides containing biofilms, 250 µl of sterile buffer solution or NucB (3µg/ml) was added and incubated for 30 min, 60 min and 90 min at 37°C. The cover slips were removed, washed once again with 250 µl sterile PBS solution three times and dried before being subjected to image analysis. For light and phase contrast microscopy, the glass cover slips were crystal violet stained and observed under a light or phase-

contrast microscope (Leica) with 40X magnification. Among the most versatile and effective of the non-destructive approaches for studying biofilms is confocal laser scanning microscopy (CLSM). CLSM reduces pre-treatments of the sample and has been used in combination with fluorescent *in situ* hybridisation techniques (Grande at al., 2010). Multiple fluorescent channels can be recorded simultaneously which record the fluorescence signal of biofilm components labelled with specific markers. Hence, the fluorescent staining of live and dead cells can detect the structural organisation of biofilms and presence of extracellular DNA can be visualised using CLSM. For CSLM, coverslips were placed inside a sterile 6-well polystyrene plate and filled with Live/Dead BacLight stain (Invitrogen, UK). Biofilms were examined using a Leica TCS SP2 confocal microscope with an argon/neon laser for visualisation of SYTOH 9 (excitation 485 nm, emission 519 nm), and propidium iodide (excitation 536 nm, emission 617 nm). For SEM, the coverslips were fixed in 2% (v/v) glutaraldehyde at 4°C overnight. Specimens were rinsed twice in 0.2M phosphate buffer and dehydrated through a series of ethanol washes as follows: 25%, 50% and 75% (30 min each) and finally in 100% absolute ethanol until dried. Samples were dried in a critical point dryer (Bal-tec), mounted on aluminium stubs and sputter coated with gold at Electron Microscopy Research Services, Newcastle University. Biofilms were visualised using a scanning electron microscope (Cambridge Stereoscan 240).

### *6.2.6 NucB and 1% NaOH treatment on mature yeast biofilms*

Yeast biofilms were formed as described in section 6.2.4 in 1ml volume in sterile glass universal bottles and incubation time of 3 days. Treatments were carried out with buffer only, with NucB only (6 µg/ml) for 1 h, with 1% NaOH only for 1 h and with NucB for 30 min followed with 1% NaOH treatment for 30 min at 37°C. The universal bottles were washed with PBS solution and the percentage reduction in biofilm biomass (dry weight) with respect to the control was determined after washing with sterile PBS and dried using a desiccator.

### *6.2.7 Comparison of biofilm dispersal efficiency of NucB, bovine DNase I and varidase on different surfaces and different biofilms*

***Biofilm formation in 96 well polystyrene plates***

Biofilms were formed and processed in 96-well microtiter plates as described in section 6.2.4. However, NucB or Varidase or bDNase I were diluted to 0, 0.5, 5, 100, 500, 5000, 10000 ng/ml in standard buffer solution (section 6.2.1) and used for treatment. Each assay was performed in triplicate.

***Biofilm formation on glass cover slips***

Individual sterile cover slips (13 mm diameter) were placed into separate wells of sterile polystyrene 12- multiwell plate (ThermoScientific Nunc™, UK). Diluted overnight cultures ($OD_{600}$ of 0.1) were added into each well (2 ml). The wells were sealed with a lid of the plates and incubated at 37°C for 24 h. The cover slip from each well was removed and the non-adherent cells were rinsed off by dipping in PBS solution at aseptic condition. NucB, bDNase I and Varidase at 3 µg/ml concentrations were used to treat the biofilms formed on the cover slips with standard buffer as control (250µl). The coverslips were incubated at 37°C for 1 h and then rinsed thoroughly with PBS solution and air dried. Biofilms were stained by submerging the cover slips in 0.1% crystal violet (CV) for 20 min. The excess dye was rinsed off by dipping each cover slip in two successive PBS solution trays, and the cover slips were then air-dried. After washing, the remaining crystal violet dye was solubilised in absolute ethanol and acetic acid mixture at ratio of 80:20 (250µl applied to each cover slip). Then, 100 µl off which were transferred to a new polystyrene plate and the absorbance at 595 nm was read in a microtiter plate reader (FLUOstar OPTIMA, BMG LABTECH). Data analysis was performed using Optima Mars analysis software and Microsoft Excel. Each assay was carried out in triplicate.

***Biofilm formation on stainless steel coupons***

Stainless steel coupons (Grade 304 stainless steel) were used as substrate for biofilm formation. The coupons were rinsed 5 times with distilled water to remove any residues. Coupons were then washed with acetone for 30 min and washed 5 times again with sterile distilled water and air dried in microbial safety cabinet. Coupons were stored in

100% ethanol and sterilised by flaming prior to testing. Individual coupons were placed into separate wells of sterile polystyrene 12-multiwell plate. Diluted overnight cultures ($OD_{600}$ of 0.1) were added into each well (2 ml) and incubated at 37°C for 24h. The culture broth was removed and the coupons were washed twice by gently pipetting with sterile 1ml PBS solution and air dried. NucB, bDNase I and varidase were used for treatment at 3 µg/ml concentration with standard buffer (section 6.2.1) as control (500µl to each coupon). The coupons were incubated at 37°C for 1 h and the rinsed thoroughly with PBS solution and air dried. Biofilms were stained by submerging the coupons in 0.1% crystal violet (CV) for 20 min. Rinsed off excess dye by dipping the coupons in PBS solution (twice), and then air dried. The excess CV was washed and remaining crystal violet dye was solubilised in 500 µl absolute ethanol and acetic acid mixture at ratio of 80:20. Then, 100 µl off which were transferred to a new polystyrene plate and the $A_{595}$ was read in a microtiter plate reader (FLUOstar OPTIMA, BMG LABTECH). Data analysis was performed using Optima Mars analysis software and Microsoft Excel. Each assay was performed in triplicate.

### *6.2.8 Measurement of biofilm dispersal efficiency*

To quantitatively measure the biofilm removal/dispersal ability of nucleases percentage reduction measure introduced by Pitts et al. (Pitts *et al.*, 2003) was used.

$$\text{Biofilm dispersal efficiency} = [(C-B)-(T-B))/(C-B)] \times 100 \quad \text{– Eq (6.1)}$$

where B denotes the average absorbance per well of blank (no biofilm, no treatment); C denotes the average absorbance per well for control (biofilms, no enzyme treatment); T denotes the average absorbance per well for treated (biofilm, enzyme treatment)

### *6.2.9 Preparation of crude microbial nucleases and determination of nuclease activity*

The isolated bacteria were sub-cultured on LB agar plates were inoculated into LB medium and 0.1 $OD_{600}$ was used to inoculate into respective optimised medium for *Bacillus* and *Streptomyces* strains. The strains were then incubated at 37°C for Bacillus and 30°C for *Streptomcyes* for 48-72 h. Culture samples (1.5 ml) were centrifuged at 13,400g for 10 min and cell pellets were discarded. The supernatants were filtered

through 0.2 µm pore size Minisart filters (Sartorius, UK) and the nuclease activity was determined in the cell-free supernatant. The nuclease activity was measured by the nuclease assay as described in Chapter 3, section 3.3.3. The crude supernatants were used as microbial nuclease sources and checked for anti-biofilm activity in 96-well polystyrene plates as described in section 6.2.4.

## 6.3 RESULTS AND DISCUSSION

### *6.3.1 Biofilm disruption potential of NucB on single species biofilms*

NucB, an extracellular DNase, showed it has the ability to disrupt the biofilms (Nijland *et al.*, 2010). To understand the extent of biofilm disruption potential of NucB on single species biofilms the effects of NucB on the diverse biofilm forming bacteria were examined. NucB efficiently disrupted biofilms formed under standard conditions (described in section 6.2.4). The biofilm dispersal potential was consistent across various single species biofilms and showed greater than 90% reduction in crystal violet stain compared to buffer only control (Figure 6.1). Hence, the capacity of strains to form biofilms bring affected at 3µg NucB per ml.

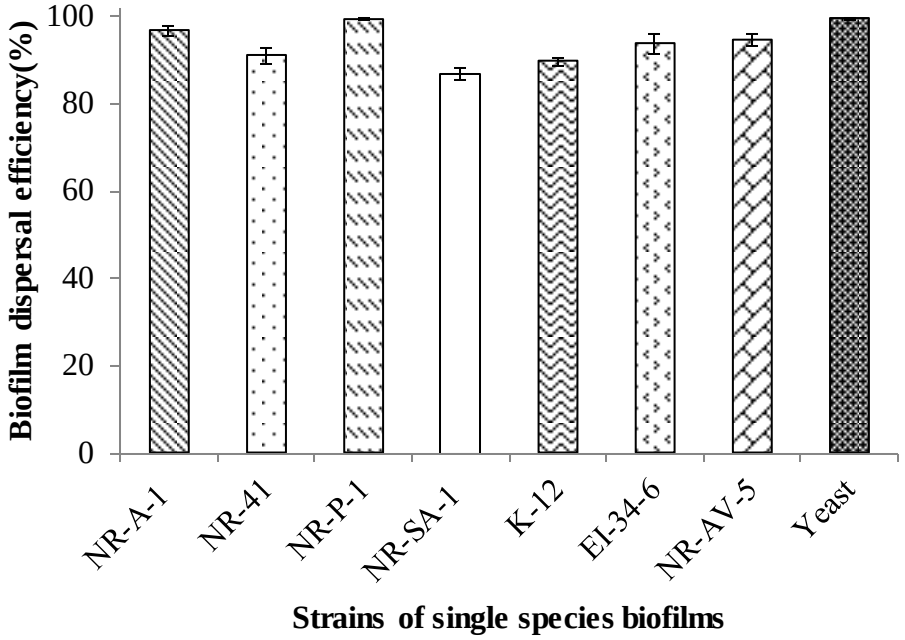



**Figure 6.1 Biofilm dispersal effect of the NucB, an extracellular nuclease over range of single species biofilms. Biofilms of Gram positive, Gram negative and yeast were formed over 24 h periods, treated in the presence and absence of NucB (3µg/ml) in 96-well microtiter plate, followed by crystal violet staining and absorbance measurement ($OD_{595}$). The various strains used in the wells are listed in Table 6.1. Bars indicate means ± standard errors for triplicate tests. The acronyms for all strains are as shown in Table 6.1.**

NucB efficacy to disrupt biofilms was reported for biofilms of clinical isolates associated to tracheoesophageal speech valve and chronic rhinosinusitis and included species belonging to genera *Staphylococcus* and *Streptococcus* (Shakir *et al.*, 2012; Shields *et al.*, 2013). The results reported in this chapter show that NucB also has the ability to disrupt biofilms of environmental isolates. This is also the first study to show that NucB can disperse biofilms formed by bacteria belonging to genera *Pseudomonas, Ruegeria, Acinetobacter*, and *Sacchromyces*.

### *6.3.2 Microscopic studies*

The efficiency of the NucB anti-biofilm activity was evaluated also by microscopic visualisation of selected strains. In the coverslip assay, the biofilm dispersal was evident and showed a disrupted biofilm for NucB treatment under light and phase contrast microscopic observations of *Bacillus* sp. and *Pseudomonas* sp. biofilms (Figure 6.2).

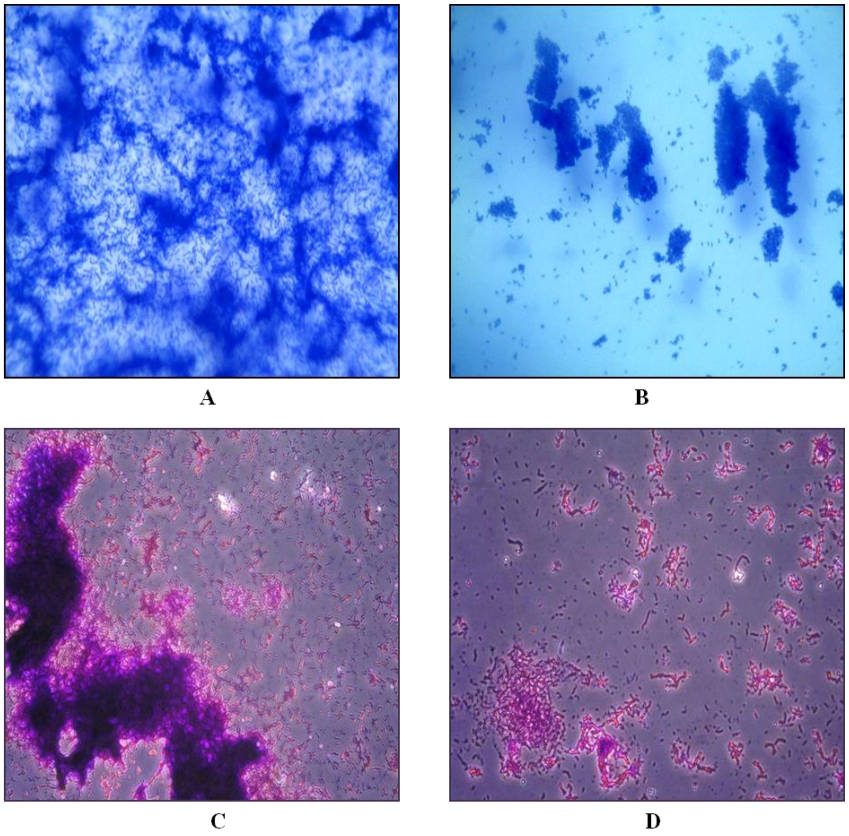



**Figure 6.2 Biofilm disruption potential of NucB against biofilm-forming bacteria under microscopic view. 40X light microscopic observation of 24 h old *Bacillus* sp. NR-AV-5 biofilms treated with buffer only (panel A) and NucB at 3µg/ml concentration (panel B) ; 40X phase contrast microscopic observation of 24 h old *Pseudomonas* sp.NR-P-1 biofilms treated with buffer only (panel C) and NucB at 3µg/ml concentration (panel D). Treatment was performed for 1h as described in section 6.2.5.**

Similar biofilm removal visualisation was observed earlier for DNase I treatment (Tetz *et al.*, 2009) and other anti-biofilm compounds (Sayem *et al.*, 2011). Data obtained here suggest that the biofilm biomass and strength was altered in the presence of NucB. This is broadly observed in the biofilms of different Gram positive and Gram negative bacteria as well as yeast. We hypothesise that this effect is a result of extracellular DNA degradation by NucB in the biofilm which releases the micro-colonies and lead to the biofilm dispersal.

To visualise the presence of extracellular DNA in the biofilm matrix, biofilms of *Bacillus licheniformis* EI-34-6 were observed under a confocal laser scanning microscopy using Live/Dead Baclight staining. The live cells stain green and dead cells stain red and the presence of extracellular DNA is observed as overlapping red over green and strong yellow. As the biofilms were treated with NucB for 30min and 60min

the area with visibility of yellow reduces and it completely disappeared after 90min treatment (Figure 6.3).

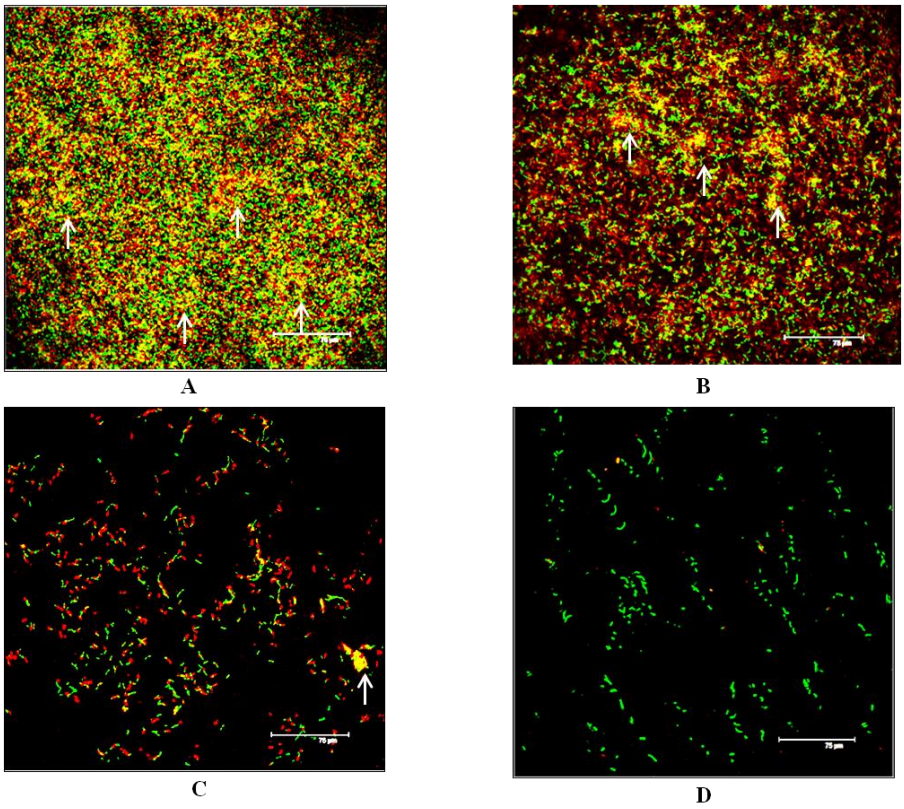



**Figure 6.3 Confocal laser scanning microscopy of 24 h biofilms of *Bacillus licheniformis* EI-34-6 without or with NucB treatment. Biofilms were formed on glass surfaces and were visualised with CLSM using Live/Dead Baclight stain, which shows dead cells in red and live cells in green. The area covered by extracellular DNA is in yellow (white arrows). Biofilms treated with A: buffer only (Control), B: NucB for 30 min, C: NucB for 60 min, D: NucB for 90 min. Scale bar – 75µm**

CLSM is widely used for eDNA visualisation and been reported in several studies earlier (Conover *et al.*, 2011). The results obtained here is similar to previously reported observation and this also confirm that biofilms contain extracellular DNA as structural component and NucB degrades the eDNA and efficiently disrupts the preformed biofilms.

Additionally, microscopic examination of the structural representation of a typical yeast biofilm is shown in Figure 6.4 (panel A and panel C). It shows aggregates and micro-colonies covered with ECM, producing compact and dense biofilm architecture. The NucB treated biofilms detached ECM from the glass coverslip during washing step, resulting in individual cells in both 30 min and 60 min incubation as shown in Figure

6.4 (panel B and panel D). This suggests that the NucB directly affects the ECM which is an important component for biofilm structural integrity.

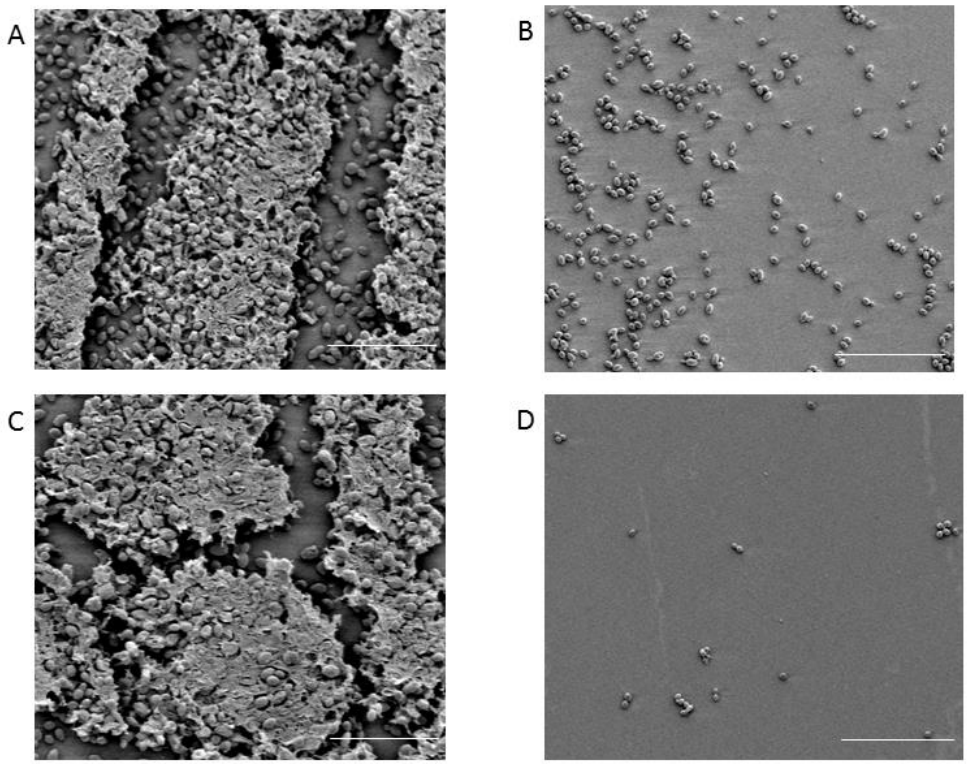



**Figure 6.4 Scanning electron microscopy of 24 h yeast biofilms treated without or with NucB. SEM Microphotographs of cells in glass slides, A and C are buffer only treated controls, while B and D are NucB (3µg/ml) treated after 30 min and 60 minutes respectively. Scale bar- 200 µm.**

The SEM images at higher magnification (×5000) are shown in Figure 6.5 where panel A represents buffer only treated yeast biofilms that clearly shows the ECM holding the cells into a biofilm on glass coverslip. On the other hand panel B represents the NucB treated yeast biofilms where ECM is absent and only individual cells are attached on the surface of coverslip.

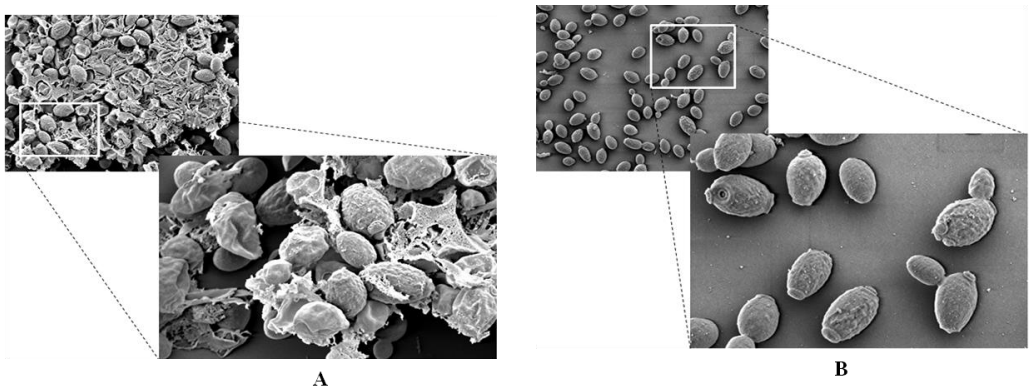



**Figure 6.5 The enlarged SEM images of the biofilms in control (panel A) showing the extracellular matrix (ECM) and NucB treated (panel B) showing only the individual cells.**

To understand whether NucB treatment can be effective on mature biofilms, the biofilms of yeast cells were grown for three days and subjected to NucB and 1% NaOH treatment. The reduction in biofilm biomass was analysed based on the dry weight determination as described in section 6.2.6. Figure 6.6 shows that there is a significant reduction in biofilm biomass measured after washing and drying compared to the control.

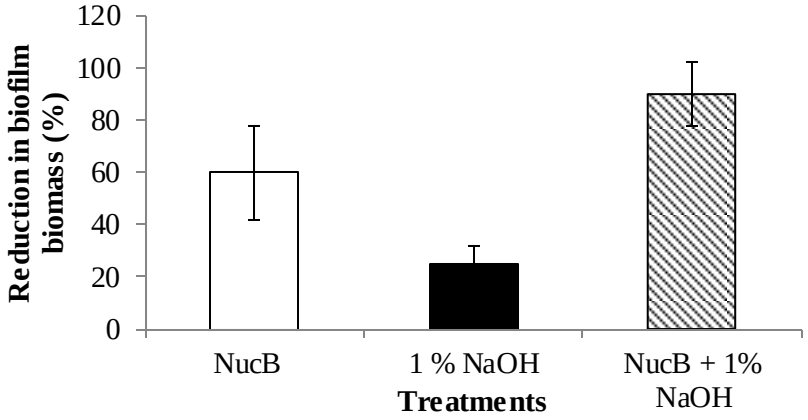



**Figure 6.6 Biofilm dispersal efficiency of NucB on mature yeast biofilms. Three days old mature yeast biofilm was subjected to NucB treatment or existing 1% NaOH treatment or NucB treatment for 30min and 1% NaOH combined treatment. The treatments were performed at 37°C under static incubation. Bars represent the mean percentage reduction in biofilm biomass ± standard error for triplicate tests.**

Figure 6.6 also shows that combining the treatments leads to ~100% biofilm biomass reduction. These results demonstrate NucB can disperse biofilms of higher organisms such as yeast. This will be a useful method to control biofilm formation in brewing industries where biofouling is a growing problem (Storgards, 2009). Also, it may be a potential and sustainable approach towards industrial cleaning processes. The enzyme activity diminished below 20% when treated above 60°C (Chapter 4), indicating enzyme deactivation process. Additionally, this can be a feasible enzymatic treatment in combination with other existing treatments which is important in breaking and reducing the yeast biofilms. Hence, industries can achieve environmental friendly and economical operating conditions such as reduced usage of chemicals, lower washing temperature and shorter cleaning time. Efficiency of the NucB enzyme was determined on the model biofilms developed in lab scale. Thus, it would be relevant to take it to an industrial scale application.

### *6.3.3 Comparison of biofilm dispersal efficiency of NucB, bovine DNase I and varidase in different substrata and different biofilms*

In order to expand the application of NucB as anti-biofilm enzyme, it is important to know how the efficacy of biofilm treatment by NucB fits with other nucleases. Since 2000, DNase I was used for eDNA degradation in various biofilm studies (Whitchurch *et al.*, 2002). Similarly, varidase which is a mixture of streptokinase and streptodornase (nuclease) was reported previously as removing *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms (Nemoto *et al.*, 2003). Therefore, we chose bovine DNase I and varidase, the known nucleases with biofilm dispersal properties to compare with NucB. Biofilms grown on different surfaces (polystyrene, glass and stainless steel) were efficiently removed using NucB (Figure 6.7 and Figure 6.8).

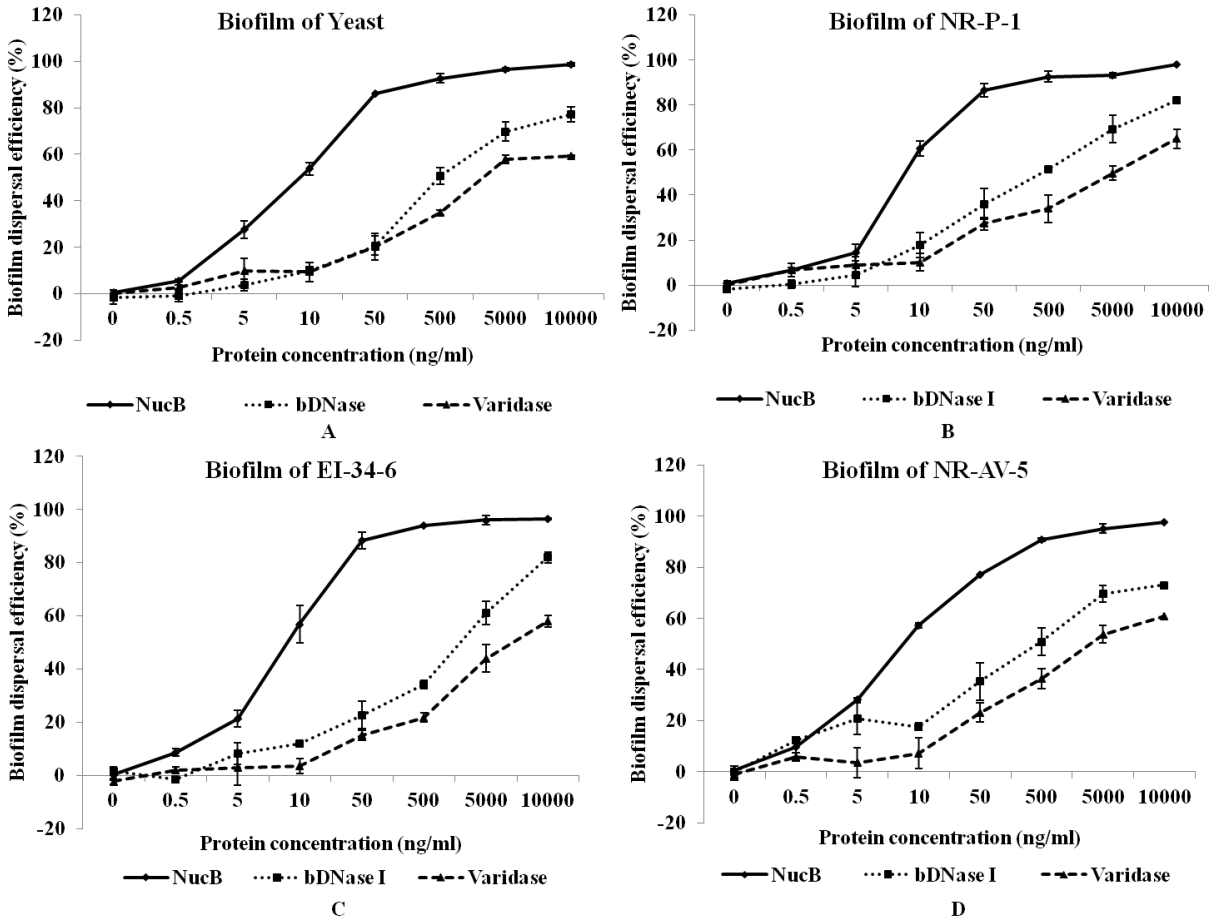



**Figure 6.7 Comparison of biofilm dispersal efficiency of nucleases. Effect of NucB, bDNase I and Varidase enzymes at a concentration of 0 - 10000 ng/ml on preformed biofilms A: yeast, B: NR-P-1, C: EI-34-6 and D: NR-AV-5 on 96-well polystyrene microtiter plates. Strain acronyms as listed in Table 6.1**

Figure 6.7, the results of microtiter plate assay shows, 10 ng/ml of NucB gave biofilm dispersal in the range 50 – 64% over the entire range of biofilms. bDNase I concentrations that gave biofilm dispersal in the range of 40– 65% were in the range of 500 – 5000 ng/ml and varidase concentrations that gave biofilm dispersal in the range 50 – 61% were in the range 5,000 – 10,000 ng/ml. NucB is able to remove 100% of a biofilm in microtiter plates whereas, at the maximum concentration used (10,000 ng/ml), bDNase I was able to remove 70 – 82% and varidase was only able to disperse 58 – 64%. In the tests on different substrates, 3 µg/ml NucB gave biofilm dispersal in the range 90 – 96% whereas bDNase I gave biofilm dispersal in the range 57 – 69% and varidase in the range 34 – 47% (Figure 6.8).

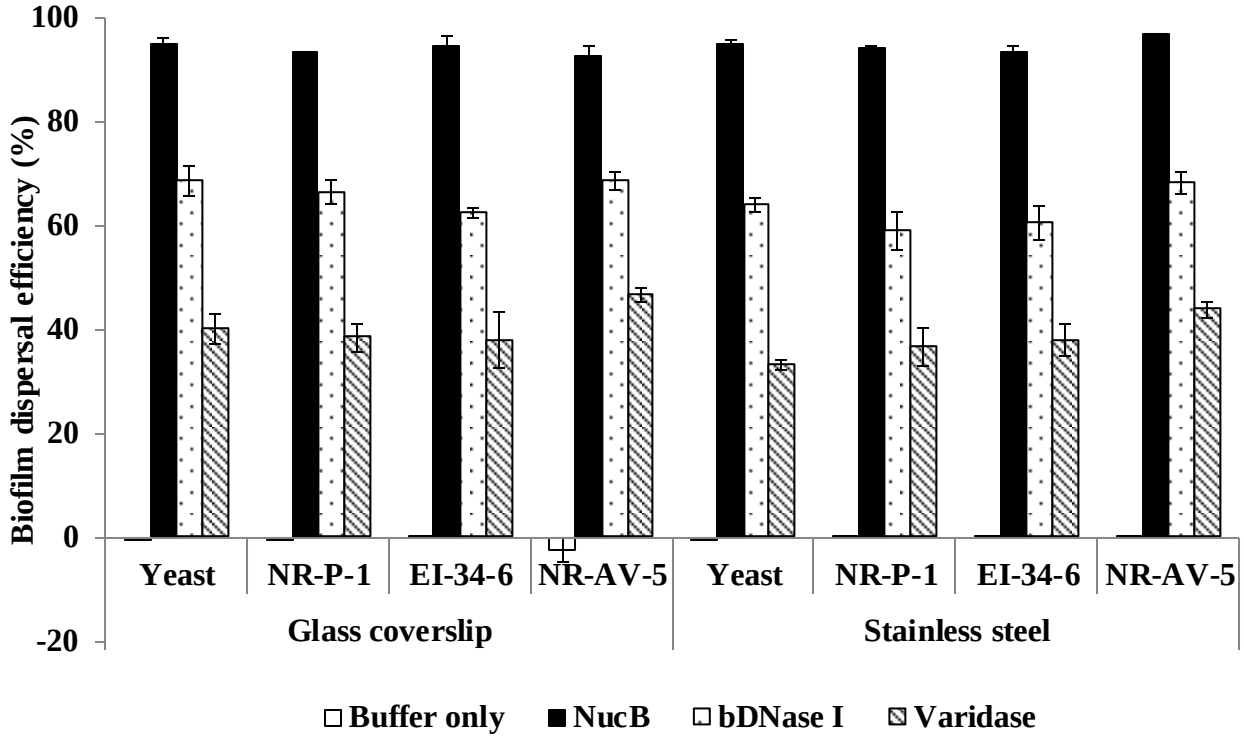



**Figure 6.8 Comparison of dispersal efficiency NucB, bDNase I and Varidase at 3µg/ml working concentration on 24h old single species biofilms grown on glass cover slips and stainless steel coupons. Biofilms were quantified by staining with crystal violet. Tests done in triplicate with buffer only control. Bars indicate means ± standard errors for triplicate tests.**

Varidase working concentration was not determined in literature in biofilm treatment. The effect of biofilm removal was shown only after treating the biofilms twice with Varidase each at 3h period (Nemoto *et al.*, 2003). However, in this study the incubation time was shortened to understand its effect in comparison with NucB. This showed that NucB is atleast 500-fold more efficient than varidase on biofilm dispersal.

DNase I working concentration was previously reported to vary between organisms, For bacteria the range was 0.01 – 0.125 mg/ml (Brown *et al.*, 2012) and for higher organisms like *Candida albicans* and *Aspergillus fumigatus* the range was 0.13 – 4 mg/ml (Martins *et al.*, 2012; Rajendran *et al.*, 2013). The results reported in this chapter on DNase I also correspond to the previous reports by showing near 80% dispersal at 0.01 mg/ml concentration and NucB is 50-fold more effective than bDNase I. Hence, the data demonstrated that the effect of NucB which was observed on 24h old biofilms formed by diverse species is consistent and concentration dependent. That is, 25% – 99% reductions in the biofilm biomass occurred at NucB concentrations ranging from 5

– 10,000 ng/ml. Thus, the potential of the NucB as an anti-biofilm agent over a wide range of non-pathogenic environmental isolates, in addition to the previous reports on clinical isolates (Shakir *et al.*, 2012; Shields *et al.*, 2013) was explored. This suggests that the enzyme might be a powerful alternative to existing bovine DNase I and varidase in the context of multispecies biofilms on different surfaces.

### *6.3.4 Biofilm dispersal efficiency of other microbial nucleases*

The crude nuclease was obtained from the supernatant of diverse bacterial strains and it was evaluated for biofilm dispersal at the nuclease activity of 100 U/ml. The crude nucleases along with NucB and bDNase I revealed notable ability to disrupt already formed 24h biofilms (Figure 6.9). Crude nucleases were able to disperse biofilms at the range of 58 – 88% against *Bacillus* sp. and *Pseudomonas* sp. biofilms. The control enzyme NucB and bDNase I biofilm dispersal range was from 78 – 95%.

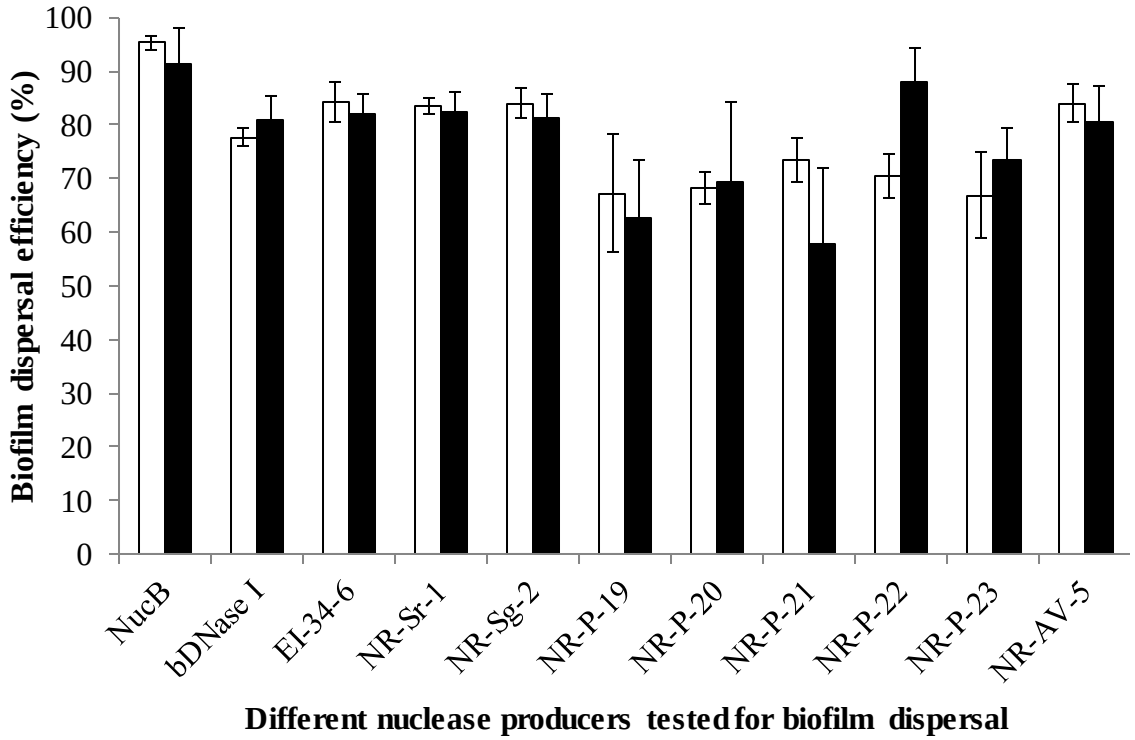



**Figure 6.9 Anti-biofilm activity of crude supernatant of nuclease producers EI-34-6 – *Bacillus licheniformis* , NR-Sr-1 – *Streptomyces* sp; NR-Sg-2- *Streptomyces* sp; NR-P-19 – *Bacillus* sp ; NR-P-20 – *Bacillus* sp; NR-P-21 – *Bacillus* sp ; NR-P-22 – *Bacillus* sp ; NR-P-23 – *Bacillus* sp ; NR-AV-5- *Bacillus* sp. with NucB and bDNase I as control at 100 U/ml concentration on preformed 24 h old *B. licheniformis* EI-34-6 (white bar) and *Pseudomonas* sp. NR-P-1 (black bar) biofilms. Bars indicate means ± standard errors for triplicate tests**

In previous reports bDNase I was used at the enzyme activity range of 100 – 140 U/ml and showed efficient biofilm removal of *S. aureus* (Seidl *et al.*, 2011; Mitchell *et al.*, 2013). Hence, microbial nucleases for this study were taken at 100 U/ml and the data proved the real potential of the microbial nucleases in disrupting the biofilms formed by Gram positive and Gram negative bacteria.

Furthermore, to assess the ability of thermophilic nuclease to reduce preformed biofilms, the thermophilic strain *Bacillus* sp. NR-T-2 (grown at 45°C) crude supernatant at 100 U/ml nuclease activity was evaluated. Good biofilm forming thermophilic strains of bacilli which showed crystal violet staining ($A_{595}$) range from 3.6-5.7 were used. The efficiency of biofilm dispersal at 45°C ranged from 60-92 % tested based on crystal violet staining.

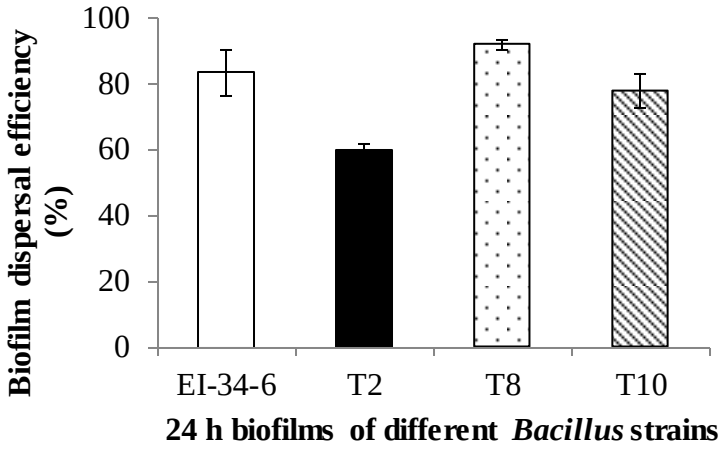



**Figure 6.10 Cell free supernatant of Thermophilic *Bacillus* sp. NR-T-2 used as a nuclease source for dispersal of other Bacillus species biofilms at 100 U/ml concentrations. EI-34-6 – *Bacillus licheniformis* biofilm grown at 37°C for 24h, T2 – *Bacillus* sp, T8 – *Bacillus* sp, T10 – *Bacillus* sp. biofilms grown at 45°C for 24 h. Bars indicate means ± standard errors for triplicate tests.**

Previously, cleaning strategies on biofilms of thermophilic bacilli on stainless steel plants required caustic wash at 75°C for 30 min and acid wash at 75°C for 30 min. Also, the change in concentration of the caustic and acid wash as well as temperature could alter the outcome of cleaning (Parkar *et al.*, 2004). This causes challenges to industry in following sustainable practises (Gunduz and Tuncel, 2006). However, the data presented here on the ability of thermophilic nucleases to remove more than 60% thermophilic bacilli at 45°C in 1 h is significant compared to existing practises. Therefore, these results suggest that the use of thermophilic enzymatic treatment of

industrial plants at lower temperature range will be more effective and reproducible. Hence, it has wider application in an industrial environment

## 6.4 SUMMARY

In this chapter, data demonstrated that microbial biofilms can be reduced by treatment with matrix degrading enzymes such as NucB and other microbial nucleases. Purified, isolated NucB was able to consistently disperse greater than 95% of preformed biofilms of Gram positive, Gram negative and yeast. NucB showed 50- to 500-fold increased effectiveness in biofilm dispersal compared to commercial nucleases. Microbial nucleases were able to disperse biofilms of different single species biofilms in the range of 78-95 %. Promising results were also shown by thermophilic extracellular nuclease of *Bacillus* sp. NR-T-2 which dispersed thermophilic biofilms in the range of 60-90%. The dispersal of bacterial biofilms with microbial nucleases may offer novel therapies to eradicate medically relevant harmful biofilms and sustainable cleaning approached in industrial biofouling problems.

# Chapter 7 Conclusions and future work

This thesis comprises of several necessary steps towards the implementation of a novel, antibiofilm technology.

- The objective of chapter 2 was achieved by studying the diversity of extracellular nucleases across microbial communities. An ecological implication of nuclease involvement in phosphate/DNA metabolism was inferred. DNA is rich in phosphates and it plays an important role in P-cycling in nature which was previously neglected. Hence, the fate of extracellular DNA in the environment was clarified in terms of nutrient cycling of phosphate in food webs.
- The objective of chapter 3 was fulfilled as the results reported the enhanced production of NucB from *B. licheniformis* through the use of a design of experiments based on the physiological understanding of the production strain's metabolism. For example, the use of manganese stimulated sporulation, to achieve increased NucB production resulted in a 10-fold increase in NucB activity (from 47 U/ml achieved in standard medium to 471 U/ml using the optimum concentration of medium constituents). The results also showed that phosphate availability had an inhibitory effect on NucB synthesis. The use of ecologically relevant information combined with classical engineering methods represents a promising new approach to increasing enzyme yield and may also be widely applicable to other microbial bioprocesses.
- The objective of chapter 4 also achieved and the results presented showed for the first time that high cell density growth of a SURE expression *B. subtilis* system can be successfully used to obtain a 68-fold enhanced level of NucB (from 3 mg/L in standard medium to 210 mg/L under optimised production conditions). NucB was produced and purified up to 95% purity and it was shown to be well-folded, non-aggregated, monodisperse, thermally stable as well as possessing refolding characteristic. To the author's knowledge, this is the first study detailing biophysical properties of NucB. Secondary and tertiary structure prediction may help to understand NucB-DNA interactions. Therefore, this characterisation can facilitate the use of this enzyme in a range of novel anti-biofilm applications, both *in vitro* and *in vivo* in clinical and industrial settings.

- The objective of chapter 5 was satisfied and the results of the diverse bacteria showed the polynomial models obtained using statistical methodology were good fit for enhanced nuclease production and proposed that optimisation of cultivation medium parameters enhance the key enzyme produced in the range of 7.5- to 19-fold (from standard medium conditions to optimum medium conditions in diverse bacteria). Thus it has been successfully demonstrated that such an optimisation strategy concentrating on operating conditions, such as temperature and medium constituents, can significantly enhance enzyme production.
- Finally, the objective of chapter 6 was achieved as reported in this thesis. It showed that several microbial nucleases had an ability to disperse preformed biofilms. Purified and isolated extracellular nuclease NucB, provided greater understanding of the biology of biofilm dispersal. NucB dispersed biofilms on different substrata and biofilm of diverse bacteria and yeast more efficiently compared to commercially available nuclease enzymes. Application of nuclease enzyme on biofilms of thermophilic strains was also tested using crude nuclease of thermophilic *Bacillus* strain.
- As a result, the overall conclusions of this research work are that microbial nucleases are widely distributed across microbial diversity, the enhanced production of specific, effective microbial nuclease (NucB) was achieved and nucleases were efficiently applied as biofilm dispersal compound. Microbial nucleases could be used in the future as a preventive measure, to avoid the establishment of a biofilm, or as a method to eradicate an already existing biofilm. Hence, microbial nucleases act as potential anti-biofilm enzyme treatments.

**Future directions**

In the present thesis, the role of extracellular nucleases in biofilm dispersal has been studied in according to the current state of knowledge. However, the findings arising from this research indicate a number of areas for future work.

Firstly, though the diversity of extracellular nucleases has been investigated by culture-based method, it is necessary to purify these nucleases in the future to explore their biotechnological applications. Also, microbial diversity study by sequence based

method identified numerous extracellular nucleases across microbial communities. Nevertheless, expanding the knowledge on individual microbial attributes such as survival in extreme conditions i.e., salinity, temperature and oxygen requirements may answer unclear processes about extracellular nuclease secretion and metabolic functions. This will also provide additional information on enzymes that work beyond the standard operating conditions which would be beneficial for industrial applications. Now that these nuclease-like gene sequences are accessible, research can be focused on building a network of multi-functional extracellular nuclease gene pathways. This will advance the study towards a synthetic biology approach, thinking beyond microbial cells, leading to synthetic multi-enzyme reaction networks.

Additionally, the present study has advanced the bioprocess development of NucB, an isolated nuclease which has potential anti-biofilm applications. Further work may involve transfer of the production technology to large scale for industrial manufacture and commercialisation. Also, existing production and purification methods can be used for research and development studies of NucB at lab scale, which includes exploring its anti-biofilm properties on biofilms grown on medical implants such as knee or hip. This is one of the growing biofilm related problems causing periprosthetic joint infections. Infection in joint replacement occurs in 0.5-5.8% of cases with devastating effects on patients. Therefore, advances might occur where NucB may be used in combination with antibiotic treatment as an *in situ* approach to eradicate implant infections. However, there is a limitation on toxicological approval for these applications which should be overcome before regulatory approvals.

Other extracellular nucleases that were optimised for production may undergo future work for the bioprocess development as described for NucB and also could be tested for various other biofilm related applications such as additives in detergents and as cleaning solution in different industries.

Therefore, novel anti-biofilm technology of extracellular nucleases is still to be fully explored and that will be the major future work derived from the research described in this thesis.

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# APPENDIX A

**Table A.1 List of nucleases surveyed across prokaryotic and eukaryotic organisms based on the location defined as secreted (+), intracellular (-) or not defined (ND)**

| Organism / Kingdom | Classification | Name of the species | Nuclease / protein name | gene/locus name | aa seq | Location |
|---|---|---|---|---|---|---|
| **Bacteria** | Acidobacteria | *Acidobacteria bacterium* | Nuclease-related protein | Acid345_4695 | 206 | ND |
| | | *Solibacter usitatus* ( Ellin6076) | S1/P1 nuclease | Acid_5845 | 261 | ND |
| | Actinobacteria (High G+C) | *Eggerthella lenta* ATCC 25559 | HNH endonuclease | - | 127 | ND |
| | | *Micrococcus sedentarius* | Predicted extracellular nuclease | - | 802 | ND |
| | | *Slackia heliotrinireducens* ATCC 29202 | HNH endonuclease | - | 97 | ND |
| | | *Streptomyces antibioticus* | 34KDa extracellular nuclease | - | 9 | + |
| | | *Streptosporangium roseum* | Extracellular nuclease like protein | Sros_3284 | 795 | ND |
| | Alphaproteobacteria | *Alteromonas macleodii* DSM17117 | Ribonuclease H | rnhA | 156 | - |
| | | *Caulobacter crescentus* | Nuclease, putative | CC_0544 | 695 | ND |
| | | *Citreicella sp.* SE45 | Extracellular nuclease | CSE45_0773 | 1183 | - |
| | | *Magnetospirillum magneticum (strain* AMB-1 / ATCC 700264) | Nuclease | amb4452 | 339 | ND |
| | | *Oceanicola batsensis* HTCC2597 | Extracellular nuclease | OB2597_06605 | 1215 | ND |
| | | *Roseovarius sp.* 217 | Extracellular nuclease | ROS217_14766 | 1682 | - |
| | | *Sagittula stellata* E-37 | Extracellular nuclease | SSE37_13468 | 1407 | - |
| | Betaproteobacteria | *Burkholderia mallei* | Extracellular nuclease, putative | BMAA0259 | 624 | ND |
| | | *Arpmatoleum aromaticum* | Putative DNA/RNA NON-specific endonuclease | nuc1 | 256 | ND |
| | | *Burkholderia mallei* | Endonuclease | nuc BMA1476 | 207 | ND |
| | Deltaproteobacteria | *Anaeromyxobacter dehalogenans* | HNH nuclease | - | 370 | ND |
| | | *Geobacter metallireducens* DSM 7210 | HNH endonuclease | - | 104 | ND |

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|---|---|---|---|---|---|---|
| | | *Sorangium cellulosum* | Putative nuclease | - | 233 | ND |
| | | *Syntrophobacter fumaroxidans* DSM 10017 | Nuclease (SNase domain protein) | - | 222 | ND |
| | | *Syntrophobacter fumaroxidans* DSM 10017 | HNH endonuclease | - | 107 | ND |
| | | *Syntrophobacter fumaroxidans* DSM 10017 | Endonuclease/exonuclease/phosphatase | - | 291 | ND |
| | Epsilonproteobacteria | *Campylobacter jejuni* RM1221 | DNA/RNA non-specific endonuclease | - | 217 | ND |
| | | *Helicobacter pylori* v225d | Endonuclease | - | 282 | ND |
| | | *Wolinella succinogenes* | Ribonuclease | - | 183 | ND |
| | Gammaproteobacteria | *Acinetobacter baumannii* | putative extracellular nuclease | A1S_1198 | 298 | ND |
| | | *Aeromonas hydrophila* | Extracellular nuclease | dns | 230 | + |
| | | *Aeromonas hydrophila* | Extracellular deoxyribonuclease | - | 227 | + |
| | | *Aeromonas salmonicida* (strain A449) | Extracellular nuclease | nucHASA_2206 | 1072 | - |
| | | *Alteromonas macleodii* DSM17117 | Extracellular ribonuclease | MADE_01224 | 1346 | ND |
| | | *Azotobacter vinelandii* ATCC BAA-1303 | Staphylococcus nuclease (SNase-like) | - | 208 | ND |
| | | *Azotobacter vinelandii* ATCC BAA-1303 | HNH endonuclease domain-containing protein | - | 116 | - |
| | | *Buchnera aphidicola* | endonuclease-1 | endA | 245 | ND |
| | | *Colwellia psychrerythraea* 34H/ATCC BAA 681 | Extracellular ribonuclease/nuclease fusion protein | CPS_2634 | 1310 | ND |
| | | *Corynebacterium glutamicum* | Predicted extracellular nuclease | **nuc** Cgl2592 cg2868 | 916 | ND |
| | | *E.coli* (K12) | Exodeoxyribonuclease 1 | sbcB | 475 | ND |
| | | *Enterococcus faecalis* | Putative uncharacterized protein | - | 192 | - |
| | | *Erwinia tasmaniensis* | Nuclease nucM | nucM | 237 | ND |
| | | *Idiomarina loihiensis* | Extracellular nuclease | IL1523 | 589 | ND |
| | | *Photobacterium profundum* | Hypothetical extracellular nuclease | PBPRA0287 | 781 | ND |
| | | *Pseudoalteromonas atlantica* | S1/P1 nulcease | - | 256 | - |
| | | *Pseudomonas* | Predicted | PA14_ | 780 | ND |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | *aeruginosa* | extracellular nuclease | 13340 | | |
| | | *Pseudomonas fluorescens biotypeA* | Extracellular endonuclease | endX | 229 | + |
| | | *Salmonella choleraesuis* | Putative DNA/RNA non-specific nuclease | nucA | 284 | ND |
| | | *Serratia marscescens* | Nuclease | nucA | 266 | + |
| | | *Shewanella oneidensis* | Extracellular nuclease, putative | SO_1844 | 948 | ND |
| | | *Shigella flexneri* | Micrococcal nuclease | nuc | 174 | ND |
| | | *Teredinibacter turnerae* ATCC 39867 | Extracellular nuclease | TERTU_0904 | 796 | ND |
| | | *Vibrio cholerae* | Extracellular nuclease | dns VC-0470 | 231 | + |
| | | *Vibrio parahaemolyticus* | Extracellular nuclease related protein | VP2799 | 984 | ND |
| | | *Xanthomonas campestris* | Putative extracellular nuclease | XCV0282 | 572 | ND |
| | | *Xanthomonas campestris* B100 | Endonuclease S1 | nucS | 270 | ND |
| | | *Yersinia mollaretti* ATCC 43969 | Nuclease nucM | - | 235 | ND |
| | Aquificae | *Aquifex aeolicus* | Endodeoxyribonuclease IV | nfo | 282 | - |
| | | *Hydrogenobaculum sp* | HNH endonuclease | - | 174 | ND |
| | | *Persephonella marina* DSM 14350 | HNH endonuclease | - | 195 | ND |
| | Bacteroidetes | *Bacteroides fragilis* | Probable endonuclease 4 | nfo | 278 | - |
| | | *Cytophaga hutchinsonii* ATCC 33406 | Nuclease | CHU_1190 | 166 | ND |
| | | *Cytophaga hutchinsonii* ATCC 33406 | Endonuclease/exonuclease/phosphatase family protein | CHU_3419 | 339 | ND |
| | | *Flavobacterium johnsoniae* | Endonuclease/exonuclease/phosphatase | Fjoh_1565 | 279 | ND |
| | | *Flavobacterium psychrophilum* | Probable extracellular ribonuclease | FP0696 | 365 | ND |
| | | *Leeuwenhoekiella blandensis* | Putative S1/P1 Nuclease | MED217_13971 | 263 | ND |
| | | *Prevotella bryantii* | NucB | nucB | 300 | ND |
| | | *Prevotella buccalis* ATCC 35310 | nuclease. EndA/nucM family | - | 516 | ND |
| | | *Prevotella melaninogenica* ATCC 25845 | Nuclease, EndA/NucM family | - | 500 | ND |
| | Chlamydiae | *Chlamydia pneumonia* | Probable endonuclease 4 | nfo | 293 | - |
| | Chloroflexi | *Dehalococcoides sp.* (strain CBDB1) | Endonuclease V | nfi | 223 | - |
| | | *Herpetosiphon aurantiacus* | DNA/RNA non-specific endonucleas | Haur_1138 | 356 | ND |

| | | | | | |
|---|---|---|---|---|---|
| Cyanobacteria | *Nostac sp. Strain* PCC7120 | Nuclease | nucA | 274 | - |
| | *Synechocystis sp.* (strain ATCC 27184 / PCC 6803 / N-1) | Extracellular nuclease | nucH | 1879 | - |
| Deferribacteres | *Deferribacter desulfuricans* DSM 14783 | Thermonuclease | DEFDS_P103 | 179 | ND |
| | *Deferribacter desulfuricans* DSM 14783 | Ribonuclease HII | rnhB | 187 | ND |
| Deinococcus-Thermus | *Deinococcus radiodurans* | Endonuclease III | DR_0289 | 225 | - |
| | *Meiothermus ruber* | Probable endonuclease 4 | nfo` | 266 | - |
| Firmicutes (low G+C) | *B.amyloliquefaciens* | Extracellular nuclease | bsn | 289 | + |
| | *B.amyloliquefaciens* DSM7 | Nuclease | nucB | 144 | |
| | *B.amyloliquefaciens* FZB42 | NucA | nucA | 145 | - |
| | *Bacillus amyloliquefaciens* FZB42 | NucB | nucB | 144 | + |
| | *Bacillus atrophaeus* 1942 | Endonuclease | nd | 145 | - |
| | *Bacillus atrophaeus* 1942 | Nuclease | - | 144 | |
| | *Bacillus cereus* AH603 | DNA entry nuclease | - | 144 | - |
| | *Bacillus cereus anthracis sp.* | Hypothetical nuclease coml family | - | 142 | |
| | *Bacillus cereus* str Q1 | Competence specific | coml | 148 | |
| | *Bacillus licheniformis* ATCC14580 | Nuclease NucA | nucA | 148 | - |
| | *Bacillus licheniformis* ATCC14580 | NucB | nucB | 142 | + |
| | *Bacillus licheniformis* ATCC14580 | Extracellular ribonuclease | yurl/ bsn BLi03441 | 270 | ND |
| | *Bacillus megatarium* | Competence specific | nucA | 146 | - |
| | *Bacillus megatarium* DSM 319 | Sporulation specific | nucB | 145 | + |
| | *Bacillus pumilus* ATCC7061 | DNA entry nuclease | - | 144 | - |
| | *Bacillus pumilus* SAFR-032 | Possible sporulation specific | nucB | 142 | + |
| | *Bacillus subtilis* 168 | DNA entry nuclease | nucA | 147 | - |
| | *Bacillus subtilis* 168 | Sporulation specific extracellular nuclease | nucB | 136 | + |
| | *Bacillus subtilis natto* BEST195 | Nuclease | nucA | 149 | - |
| | *Bacillus subtilis* | Endonuclease | nucA | 145 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | spiz W23 | | | | |
| | | *Bacillus thuringensis konkukian sp.* | possible competence specific nuclease | - | 144 | - |
| | | *Bacillus subtilis* | Extracellular ribonuclease | bsn yurI BSU3 2540 | 288 | + |
| | | *Bacillus subtilis spiz* W23 | Nuclease | nucB | 136 | ND |
| | | *Brevibacillus brevis* NBRC 10059 | Probable nuclease | - | 142 | ND |
| | | *Desulfotomaculum acetoxidans* | Endonuclease III | Dtox_ 0804 | 219 | - |
| | | *Eubacterium eligens* | Endonuclease | encode d pEubel i2 | 316 | ND |
| | | *Finegoldia magna* ATCC 53516 | Endo/exonuclease | - | 91 | - |
| | | *Geobacillus sp.*Y412MC10 | Nuclease | - | 146 | - |
| | | *Lactobacillus johnsonii* | Extracellular/ cell surface nuclease | endA | 282 | + |
| | | *Paenibacillus polymyxa* SC2 | DNA entry nuclease | - | 245 | + |
| | | *Staphylococcus aureus* | Staphylococcal/micro coccal/thermococcal nuclease | nuc | 231 | Nuc A +, Nuc B - |
| | | *Staphylococcus aureus* MW2 | Thermonuclease | nuc | 228 | + |
| | | *Staphylococcus hyicus* | Thermonuclease | nucH | 169 | + |
| | | *Staphylococcus intermedius* | Thermonuclease | nucI | 168 | + |
| | | *Streptococcus equi* | Extracellular nuclease | Sez_0 781 | 926 | + |
| | | *Streptococcus sanguinis* | Extracellular nuclease, putative | SSA_1 750 | 749 | ND |
| | | *Thermincol potens* | Endonuclease III | TherJ R_236 8 | 208 | - |
| | Fusobacteria | *Fusobacterium ulcerans* ATCC 49185 | Nuclease | SNc | 156 | ND |
| | | *Fusobacterium nucleatum* ATCC 23726 | Family 2 AP endonuclease | HMPR EF039 7_010 9 | 316 | ND |
| | | *Streptobacillus moniliformis* ATCC 14647 | Endonuclease/exonuc lease/phosphatase family | | 277 | ND |
| | Nitrospirae | *Candidatus Nitrospira defluvii* | Putative HNH endonuclease | - | 181 | ND |
| | | *Candidatus Nitrospira defluvii* | Putative Endonuclease | - | 82 | - |
| | Planctomycetes | *Rhodopirellula baltica* | probable extracellular nuclease | RB437 5 | 3056 | - |
| | | *Candidatus Kuenenia* | Highly similar to nuclease | nucI | 175 | ND |

| | | | | | |
|---|---|---|---|---|---|
| | *stuttgartiensis* | | | | |
| | *Cloacamonas acidaminovorans* | Putative micrococcal nuclease | CLOAM1654 | 251 | ND |
| | *Pirellula staleyi* ATCC 27377 | Nuclease (SNase domain protein) | Psta_2998 | 178 | ND |
| | *Pirellula staleyi* ATCC 27377 | Endonuclease/exonuclease/phosphatase | Psta_4178 | 290 | ND |
| Spirochaetes | *Borrelia afzelii (strain Pko)* | Endonuclease | nucA | 293 | ND |
| | *Leptospira biflexa serovar Patoc* | HNH nuclease | LBF_1075 | 97 | ND |
| | *Leptospira interrogans* | Nuclease-like protein | LIC_10505 | 325 | ND |
| | *Treponema pallidum* | Endonuclease/exonuclease/phosphatase family | TPChic_0544 | 622 | ND |
| | *Treponema vincentii* | HNH endonuclease domain protein | TREVI0001_0568 | 172 | ND |
| Thermotogae | *Thermotoga maritime* | Endonuclease V | nfi | 225 | - |
| Verrucomicrobia | *Methylacidiphilum infernorum* | NurA Nuclease | - | 384 | ND |
| | *Methylacidiphilum infernorum* | predicted endonuclease | - | 78 | ND |
| | *Methylacidiphilum infernorum* | HNH nuclease | - | 221 | ND |
| Tenericutes (no wall) | *Mycoplasma agalactiae* | Nuclease | - | 164 | ND |
| | *Mycoplasma mobile* | Staphylococcal nuclease | - | 200 | ND |
| | *Ureaplasma urealyticum serovar* 10 ATCC 33699 | Endonuclease/exonuclease/phosphatase family protein | - | 1134 | ND |
| Fibrobacteres | *Fibrobacter succinogenes* ATCC 19169 | Deoxyribonuclease I | endA | 247 | ND |
| Gemmatimonadetes | *Gemmatimonas aurantiaca* DSM 14586 | Endonuclease | - | 285 | ND |
| Nitrospirae | *Thermodesulfovibrio yellowstonii* ATCC 51303 | Thermonuclease | - | 183 | ND |
| Synergistetes | *Aminobacterium colombiense* DSM 12261 | Endonuclease/exonuclease/phosphatase | - | 359 | ND |
| | *Aminobacterium colombiense* DSM 12261 | Nuclease (SNase domain protein) | - | 264 | ND |
| | *Anaerobaculum hydrogeniformans* ATCC BAA-1850 | Thermonuclease | - | 282 | ND |
| | *Anaerobaculum hydrogeniformans* ATCC BAA-1850 | Endonuclease/exonuclease/phosphatase family protein | - | 240 | ND |
| | *Anaerobaculum hydrogeniformans* ATCC BAA-1850 | AP endonuclease, family 2 superfamily | - | 260 | ND |
| | *Dethiosulfovibrio peptidovorans* | Nuclease (SNase domain protein) | - | 258 | ND |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | DSM 11002 | | | | |
| | Dictyoglomi | *Dictyoglomus thermophilum* ATCC 35947 | HNH endonuclease domain protein | - | 166 | ND |
| **Archaea** | Crenarchaeota | *Sulfolobus solfataricus* | Flap structure-specific endonuclease | - | 349 | ND |
| | | *Ignicoccus hospitalis* DSM 18386 | Endonuclease (RecB family)-like protein | - | 195 | ND |
| | | *Ignicoccus hospitalis* DSM 18386 | Nuclease (SNase domain protein) | - | 227 | ND |
| | Euryarchaeota | *Methanobrevibacter ruminantium* ATCC 35063 | Hef nuclease | - | 842 | ND |
| | | *Methanobrevibacter smithii* ATCC 35061 | Nuclease, Staphylococcus nuclease-like family | - | 161 | ND |
| | | *Thermococcus onnurineus* | Staphylococcus nuclease | - | 152 | ND |
| | | *Thermoproteus neutrophilus* DSM 2338 | Secreted endonuclease-like protein | - | 215 | + |
| | | *Methanosarcina acetivorans* | Hnh endonuclease | - | 279 | ND |
| | | *Halobacterium marismortui* | putative nuclease | - | 94 | - |
| | | *Methanococcus maripaludis* | Staphylococcus nuclease (SNase-like):Thermonuclease | - | 185 | ND |
| | | *Thermoplasma sp.* P61 | Flap endonuclease-1 | fen-1 | 328 | ND |
| | Korarchaeota | *Korarchaeum cryptofilum* | predicted nuclease | - | 183 | ND |
| | Nanoarchaeota | *Nanoarchaeum equitans* Kin4-M | Flap structure-specific endonuclease | fen | 339 | ND |
| | Thaumarchaeota | *Nitrosopumilus maritimus* SCM1 | Ribonuclease | - | 205 | - |
| | | *Cenarchaeum symbiosum* | Micrococcal nuclease-like protein | - | 637 | ND |
| | | *Cenarchaeum symbiosum* | HNH nuclease | - | 338 | ND |
| **Fungi** | Ascomycota | *Aspergillus niger* | nuclease | - | 334 | ND |
| | | *Aspergillus oryzae* | Nuclease S1 | nucS | 287 | ND |
| | | *Aspergillus fumigates* | Staphylococcal nuclease protein domain | - | 296 | ND |
| | | *Penicillum citrinum* | Nuclease P1/ Dnase P1/Endonuclease P1 | - | 270 | + |
| | | *Penicillium sp.* | Nuclease PA3 | - | 270 | + |
| | | *Candida albicans* | Endo-exonuclease NUCR | - | 551 | ND |
| | | *Candida dubliniensis* | nuclease, putative | - | 235 | ND |
| | | *candida tropicalis* | mitochondrial nuclease | - | 375 | ND |
| | | *Schizosaccharomyces pombe* | AP endonuclease 2 | apn2 | 523 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | *Syncephalastum racemosum* | Nuclease/ Sr-nuclease | - | 320 | + |
| | Basidiomycota | *Armillaria mellea* | endo-exonuclease | - | 164 | ND |
| | Zygomycota | *Cunninghamella echinulata var. echinulata* | Nuclease1 | NUC1 CE | 252 | + |
| | | *Rhizopus niveus* | Ribonuclease Rh | - | 238 | ND |
| **Eukaryota** | Chordata | *Homospiens* | DNA-(apurinic or apyrimidinic site) lyase | APEX 1 | 318 | - |
| | | *Homospiens* | Nuclease EXOG, mitochondrial/ Endonuclease G-like 1 | EXOG | 368 | - |
| | | *Syncephalastum racemosum* | Nuclease/ Sr-nuclease | | 320 | + |
| | | *Mus musculus* (Mouse) | E-NPP2/autotaxin | Enpp2 Npps2 Pdnp2 | 862 | + |
| | | *Bos Taurus (Bovine)* | Deoxyribonuclease | DNASE1 | 282 | + |
| | Magnoliaphyta | *Solanum lycopersicum* (Tomato) | Extracellular ribonuclease LE | | 230 | + |
| | Chlorophyta | *Ostreococcus tauri* | probable extracellular nuclease | | 1490 | + |
| | | *Paralithodes camtschaticus* (Red king crab) | Duplex-specific nuclease | NUC | 407 | ND |
| | | *Naegleria gruberi* (Amoeba) | probable extracellular nuclease | | 1888 | - |
| | | *Hordeum vulgare* (Barley) | Nuclease I | Bnuc1 | 290 | ND |
| | Apicomplexa | *Plasmodium yoelii yoelii* | AP endonuclease 1 | | 460 | - |
| | Metazoa | *Danio rerio* (Zebra fish) | APEX nuclease | apex1 | 310 | - |
| **Virus** | | *Enterobacteri phage* | Exodeoxyribonuclease | D15 | 291 | ND |
| | | *Human herpes virus* | alkaline exonuclease | BGLF5 | 470 | ND |

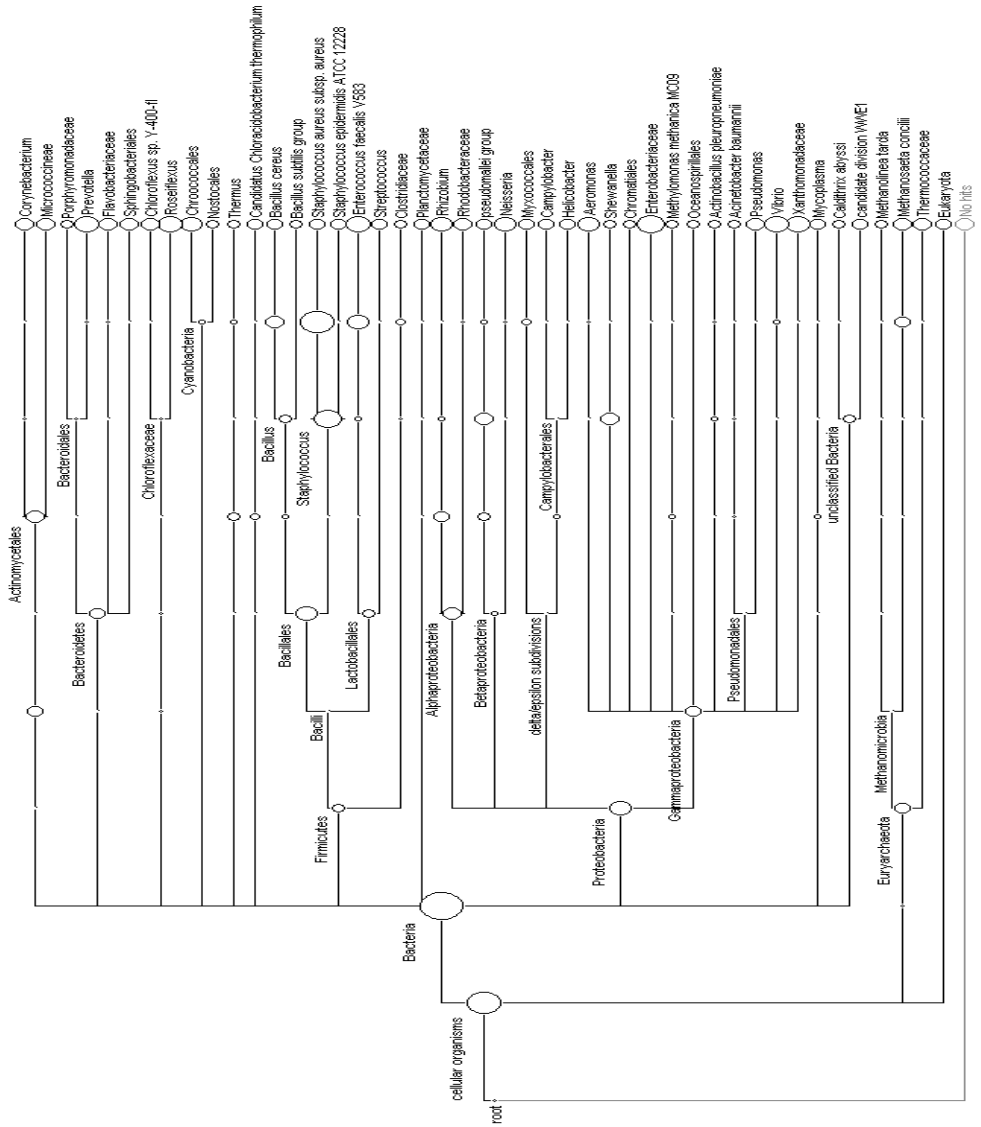


**Figure A.1 Occurrence of the genetic potential of taxa to produce nuclease-like enzymes shown within each phylum which is observed for majority of bacterial lineages**

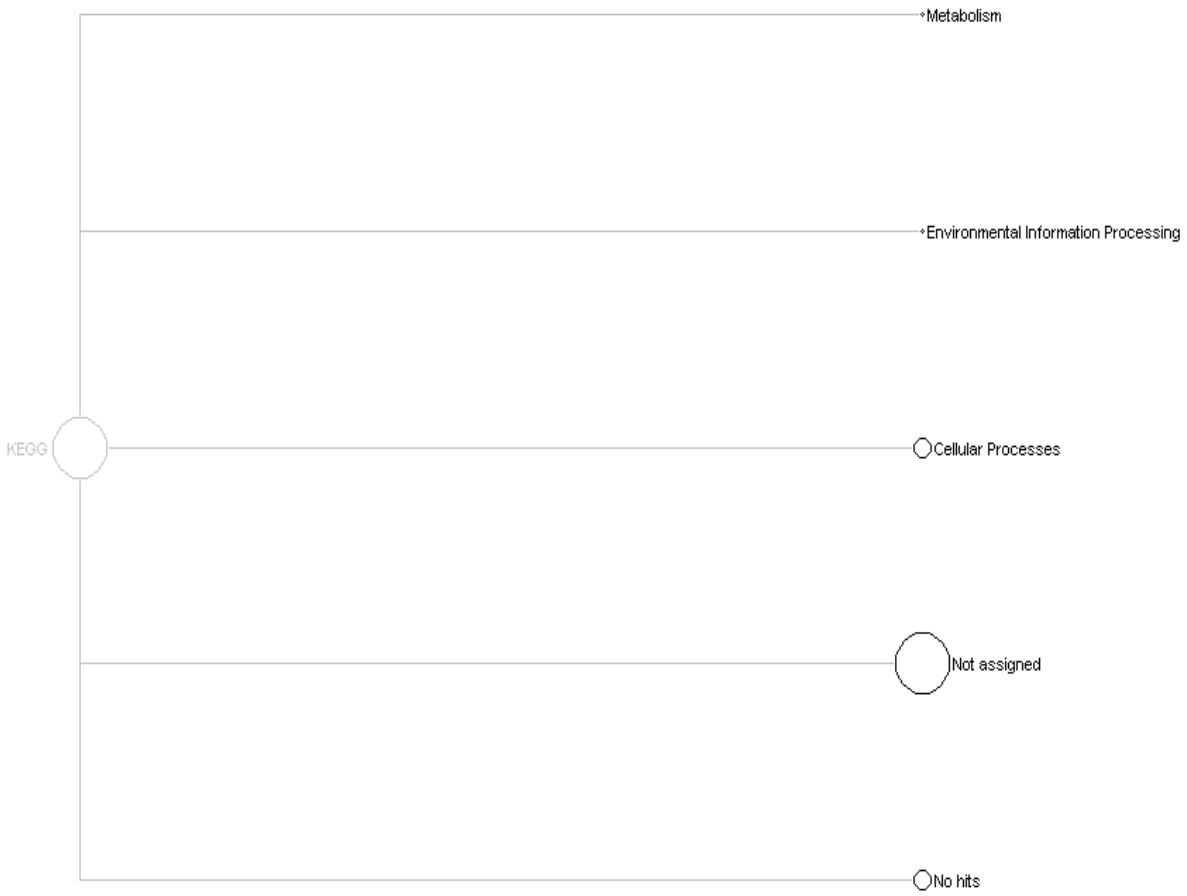


**Figure A.2 KEGG-based analysis of nucleases-like gene reads. Each item represents the general processes assigned by gene reads within the environments.**

Sections A.1, A.2. and A.3 are included electronically in CD. Section A.1 is the dataset of the BLST2GO results of 832 nuclease-like gene reads. Section A.2 showed the biological process involved by nuclease resulting in sporulation. Section A.3 showed the biological process involved by nuclease resulting in metabolism of nitrogen, phosphorous, purine, small molecules such as tRNA and response to stress stimulus.

## APPENDIX B

**Table B.1 ANOVA for the selected quadratic model (Eq. 5.2) based on the CCD data. Df represents degrees of freedom**

| Variables | Sum of squares | df | Mean square | *F*-value | *p*-value (Prob > *F*) |
|---|---|---|---|---|---|
| Model | 72648.8 | 5 | 14529.9 | 41.8 | < 0.0001* |
| Yeast extract (A) | 9833.9 | 1 | 9833.9 | 28.3 | 0.001* |
| Hysoy (*B*) | 6775.8 | 1 | 6775.8 | 19.5 | 0.003* |
| *AB* | 1269.1 | 1 | 1269.1 | 3.65 | 0.098 |
| $A^2$ | 40760.6 | 1 | 46486 | 133.8 | < 0.0001* |
| $B^2$ | 14009.3 | 1 | 14009.3 | 40.3 | < 0.0001* |
| Residual | 2432.3 | 7 | 347.5 | | |
| Lack of Fit | 1528.6 | 3 | 509.5 | 2.3 | 0.224 |
| Pure Error | 903.7 | 4 | 225.9 | | |
| Corrected total | 75081.1 | 12 | | | |

*$p$-values < 0.05 is significant

**Table B.2 ANOVA for the selected quadratic model (Eq. 5.3) of CCD. Df represents degrees of freedom**

| Variables | Sum of squares | df | Mean square | *F*-value | *p*-value (Prob > *F*) |
|---|---|---|---|---|---|
| Model | 45662 | 9 | 50518 | 38.25 | <0.0001* |
| Hysoy (*A*) | 27731 | 1 | 27731 | 21 | 0.001* |
| Glucose (*B*) | 19654 | 1 | 19654 | 14.88 | 0.003* |
| Yeast extract (*C*) | 9256 | 1 | 9256 | 7.01 | 0.024* |
| *AB* | 1800 | 1 | 1800 | 1.36 | 0.095 |
| *BC* | 8450 | 1 | 8450 | 6.4 | 0.27 |
| *AC* | 800 | 1 | 800 | 0.61 | 0.03* |
| $A^2$ | 157933 | 1 | 157933 | 119.59 | <0.0001* |
| $B^2$ | 185150 | 1 | 185150 | 140.2 | <0.0001* |
| $C^2$ | 119532 | 1 | 119532 | 90.51 | <0.0001* |
| Residual | 13206 | 10 | 1321 | | |
| Lack of Fit | 12489 | 5 | 2498 | 17.41 | 0.4 |
| Pure Error | 718 | 5 | 144 | | |
| Corrected total | 467868 | 19 | | | |

*$p$-values < 0.05 is significant