Acknowledgments

This master thesis was only possible due to the contribution of several people, which directly or indirectly helped me on this project and I would like to acknowledge them and thank them for their support.

Firstly, I would like to thank my ISEL supervisor Doctor Maria Celeste Serra, by her knowledge, enthusiasms, wisdom and for having always an open door to help me solve all the problems that have arisen.

To my IST supervisor Doctor Carla de Carvalho, who was tireless throughout this journey and for always showing an extraordinary availability and patience, but also for her wisdom and knowledge. The best supervisor and friend anyone could ask for, that helped me grown personally and professionally.

To Professor Joaquim Sampaio Cabral, for allowing me all the resources needed to complete this thesis, and for the great insights and wisdom given throughout the years. A great friend.

To Doctor Pedro Fernandes I would like to thank for his availability, help, wisdom and friendship.

I would like to thank Doctor Luis Santos for all the availability demonstrated with FTIR, to Doctor Ricardo Santos for the DNA identification analysis, to Flávio Ferreira and José Silvares for their support at some of the analytical data presented in the thesis.

My gratitude goes also to my office companions, Teresa Esteves, Teresa Cesário, Marisa Santos, Nuno Faria, and to Filipe Carvalho, Sofia Duarte, Cláudia Alves and Carlos Rodrigues for all the good dispositions and helpful discussion that helped make things easier. A special thanks goes also to the friends on Tagus Park, Carlos Rodrigues, and Ana Fernandes for all the good dispositions. For Marco Marques, Salomé Magalhães and Nuno Lourenço for the great laughs given during work and lunch breaks that helped in many ways. To all the professors from ISEL and IST that gave me the tools and friendship for being wright at this moment.

A special thanks goes to D. Rosa, for being tireless laboratory companion and friend, and Rosa Maria (my “mom” from Técnico), both made possible the realization of this thesis by helping me in my many task during a day’s work. Thank you for being there.

To my new extended family, for their joyfulness, friendship and good advice, but specially for accepting me as one of them.
To my mother, father and brother for being there for me throughout the good and bad times, for believing always even when I doubt myself, and for the patient task that took. Thank for your kind words and the lessons I needed to ear. Thank you for your love.

And finally to my best friend, companion, girlfriend and wife, Diana, for being the light of my day every day, for the love that she gives me always, for being my best supporter, and my most fierce believer, she never doubted that I could do it. Thank you my love for being in my life.

The study was partially supported by project IF/01203/2013/CP1163/CT0002 awarded by Fundação para a Ciência e a Tecnologia (Portugal) and by project INMARE (no. 634486) awarded by the EU Research and Innovation programme Horizon 2020 of the European Commission.
Index

Abbreviations .............................................................................................................................................. V
List of Figures ................................................................................................................................................ VII
List of tables .................................................................................................................................................. XI

1. Abstract ...................................................................................................................................................... 1
2. Resumo ....................................................................................................................................................... 3
3. Introduction ................................................................................................................................................ 5

  3.1. Biotechnology ......................................................................................................................................... 6
  3.2. Marine Biotechnology .......................................................................................................................... 8
    3.2.1. The bio-economics of marine biotechnology ................................................................................ 9
    3.2.2. Marine based bioprocesses ........................................................................................................... 11
  3.3. Isolation of marine microorganisms .................................................................................................... 14
  3.4. Media used in the cultivation of marine microorganisms ..................................................................... 15
  3.5. Adaptation to the marine environment ................................................................................................ 15
  3.6. Marine bacteria characteristics .......................................................................................................... 17
  3.7. Prodigines and Porphyrins .................................................................................................................. 18

4. Materials and Methods ................................................................................................................................ 23

  4.1. Bacterial identification ........................................................................................................................... 23
  4.2. Biomass and product production .......................................................................................................... 23
    4.2.1. Biomass quantification .................................................................................................................. 23
    4.2.2. Agar plates ...................................................................................................................................... 24
    4.2.3. Microtiter plates with Oxygen and pH monitoring ...................................................................... 24
    4.2.4. 15 mL test tubes .......................................................................................................................... 25
    4.2.5. Shaken flasks .................................................................................................................................. 25
    4.2.6. Solid state reactor ......................................................................................................................... 26
    4.2.7. Bioreactor ....................................................................................................................................... 26
  4.3. Bacterial Adaption .................................................................................................................................. 26
    4.3.1. Zeta potential ................................................................................................................................. 26
    4.3.2. Lipid Composition ........................................................................................................................ 27
  4.4. Antibacterial properties of the product ................................................................................................. 27
  4.5. Scale Up .................................................................................................................................................. 27
    4.5.1. $k_L a$ determination in shake flask ............................................................................................. 27
    4.5.2. From 100 mL to 1 L Shake flask ................................................................................................. 28
4.5.3. From a 100 mL shake flask to a 2 L bioreactor ........................................ 28
4.6. Biomass and product production in 2 L bioreactors ........................................ 28
4.7. Product analysis ........................................................................................................ 29
   4.7.1. Extraction procedure .............................................................................................. 29
   4.7.2. UV/Visible spectroscopy ......................................................................................... 29
   4.7.3. Thin layer chromatography .................................................................................... 30
   4.7.4. Fluorescence Spectroscopy and Microscopy .......................................................... 30
   4.7.5. Fourier transform infrared spectroscopy (FTIR) ..................................................... 30
   4.7.6. $^1$H-NMR ............................................................................................................. 31
   4.7.7. GC-MS ................................................................................................................ 31
5. Results and discussion ..................................................................................................... 33
   5.1. Bacterial identification ............................................................................................... 33
   5.2. Assessing growth conditions ....................................................................................... 35
      5.2.1. Carbon sources .................................................................................................... 35
      5.2.2. Nitrogen sources ................................................................................................ 40
      5.2.3. Effect of salinity .................................................................................................. 42
      5.2.4. Effect of growth temperature .............................................................................. 43
      5.2.5. Absence of light exposure .................................................................................. 46
      5.2.6. Nutrient depletion ............................................................................................... 49
      5.2.7. Effect of pH ......................................................................................................... 51
      5.2.8. Oxygen availability ............................................................................................. 55
      5.2.9. Metal ions ............................................................................................................ 58
      5.2.10. Growth in biofilms ............................................................................................ 60
         5.2.10.1. Net surface charge ...................................................................................... 60
      5.2.11. Cell adhesion to surfaces ................................................................................ 62
   5.3. Scale Up .................................................................................................................... 64
      5.3.1. Maintenance of reactor geometry ....................................................................... 64
      5.3.2. Maintenance of $k_L a$ ....................................................................................... 65
         5.3.2.1. Mathematical models for $k_L a$ determination .............................................. 66
         5.3.2.2. Dynamic method ......................................................................................... 68
   5.4. Improving fermentation conditions ........................................................................... 69
      5.4.1. Agitation .............................................................................................................. 69
      5.4.2. Media ................................................................................................................ 72
5.4.3. Fermenter geometry ................................................................. 73
5.4.4. Solid State Fermentation (SSF) .............................................. 76
5.5. Product characterisation and identification ........................................ 77
  5.5.1. Product extraction and purification ...................................... 77
5.5.2. Fluorescence spectroscopy .................................................... 80
5.5.2.1. FTIR .................................................................................. 82
5.5.2.2. GC-MS ............................................................................ 83
5.5.2.3. H1-NMR ........................................................................... 84
5.5.3. Product activity ................................................................. 85
5.5.4. Product stability ............................................................... 85
6. Conclusion ................................................................................. 87
7. Future Work ............................................................................... 89
8. Bibliography ............................................................................... 90
Annex 1 .........................................................................................
Annex 2 .........................................................................................
**Abbreviations**

BA – Blood Agar  
CIESM - Mediterranean Science Commission  
CL - Cardiolipin  
CP – Casein Peptone  
CycloBFA – Cyclopropyl Branched Fatty Acids  
FAs – Fatty Acids  
FAMEs – Fatty Acids Methyl Esters  
FDA – Food and Drugs Administration  
FTIR - Fourier transform infrared spectroscopy  
GC-MS – Gas Chromatography – Mass Spectrometry  
Gly – Glycerol  
HSFA – Hydroxisubstituted Fatty Acids  
\( K_d \) – cellular death, (h\(^{-1}\))  
\( k_{La} \) – Oxygen transfer Coefficient  
LB – Luria-Bertani Broth  
MA – Marine Agar  
MB – Marine Broth  
MBFA – Methyl Branched Fatty Acids  
MH – Mueller Hinton Broth  
MM – Mineral medium  
MiM – Minimal medium  
MP – Meat Peptone  
MTBE – Methyl Tert-Butyl Ether  
MTP – Microtiter Plates  
MUFA – Mono Unsaturated Fatty Acids  
\( \mu_{max} \) – Maximum cellular growth, (h\(^{-1}\))  
NMR – Nuclear Magnetic Resonance
NO$_2^-$ - Nitrites

OD – Optical Density

OECD - Organization for Economic Cooperation and Development

PE - Phosphatidylethanolamine

PG - Phosphatidylglycerol

PS - Polystyrene

PUFA – Polyunsaturated Fatty Acids

rRNA – Ribosomal ribonucleic acid

RNA – Ribonucleic Acid

SBFA – Saturated Branched Fatty Acids

SSFA – Saturated Sheath Fatty Acids

SP – Soy Peptone

TB – Terrific Broth

TMAO - Trimethylamine N-oxide

Tryp - Tryptone

TSA – Triptic Soy agar

TSB – Tryptic Soy Broth

UV-VIS – Ultraviolet - Visible

X$_0$ - Biomass at t=0, (g/L)

X - Biomass at t=n, (g/L)

YE – Yeast Extract

Y$_{PX}$ – Product per biomass Yield

Vvm – Volume flow of air per volume of liquid in minutes

ZP – Zeta Potential
List of Figures

**Figure 1** – Biotechnology evolution. Adapted from [16]................................................................. 8

**Figure 2** – Typical development chain in marine biotechnology. Adapted from [14]...................... 12

**Figure 3** – Bacterial strain used in the study. Magnification: 1500x. Horizontal field width = 0.8 mm; vertical field width = 0.6 mm. ................................................................. 18

**Figure 4** – Possible prodiginine sequence of products, a) pyrrole, b) prodigiosin, c) porphyrin. Adapted from [74,103].................................................................................. 21

**Figure 5** – Cell Identification in a) TSA and b) BA, using the Sherlock MIS Software............. 33

**Figure 6** – FASTA sequence of the strain of interest................................................................. 34

**Figure 7** – Phylogenetic tree obtained by comparing fatty acid profiles. a) Method ITSA1. b) Method IBA1. ............................................................................................................. 35

**Figure 8** – Phylogenetic tree of the strain being study based on its FASTA sequence and interpretation by Moleblast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Lcl|Query_10001 is the strain of the study. ............................................................................................................. 35

**Figure 9** – Biomass production in salted MM and in MB supplemented with different carbon sources. Control represents the media without any supplementation from a carbon source.36

**Figure 10** - Analysis of product produced in salted MM and in MB with different carbon sources. Right – Product produce in MM; left – product produced in MB. ......................... 37

**Figure 11** – The degree of saturation of the cells membrane grown on different carbon sources. a) MM; b) MB................................................................................................................. 38

**Figure 12** - Analysis of a) biomass growth and b) product production with different lipids used as carbon sources after a 24 h growth. ......................................................................... 38

**Figure 13** – Lipid profile of the membrane of cells grown on different lipid sources in the presence and absence of salt................................................................. 39

**Figure 14** – Product-to-biomass yield when the cells grew on media prepared in-house and in commercially available media.................................................................................. 40

**Figure 15** – Influence of different nitrogen sources on a) biomass and product production in MB. b) $Y_{px}$ obtained in each sample. Control (MB medium not supplemented), NO$_2$ (nitrites), YE (yeast extract) MP (meat peptone), SP (soy peptone); CP (casein peptone)........... 41

**Figure 16** – Fatty acids profile of the cellular membrane of the strain influenced by the nitrogen source used. Control is the MB medium without supplementation. ................... 41

**Figure 17** – The importance of salt in the growth of N-RED. Results obtained after 24 h of growth................................................................................................................. 42

**Figure 18** – MA plates incubated at different temperatures .......................................................... 44

**Figure 19** – Strain growth curves at different temperatures in MB medium ....................... 44

**Figure 20** – Biomass and product concentrations in MB after 24h of cultivation. ................. 45

**Figure 21** – Influence of temperature in cellular membrane: a) FAMES profile; b) degree of saturation of the fatty acids of the cellular membrane........................... 45

**Figure 22** – Effect of light and temperature on growth and product production. ................. 47

**Figure 23** – $Y_{px}$ for cells growing on MB (left side) and MB supplemented with MP (right side)............................................................................................................. 48

**Figure 24** – Fatty acid profile of cells grown under different light and temperature conditions. ......................................................................................................................... 48

**Figure 25** – Lipid composition of the cells after 48 h at the conditions stated in Figure 23... 49
Figure 26 – Oxygen concentration consumed by N-RED diluted up to 40 times during 30 h of growth................................................................. 50
Figure 27 – Maximum growth achieved at different nutrient concentration............ 50
Figure 28 – Biomass and product concentrations in diluted MB + MP media............. 51
Figure 29 – Dissolved oxygen concentration and pH value during cultivation of strain N-Red monitored by the PreSense System. a) and b) MB+MP medium; c) and d) TB medium.............. 52
Figure 30 – Effect of pH on maximum oxygen consumption rate. a) MB+MP and b) TB ....... 53
Figure 31 – Biomass and product concentrations obtained after 24 h by N-RED in the PreSense System. a) and c) Biomass and product produced in MB+MP medium; b) and d) biomass and product produced in TB medium. ................................................................. 54
Figure 32 - Effect of pH on the lipid profile of strain N-Red. a) MB+MP and b) TB ........ 55
Figure 33 – Shaken flask used to assess simultaneous aerobic and anaerobic conditions at 30 °C and 150 rpm in MB medium. ........................................................................ 56
Figure 34 – Biomass and product concentration during growth, in MB medium. Available volume of air: a) 80%, b) 60%, c) 40%. ................................................................. 57
Figure 35 – Product per biomass yield after 24h and 48h of growth in MB medium with an different metal ion. ........................................................................ 58
Figure 36 – Lipid profile of cells grown in MB medium supplemented with metal ions, after a) 24h and b) 48h ................................................................ 59
Figure 37 – Influence of different concentrations of Fe (III) on Ypx in MB medium........ 60
Figure 38 – Zeta potential of the cells grown at different temperatures....................... 61
Figure 39 – Adhesion of N-RED cells to: a) glass slids, b) glass beads fully submerged, c) sea sand in polystyrene (PS) T-flask. ........................................................................ 62
Figure 40 – FAMES profile of the cells in the three different adhesion bioreactors........ 63
Figure 41 – Biomass and product concentration in the supematant in the different bioreactors after 24 h. ................................................................. 63
Figure 42 - Biomass and product curves followed for 48 h, in MB medium in different size shake flasks with different working volumes.................................................. 64
Figure 43 – Variation of k_{La} with agitation, in the Electrolab and Infors bioreactors using a) double distilled water and b) 30 g/L NaCl solution. ................................................................. 68
Figure 44 - Influence of different stirring speeds on a) biomass and b) prodiginine production. ........................................................................ 69
Figure 45 - Biomass and product concentrations in a two stage fermentation. .......... 70
Figure 46 – Fatty acids profile of a 26 h fermentation in a bioreactor......................... 71
Figure 47 – Influence of medium composition on a) biomass and b) product production... 72
Figure 48 - Influence of different medium composition and fermenter geometry on a) biomass and b) product formation................................................................. 74
Figure 49 – Production difference between N-RED cells grown in a) Electrolab and b) Infors bioreactors, in cultivation media supplemented with Fe (III), may be assessed but the red colour which is characteristic of prodiginines. .................................................. 75
Figure 50 – Biomass and product concentration on T-flask with different volumes of MB+ MP medium, after 10 days with rocker agitation. a) and b) show the T-flask with 35 and 50 mL medium at the beginning of the study and c) the red sand resulting from the adhesion of product............................................................................... 76
Figure 51 – Product per biomass yielded in the T-flasks, after 10 days of incubation at room temperature. 77
Figure 52 – UV-Vis Spectrum of the red compound extracted with different organic and inorganic solvents. 78
Figure 53 – UV-VIS spectrum of the purified product present in the band observed in the TLC plate. 79
Figure 54 – 2D TLC of the compound produced by N-RED. Photographs taken under visible (grey hue) and UV light (green hue). 79
Figure 55 – Spectra of the compound produced by N-RED at different pH values. 80
Figure 56 – Crystal of the red product observed under visible (left) and fluorescent (right) light. Magnification: 1000x. 81
Figure 57 – Fluorescence excitation spectra runs at 400 nm of the red compound dissolved in ethyl acetate and methanol. 81
Figure 58 – FTIR analysis of ethyl acetate and of the compound purified by Hubbrad’s (orange product) and Cox’s (pink product) methods. 82
Figure 59 – GC-MS spectrum of the prodiginines precursors and their structure. 84
Figure 60 – Loss of product over time when conserved in a refrigerated environment. 86
List of tables

Table 1 – Constant parameters used for the application of the Klöckner-Büchs model at the operation temperatures. ........................................................................................................................................ 28

Table 2 – Composition of the medium used in each well of the 24 well plate shown on Figure 17. K→Na: potassium salts substituted by sodium salts................................................................................ 43

Table 3 – Growth parameters determined for each headspace tested............................................ 58

Table 4 - Growth parameters and oxygen mass transfer coefficient determined for each shaken flask. ........................................................................................................................................ 65

Table 5 - $k_La$ determination, according to Equation 3, for the growth of strain N-RED in shaken flasks. ........................................................................................................................................ 66

Table 6 – Calculation of Ni needed to maintain the same $k_La$ values as in shaken flask, by Doran’s empirical model for $P_g$. ........................................................................................................................................ 67

Table 7 – Calculation of $k_La$ by Michel and Miller's empirical model for $P_g$. .............................. 67

Table 8 – Growth parameters and the best productivities achieved for biomass and product at different agitations speeds........................................................................................................ 70

Table 9 – Growth parameters and the best productivities achieved for biomass and product at different agitations speeds........................................................................................................ 71

Table 10 – Growth parameters and best productivities achieved for biomass and product with different medium in 2 L fermenters........................................................................................................ 73

Table 11 – Bioreactor geometry influence on growth parameters and productivities ............ 75
1. Abstract

Marine biodiscovery is a promising field for novel compounds and materials. For the application of blue biotechnology, the development of new strategies is paramount to shorten the pipeline between enzyme and metabolite discovery and application, i.e. to increase of efficiency of the bioprocess to obtain industrial relevant yields. By understanding the mechanisms and stress conditions in the marine environment that favoured the production of a given compound by marine bacteria, high production levels of commercially interesting compounds should be achieved. The goal of this thesis was to develop a bioprocess, at laboratory scale, using marine bacteria for the production of prodiginine compound(s), which have applications in different fields including the pharmaceutical and food industries. The selected strain and product(s) were characterized using, respectively, i) 16S rRNA and lipid profile, and ii) UV-VIS, NMR, FTIR, and GC-MS techniques. Through an intense screening process in shaken flasks, using culturomics and lipidomics approaches, the growth conditions leading to the highest yields were assessed. Several parameters were evaluated, including e.g. nature of the carbon and nitrogen sources, presence of metals ions, light exposure, temperature, pH, agitation, and oxygen concentration. Additionally, the effect of specific stresses such as nutrient depletion and the growth of the cells in biofilms were also evaluated. Once the best medium composition and cultivation conditions were determined, the bioprocess was scaled up to 2 L bioreactors for further improvement of the bioprocess. The maintenance of the oxygen transfer coefficient ($k_La$) was used as scale-up criterion, and Büch’s, Doran’s, Michels and Miller’s Equations were firstly employed for the determination of $k_La$. The effect of stirring conditions, medium composition and $k_La$ on biomass and prodiginine production was further assessed at this scale. It was found that supplementation of marine broth (MB) with appropriate nitrogen sources and metal ions increased to 200 mg/(L.h) both biomass and prodiginine productivities.

Keywords: Bioprocess, Bioprospecting, Fermentation, Marine bacteria, Prodiginine compounds, Scale-up, Screening
2. Resumo

A descoberta de novos compostos e materiais de origem marinha é um promissor campo de investigação. Para que possa haver a aplicação da biotecnologia azul a nível industrial torna-se fundamental o desenvolvimento de novas estratégias para diminuir o tempo entre a descoberta e a aplicação destes novos compostos, e para aumentar a eficiência do processo para rendimentos suficientemente altos para aplicação industrial. A compreensão dos mecanismos, e das condições de stress a que as bactérias marinhas estão sujeitas e que as levam a produzir um determinado composto, permite que este composto possa ser produzido em larga escala. O objectivo desta tese foi o desenvolvimento de um bioprocesso à escala laboratorial usando bactérias marinhas para a produção de prodigininas. Estes produtos têm várias aplicações em diferentes sectores industriais, tais como a indústria farmacêutica e a alimentar. A estirpe selecionada e o produto por ela produzido foram caracterizados por i) 16S rRNA e pelo seu perfil lipídico, e por ii) UV-VIS, NMR, FTIR, e GC-MS, respectivamente. Através de um intenso processo de análise em balão Erlenmeyer, e usando técnicas de culturomica e lipidomica, as condições de crescimento que levam a elevados rendimentos de biomassa e de produto foram determinados. Os parâmetros avaliados foram vários, e incluíram o tipo de fontes de carbono e azoto, a presença de iões metálicos, presença de luz, temperatura, pH, agitação e concentração de oxigénio no meio. Condições específicas de stress, como baixas concentrações de nutrientes, e o crescimento celular em biofilme foram avaliadas. Após a selecção da composição do meio e das melhores condições de crescimento, realizou-se o aumento de escala do bioprocess para fermentadores de 2 L. O coeficiente de transferência de oxigénio ($k_La$) foi escolhido como critério de aumento de escala, e os modelos de Büch’s, Doran’s, Michels e Miller foram aplicados na sua determinação. O efeito da agitação, composição do meio e do $k_La$ na concentração de biomassa e produto foi estudado a esta escala. Determinou-se que através do enriquecimento de meio marinho (MB) com fontes de azoto e de iões metálicos apropriados, a produtividade em biomassa e prodigininas aumentou para aproximadamente 200 mg/(L.h).

Palavras chave: Aumento de escala, Bactérias marinhas, Bioprocesso, Bioprospecção, Fermentação, Prodigininas, Selecção
Note: The remaining sections of the Thesis will not be made public during the Confidentiality Period.