Optimization of antifungal production by *Bacillus* species in the presence of nanoparticle supplementation and preliminary scale-up

Submitted in partial fulfilment of the academic requirements of

Master of Science

by

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in

Microbiology

School of Life Sciences

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Pietermaritzburg

South Africa

July 2023

PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa.

The contents of this work have not been submitted in any form to another University and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION 1: PLAGIARISM

I, Sikhulile Nosimo Nzimande, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work.

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written, but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, to be published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the references sections.

Signed: Sikhulile Nosimo Nzimande

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DECLARATION 2: MANUSCRIPTS

This dissertation consists of two manuscripts. The first author (Sikhulile N. Nzimande) contributed towards the experimental work, data collection and manuscript and was guided by the second, third and fourth author/supervisor (I. Sanusi, E.B. Gueguim Kana & Santosh Ramchuran).

1. Sikhulile N. Nzimande, Isaac A. Sanusi, Santosh Ramchuran, Gueguim E. B. Kana. Process development for antifungal production by *Bacillus subtilis* BS20: Optimization and nanoparticle supplementation (Chapter 3).
2. Sikhulile N. Nzimande, Isaac A. Sanusi, Santosh Ramchuran, Gueguim E. B. Kana. Preliminary scale up studies of antifungal production by *Bacillus subtilis* BS20 in a stirred stainless bioreactor (Chapter 4).

Signed: Sikhulile Nosimo Nzimande

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CONFERENCE CONTRIBUTIONS

1. Nzimande, S N., Ramchuran S, Sanusi I. Gueguim Kana, E.B. Optimization of biosurfactant production from *Bacillus* sp. BS20 and assessment for antifungal activity, Flash presentation, 10 – 11 December 2020, College of Agriculture, Engineering & Science Postgraduate Research & Innovation Symposium, University of KwaZulu-Natal, South Africa.

ABSTRACT

The intensive agricultural practices used to meet global crop production demands have resulted in the rigorous use of chemical pesticides. These ultimately compromise crop production as well as the environment. In order to alleviate this, cheaper and environmentally friendly, biocontrol agents have been considered as an alternative to chemical pesticides. Biosurfactants are a promising alternative to chemical pesticides due to their higher biodegradability, lower toxicity, and environmental friendliness. Amongst the many bacterial and fungal biosurfactant producers, biosurfactants from *Bacillus* species show promise as biocontrol agents. These biosurfactants are known for their wide biotechnological use in agricultural, industrial, and medicinal fields. However, large scale production is still faced with challenges such as low yields and high production cost thus raising the need for modelling, optimization, catalytic and scale up investigations. Hence, a study was undertaken with the aim of enhancing biosurfactant production through process modelling and optimization with subsequent assessment of the scale up potential of the optimized process. A Response Surface Methodology (RSM) using box Behnken design was used to investigate the optimal process conditions for improved biosurfactant production from *B. subtilis* BS20. The investigated process parameters included glucose concentration (10 – 30 g/L), incubation temperature (25 – 45°C) and incubation time (24 – 96 h). The developed model gave a high coefficient of determination (R^2) = 0.86, p-value of 0.0279 and F-value of 4.62 for the modelled biosurfactant production. Optimized process conditions of 11.5 g/L glucose concentration, 24 h incubation time and 41°C for incubation temperature were obtained and produced a maximal antifungal activity of 68 mm. Moreover, supplementary inclusion of seven (7) different nanoparticles as a biocatalyst in the cultivation of *B. subtilis* BS20 was carried out using the optimal process condition to further improve antifungal (biosurfactant) production. The inclusion of nanoparticles favored increased biomass yield, but biosurfactant with high antifungal activity was not obtained.

Moreover, when it comes to commercializing new bioprocess and bioproduct developments, bio – process scale-up in the biotechnology industry is an essential stage.

This study therefore evaluated the scale up of biosurfactant production based on constant power consumption, Reynold number and impeller tip speed. The stirrer speed (n), impeller diameter (di), number of impellers (N), power number (N_p), broth density (ρ), working volume and geometric factor (f_c) were correlated with impeller tip speed (V_{tip}), Reynolds number (Re) and power consumption rate (P/V) to obtain the most suitable criterion for biosurfactant production in a 10 L bioreactor. Implementing constant V_{tip} value from the 1 L scale: 93 rpm, Reynold number (Re) 5.9E - 04, Power (P) 0.32 W, Power to Volume ratio (P/V_L) 160 W/m^3, circulation

time (t_c) 5.2 s and shear stress (γ) 15.5 S^{-1}, at 41 °C, gave the highest antifungal activity of 65 mm zone of inhibition in the 10 L scale bioreactor. The antifungal activity obtained for constant V_{tip} were comparable to those obtained at 1L bioreactors (57 mm), this showed that the bioprocess dynamics for achieving high antifungal activity are available, further paving the way for feasible commercialization strategies.

This study has elucidated the optimum process conditions for *B. subtilis* BS20 metabolism for improved biosurfactant production resulting in significant antifungal activity. Furthermore, findings showed that the inclusion of nanoparticles biocatalyst to the process enhanced biomass yields. Process scale up provided preliminary data for large scale production of biosurfactant production from *B. subtilis* BS20

ACKNOWLEDGMENTS

First and foremost, I would like to acknowledge and be thankful to God and my ancestors for allowing me to enter such a space in my life. Akwandele bani loku. Thank you.

To my supervisors:
Professor E. B. Gueguim Kana, thank you for allowing me into your circle of students, thank you for always offering sound judgement and advice.

To Dr Santosh, thank you for your generosity with supplying me with the *Bacillus* sp. BS20 and funding for my study.

Dr Sanusi, you have been a great friend, mentor, and supervisor, I cannot thank you enough. In IsiZulu we say, Izandla zedlula ekhanda.

To lab 117 family (special shout out to Daneal), you have made bad days bearable, thanking you for always cracking jokes and being amazing people.

To my family, thank you for the sacrifice and compromises you had to make on my behalf, none of it is taken lightly.

To my friends, thank you for listening to me vent, complain and cry just releasing my frustrations on you.

Last but not the least, thank you to me. You did it!

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CHAPTER 1

1. General Introduction

The cost and environmental concerns of using traditional chemical fertilizers and agricultural pesticides are problems that affect crop productivity, food availability and accessibility (Tredgold, 2021). Hence environmentally friendly practices are crucial for the achievement of sustainable development in large scale crop production. In addition, there is global increase in food demand due to overall growth in world population. Currently, the world's population has increased to over 7.5 billion, and the United Nations estimates that the world population will increase to ~10 billion people by the year 2050 (United Nations, 2022). This has made the development of an efficient and sustainable control of food loss an imperative approach, as well as mitigating the negative impact of chemical fertilizers and pesticides (Barbir and Veziroglu, 1990; Faloye, 2015).

The global issue of microbial food spoilage has resulted in major effects such as food waste, customer dissatisfaction and foodborne illnesses (Alegbeleye., 2022). Approximately, 600 million cases of foodborne illness and 40 000 deaths occur annually across the globe (FAO, 2022). Unsafe food is a threat to human health and sufficient supply of nutritious and safe food is essential to meet nutritional requirement for healthy living. Unfortunately, chemical pesticides pose a danger to the quality and quantity of nutritious food, as well as safe food supply. Chemical pesticides are toxic, non-biodegradable and the chemical residue left on the crop causes human health issues (Borriss, 2011). Although, synthetic chemical pesticides are efficient in food pathogen, their use is not sustainable due to environmental, and health concerns (Mardanova *et al*., 2017). The annual FAO report (2021) reported that the global application of pesticides went up 36%. Thus, to achieve sustainable environmental health and alleviate the threat to food security the development of alternative methods in food pathogen control is imperative.

Utilizing microbial species such as *Bacillus* spp. as biofertilizers and biopesticides is one alternative crop management strategy that modern agriculture practices are progressively promoting (Comapant *et al*., 2005; Tredgold, 2021). The family *Bacillaceae* and the genus *Bacillus* in particular are aerobic endospore-forming bacteria that play significant roles in plant health, disease antagonism and pathogen control (Borriss. 2011). Members of the *Bacillus* genus have been the focus of several ecological and biocontrol research. Several species of this genus are frequently found in soil and in close proximity to plants (Borriss, 2011). Numerous commercial *Bacillus*-based solutions for the control of crop pathogens have been developed as an alternative to expensive agrichemicals, which are also, not environmentally friendly (Cawoy *et al*., 2011; Tredgold, 2021). The use of *Bacillus*-based pesticide is desirable due to their prevalence and competitiveness in plant-associated habitat, favorable plant-health-promotion activities, and simplicity of formulation (Govindasamy *et al*., 2010; Borriss, 2011; Cawoy *et al*., 2011). In addition to increasing plant development and productivity in the face of illnesses, bio-fertilizers and bio-pesticides can be utilized as substitutes for chemical fertilizers and pesticides (Choudhary, 2011).

Bacillus spp. can be recovered from all niches in the environment due to their ubiquitous nature (Radhakrishnan *et al*., 2017). These species have also been used to prepare medicinal, industrial and agricultural products (Lyngwi and Joshi, 2014). The application of *Bacillus*-based fertilizers to soil can also enhance plant-available forms of nutrients in rhizospheres, control disease-causing pathogenic microbial growth and induce pest defense systems (Garcia-Fraile *et al*., 2015; Kang *et al*., 2015). Similarly, several compounds secreted by *Bacillus* species stimulate plant development and protect against disease invasion (Radhakrishnan *et al*., 2017). As a result, *Bacillus* spp. have gained considerable biotechnological and medicinal interest as biosurfactants and antibiotics. Moreover, the plant-beneficial *Bacillus* spp. associated with roots or rhizospheres usually develop biofilms to increase plant growth (Beauregard *et al*.,

2013). Almost 4-5% of the genome of *Bacillus* species is devoted to the synthesis of structurally varied antimicrobial compounds that have shown variable antagonism for bacterial and fungal phytopathogens (Kespar *et al*., 2019). Cyclic-lipopeptides (CLPs), which make up iturins, fengycins, and surfactins and are crucial for *Bacillus* spp. to colonize plant roots, are the most significant of these antimicrobials (Kespar *et al*., 2019; Shahid *et al*., 2021). By combating plant diseases and fostering plant development, they have become popular as biological control agents in agriculture. *Bacillus*-based biocontrol agents can be applied directly to soil to improve the plant-available forms of nutrients in rhizospheres, control disease-causing pathogenic bacteria growth, and stimulate pest defense mechanisms in plants (Radhakrishnan *et al*., 2017; Shahid *et al*., 2021). Many research studies have investigated the biofertilizer and biocontrol potential of *Bacillus* spp., however, there exists certain challenges with lipopeptide production, such as choice of species, low yield, and high production cost. Moreover, there is scarcity of information on bio-fertilizers and bio-pesticides formulation and optimization strategies, large scale production and marketability of the products.

Several modelling and optimization algorithms have been used for bioprocess development (Nikzad *et al*., 2015). These include the one variable at a time (OVAT), factorial design of Experiment (DOE), response surface methodology (RSM), genetic algorithm and artificial neural networks (ANN) (Venkata-Mohan *et al*., 2009; Anwar *et al*., 2012; Nikzad *et al*., 2015). Conventionally, the one variable at a time (OVAT) can be used to examine and/or develop a process/product (Czitrom, 1999; Wahid and Nadir, 2013). However, OVAT has the potential to be ineffective, unreliable, and produce erroneous optimal conditions (Wahid and Nadir, 2013). Furthermore, the analysis of a large number of samples used in OVAT experiment is laborious and time-consuming. It does not take into account the interactions between process variables, the sensitivity of a variable, or the influence of process variables on the quality of the final product (Wahid and Nadir, 2013). On the other hand, RSM as a modelling tool has

been employed to improve several fermentation processes (Anwar *et al*., 2012; Nikzad *et al*., 2015; Sanusi *et al*., 2020). RSM allows for the identification of many factors and their interactive effects on the process yield and has been reported in the optimization of various bioprocesses (Rorke and Kana, 2016; Sanusi *et al*., 2020). Hence, to develop and validate an intelligent model based on response surface methodology in biosurfactant production under different bioprocessing conditions is necessary such as choice strain, pH, substrate concentration, temperature, and media type.

Furthermore, kinetic studies on biosurfactant production should be assessed to improve the product yield, quality, and productivity. Presently, there is a dearth of information regarding kinetic assessment of biosurfactant and biopesticides production from *Bacillus* spp. Kinetic modelling enables assessment of the biochemical characteristics of a biological process (Phukoetphim *et al*., 2017). One of such models, the logistic models are used to study the cell growth and provide behavioral data of microorganism in response to the process (Manikandan *et al*., 2008; Phukoetphim *et al*., 2017; Moodley and Kana, 2017). The application of these models offers a solid framework for process design, control, and optimization, which obviously lessens the difficulties encountered during process scale up (Linville *et al.,* 2013; Rorke and Kana, 2017).

Process scale-up study is usually required for processes that has been optimized and has the potential for commercialization (Bonvillani *et al*., 2006). Understanding the dynamics of a bioprocess during scale up is crucial when laboratory scale production is to be translated to efficient industrial production scale (Faloye *et al*.,2014). The variables used in process scale up are typically connected to the bioreactor's geometry, mass transfer, mixing activity, power consumption, bulk rheology, cell viability, micro-conditions in the bioreactor and product yield. Consequently, the establishment of appropriate process parameters that directly relate to improved productivity and scaling up capability is crucial. The basic problem of bioprocess

scale up is its adverse impact on the cell kinetic mechanism resulting from heterogeneous condition in the large-scale bioreactor (Xia *et al*., 2015). Large scale bioreactors constantly face different challenges such as mixing problem, heterogeneous environment, contamination, and variability (Xia *et al*., 2015; Qazizada, 2016). Mass and heat transfer can be adversely affected leading to local substrate-nutrient concentration and unfavorable temperature-gradients in the bioreactor (Deniz *et al*., 2015; Qazizada, 2016). Similarly, the cell immediate microenvironment and the cell physiology might be influenced, resulting in critical metabolic alterations. Hence, knowledge on the relationship between the fluid movement, the impeller speed, and the power consumption will be required to achieve effective mixing regime from suitable combination of parameters toward substrate-nutrient concentration and temperature-gradient homogeneity. Thus, experimental investigation on process scaling up is necessary to provide more insights on these issues.

1.1 Research motivation

The industrialization of the agriculture industry has made the natural ecosystem more vulnerable to chemical pollution (Nicolopoulou-Stamati *et al*.,2016). The extensive use of agricultural chemicals and pesticides to protect plants from pest, weed or diseases have been associated with foodborne illness and soil toxicity (Aktar *et al*., 2009; Nicolopoulou-Stamati *et al*.,2016; Yadev *et al*.,2020). The development and implementation of sustainable and ecological approach has become imperative to alleviate the risk that chemical pesticides pose to humans and the environment. Eco-friendly alternatives such as biological control agents have gained considerable attention; the implementation of beneficial microorganisms such as *Bacillus* spp. in this regard is considered one of the most promising methods for safe crop management practices (Radhakrishnan *et al*., 2017).

Bacillus spp. are considered microbial factories due to their broad array production of biologically active compounds that are inhibitory to phytopathogen growth (Ongena and

Jacques, 2008; Kespar *et al*., 2019). Among these biologically active compounds are antimicrobial cyclic lipopeptides biosurfactants of the surfactin, fengycin and Iturin family (Ongena and Jacques, 2008; Dimkić *et al*., 2017). These lipopeptide family are structurally different and exude different qualities, however, they are known to work in a synergistic manner (Wang *et al*., 2015; Ongena and Jacques, 2008). Biologically produced antimicrobial compounds offer great advantage over chemical pesticides such as less toxicity, biodegradability; have the ability to slow down pest resistance and align with the environmental and health regulations (Malviya *et al*., 2020). Despite the intensive research on *Bacillus*-based biocontrol processing, its commercialization is still faced with major challenges of high production costs and low yields, thus the need to come up with efficient and economically feasible strategies. Though, numerous studies have focused on the production of biosurfactant and biopesticide via strain selection using various screening approach (Sun *et al*., 2009; Jung *et al*., 2012), however, there is a dearth of studies on the optimization and scaling up of biosurfactant production.

Generally, biosurfactant yields are affected by process conditions such as culture media, nutrient supplementation, pH and temperature (Ebadipour *et al*., 2016). Only a few studies have reported on the impact of operating conditions on the dynamic behavior of biosurfactant production (Ghribi and Ellouze-Chaabouni, 2011; Mouafi *et al*., 2016; Heryani and Putra, 2017). Little is known on the interactive effect of these process conditions on biosurfactant production. Hence, biosurfactant lipopeptides production optimization would be essential to optimize these operational conditions such that high productivity could be achieved.

Therefore, to alleviate concerns regarding low yield biosurfactant lipopeptides production, further process optimization is required for more efficient biosurfactant lipopeptides production strategies. This could be realized by response surface methodology to capture the complex interactions which link the process conditions to biosurfactant lipopeptides production

as well as explore the supplementation of nano biocatalyst for enhance metabolic rates that could lead to greater antifungal activity production. Furthermore, using kinetic models such as logistic function models will help to further understand the process and increase the quality of the biosurfactant lipopeptides production. These findings could therefore contribute to industrial scale biosurfactant lipopeptides productions from *Bacillus* spp.

1.2 Aims and objectives:

This study aimed to assess the impact of process parameters on *Bacillus* sp. metabolism for improved biosurfactant lipopeptides production. The impact of nanoparticle inclusion was assessed for enhanced Bacillus spp. growth and lipopeptide biosurfactant production and preliminary scale up studies were undertaken to assess the feasibility of large-scale production, to achieve this aim, the following specific objectives were carried out:

1. Modelling and optimization of biosurfactant production using *Bacillus subtilis* BS20 on process parameters of substrate concentration (g/L), temperature (ºC), and incubation time(h).
2. The potential of nanoparticle supplementation (Fe_2O_3, Fe_3O_4, CoO, CuO ZnO, NiO and MnO_2) to enhance the optimized process.
3. Preliminary scale up studies for biosurfactant production at a semi-pilot scale using scale criterion of impeller tip speed (V_{tip}), Reynolds number (Re) and power consumption rate (P/V).

1.3 Thesis outline:

This thesis comprises an introductory chapter, literature review chapter and two experimental chapters presented in research paper format. Each experimental chapter is independent, containing an introduction, materials and methods, results and discussion, conclusion, and references:

- Chapter two presents a literature review that explores the plant phytopathogen infection modes, the use of *Bacillus* spp. as a biocontrol agent, the synthesis and production of biologically active compounds as well as various factor to consider when developing a bioprocess for the commercialization of such biocontrol agents.
- Chapter three focuses on the modelling and optimization of key operational parameters for biosurfactant production using response surface methodology. Moreover, the impact of these parameters as well as the potential of nanoparticle inclusion for improved biosurfactant production were discussed.
- Chapter four presents the findings of a preliminary scale up of biosurfactant production undertaken. The geometrical, rheological and hydrodynamic parameters of the bioreactors were used to evaluate the viability of the scale up process based on constant power consumption, constant impeller tip speed and constant Reynolds number. In addition, kinetic modelling of biosurfactant production using *B. subtilis* BS20 was undertaken to determine the dynamics and thus, predict *Bacillus* BS20 behavior based on factors such as the specific growth rate.
- The last chapter, Chapter 5, integrates the significant findings of the research and highlights major conclusions obtained from this research. Also, provided are recommendations for future studies.

References

Aktar, W., Sengupta, D. and Chowdhury, A., 2009. Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology*, *2*(1), pp.1-12.

Alegbeleye, O., Odeyemi, O.A., Strateva, M. and Stratev, D., 2022. Microbial spoilage of vegetables, fruits and cereals. *Applied Food Research*, p.100122.

Anwar, Z., Gulfraz, M., Imran, M. and Qureshi, R. Optimization of dilute acid pretreatment using response surface methodology for bioethanol production from cellulosic biomass of Rice Polish. *Pakistan Journal of Botany* 2012. 44(1): 169-176.

Barbir, F., Veziroğlu, T.N. and Plass Jr, H.J., 1990. Environmental damage due to fossil fuels use. *International journal of hydrogen energy*, *15*(10), pp.739-749.

Beauregard, P.B., Chai, Y., Vlamakis, H., Losick, R. and Kolter, R., 2013. *Bacillus subtilis* biofilm induction by plant polysaccharides. *Proceedings of the National Academy of Sciences*, *110*(17), pp. E1621-E1630.

Bonvillani, P., Ferrari, M.P., Ducrós, E.M. and Orejas, J.A., 2006. Theoretical and experimental study of the effects of scale-up on mixing time for a stirred-tank bioreactor. *Brazilian Journal of Chemical Engineering*, *23*, pp.1-7.

Borriss, R., 2011. Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. *Bacteria in agrobiology: Plant growth responses*, pp.41-76.

Cawoy, H., Bettiol, W., Fickers, P. and Ongena, M., 2011. *Bacillus*-based biological control of plant diseases. *Pesticides in the modern world-pesticides use and management*, pp.273-302.

Choudhary, D.K., 2011. Plant growth-promotion (PGP) activities and molecular characterization of rhizobacterial strains isolated from soybean (Glycine max L. Merril) plants against charcoal rot pathogen, *Macrophomina phaseolina*. *Biotechnology letters*, *33*(11), pp.2287-2295.

Compant, S., Duffy, B., Nowak, J., Clément, C. and Barka, E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and environmental microbiology*, *71*(9), pp.4951-4959.

Czitrom, V., 1999. One-factor-at-a-time versus designed experiments. *The American Statistician*, *53*(2), pp.126-131.

Deniz, I., Imamoglu, E. and Sukan, F.V., 2015. Evaluation of scale-up parameters of bioethanol production from *Escherichia coli* KO11/[*Escherichia coli* KO11 suşundan biyoetanol üretimi için ölçek büyütme parametrelerinin değerlendirilmesi]. *Turkish Journal of Biochemistry*, *40*(1), pp.74-80.

Dimkić, I., Stanković, S., Nišavić, M., Petković, M., Ristivojević, P., Fira, D. and Berić, T., 2017. The profile and antimicrobial activity of *Bacillus* lipopeptide extracts of five potential biocontrol strains. *Frontiers in microbiology*, *8*, p.925.

Ebadipour, N., Lotfabad, T.B., Yaghmaei, S. and RoostaAzad, R., 2016. Optimization of low-cost biosurfactant production from agricultural residues through response surface methodology. *Preparative Biochemistry and Biotechnology*, *46*(1), pp.30-38.

Faloye, F.D., 2015. *Optimization of biohydrogen production inoculum development via hybrid pre-treatment techniques: semi pilot scale production assessment on agro waste (potato peels)* (Doctoral dissertation).

Faloye, F.D., Kana, E.G. and Schmidt, S., 2013. Optimization of hybrid inoculum development techniques for biohydrogen production and preliminary scale up. *International journal of hydrogen energy*, *38*(27), pp.11765-11773.

FAO. 2021. World Food and Agriculture - Statistical Yearbook 2021. Rome. https://doi.org/10.4060/cb4477en.

FAO. 2022. Voluntary Code of Conduct for Food Loss and Waste Reduction. Rome. https://doi.org/10.4060/cb9433en.

Fira, D., Dimkić, I., Berić, T., Lozo, J. and Stanković, S., 2018. Biological control of plant pathogens by *Bacillus* species. *Journal of biotechnology*, *285*, pp.44-55.

García-Fraile, P., Menéndez, E. and Rivas, R., 2015. Role of bacterial biofertilizers in agriculture and forestry. *AIMS Bioeng* 2: 183–205.

Ghribi, D. and Ellouze-Chaabouni, S., 2011. Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnology research international*, 2011.

Govindasamy, V., Senthilkumar, M., Magheshwaran, V., Kumar, U., Bose, P., Sharma, V. & Annapurna, K. (2010) *Bacillus* and *Paenibacillus* spp.: Potential PGPR for sustainable agriculture. Plant Growth and Health Promoting Bacteria (Maheshwari, D. K., ed.) pp. 333–364. Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-13612-2.

Heryani, H. and Putra, M.D., 2017. Kinetic study and modeling of biosurfactant production using *Bacillus* sp. *Electronic Journal of Biotechnology*, *27*, pp.49-54.

Jung, J., Yu, K. O., Ramzi, A. B., Choe, S. H., Kim, S. W., & Han, S. O. (2012). Improvement of surfactin production in *Bacillus subtilis* using synthetic wastewater by overexpression of specifc extracellular signaling peptides, comX and phrC. Biotechnology and Bioengineering, 109, 2349–2356. 20.

Kang, S.M., Radhakrishnan, R. and Lee, I.J., 2015. *Bacillus amyloliquefaciens* subsp. plantarum GR53, a potent biocontrol agent resists Rhizoctonia disease on Chinese cabbage through hormonal and antioxidants regulation. *World Journal of Microbiology and Biotechnology*, *31*(10), pp.1517-1527.

Kanchan Yadav, Lavleesh Garg and Lakhwinder Kaur. 2020. Pesticides Usage: Effect on Human Health. *Int. J. Curr. Microbiol. App. Sci.* 9(8): 2605-2615.

Kaspar, F., Neubauer, P. and Gimpel, M., 2019. Bioactive secondary metabolites from *Bacillus subtilis*: a comprehensive review. *Journal of natural products*, *82*(7), pp.2038-2053.

Linville, J. L., Rodriguez Jr., M., Mielenz, J. R. and Cox, C. D. Kinetic modeling of batch fermentation for Populus hydrolysate tolerant mutant and wild type strains of *Clostridium thermocellum*. *Bioresource Technology* 2013. 147: 605-613.

Lyngwi, N.A. and Joshi, S.R., 2014. Economically important *Bacillus* and related genera: a mini review. *Biology of useful plants and microbes*, *3*, pp.33-43.

Manikandan, K., Saravanan, V., Viruthagiri, T., 2008. Kinetics studies on ethanol production from banana peel waste using mutant strain of *Saccharomyces cerevisiae. Indian. J. Biotechnol*. 17, 83–88.

Malviya, D., Sahu, P.K., Singh, U.B., Paul, S., Gupta, A., Gupta, A.R., Singh, S., Kumar, M., Paul, D., Rai, J.P. and Singh, H.V., 2020. Lesson from ecotoxicity: revisiting the microbial lipopeptides for the management of emerging diseases for crop protection. *International journal of environmental research and public health*, *17*(4), p.1434.

Mardanova, A. M., Hadieva, G. F., Lutfullin, M. T., Khilyas, I. V. E., Minnullina, L. F., Gilyazeva, A. G., Bogomolnaya, L. M. & Sharipova, M. R. 2017. *Bacillus subtilis* strains with antifungal activity against the phytopathogenic fungi. *Agr Sci*, 8, 1-20.

Moodley, P., 2017. *Strategies for enhancing sugar recovery from sugarcane leaf waste and kinetic modelling for bioethanol production using saccharomyces cerevisiae BY4743* (Doctoral dissertation).

Moodley, P. and Kana, E.G., 2017. Bioethanol production from sugarcane leaf waste using *Saccharomyces cerevisiae* BY4743: Effect of various optimized pretreatments and fermentation conditions on process kinetics. p.95.

Mouafi, F.E., Elsoud, M.M.A. and Moharam, M.E., 2016. Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. *Biotechnology Reports*, *9*, pp.31-37.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P. and Hens, L., 2016. Chemical pesticides and human health: the urgent need for a new concept in agriculture. *Frontiers in public health*, *4*, p.148.

Nikzad, M., Movaghernajed, K., Talebnia, F. and Mighani, M. Modeling of Alkali Pretreatment of Rice Husk Using Response Surface Methodology and Artificial Neural Network. *Chemical Engineering Communications* 2015. 202(6): 728-738.

Ongena, M. & Jacques, P. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in microbiology*, 16, 115-125.

Phukoetphim, N., Salakkam, A., Laopaiboon, P., Laopaiboon, L., 2017. Kinetic models for batch ethanol production from sweet sorghum juice under normal and high gravity fermentations: Logistic and modified Gompertz models. J. Biotech. 243, 69-75.

Qazizada, M.E., 2016. Design of a batch stirred fermenter for ethanol production. *Procedia Engineering*, *149*, pp.389-403.

Radhakrishnan, R., Hashem, A. and Abd_Allah, E.F., 2017. *Bacillus*: A biological tool for crop improvement through bio-molecular changes in adverse environments. *Frontiers in physiology*, *8*, p.667.

Rorke, D.C.S., 2017. *The potential of waste sorghum (sorghum bicolor) leaves for bioethanol process development using Saccharomyces cerevisiae BY4743* (Doctoral dissertation).

Sanusi, I.A., Suinyuy, T.N., Lateef, A. and Kana, G.E., 2020. Effect of nickel oxide nanoparticles on bioethanol production: process optimization, kinetic and metabolic studies. *Process Biochemistry*, *92*, pp.386-400.

Shahid, I., Han, J., Hanooq, S., Malik, K.A., Borchers, C.H. and Mehnaz, S., 2021. Profiling of metabolites of *Bacillus* spp. and their application in sustainable plant growth promotion and biocontrol. *Frontiers in Sustainable Food Systems*, *5*, p.605195.

Sun, H., Bie, X., Lu, F., Lu, Y., Wu, Y., & Lu, Z. (2009). Enhancement of surfactin production of *Bacillus subtilis* fmbR by replacement of the native promoter with the Pspac promoter. *Canadian Journal of Microbiology*, 55, 1003–1006. 21.

Tran, C., Cock, I.E., Chen, X. and Feng, Y., 2022. Antimicrobial *Bacillus*: Metabolites and Their Mode of Action. *Antibiotics*, *11*(1), p.88.

Tredgold, H.R., 2021. *Diversity of quorum sensing pherotypes amongst ecotypes of plant-associated Bacillus subtilis sensu lato isolates* (Doctoral dissertation).

United Nations Department of Economic and Social Affairs, Population Division (2022). World Population Prospects 2022: Summary of Results. UN DESA/POP/2022/TR/NO. 3.

Wahid, Z. and Nadir, N., 2013. Improvement of one factor at a time through design of experiments. *World Applied Sciences Journal*, *21*(1), pp.56-61.

Wang, T., Liang, Y., Wu, M., Chen, Z., Lin, J. & Yang, L. 2015. Natural products from *Bacillus subtilis* with antimicrobial properties. *Chinese Journal of Chemical Engineering*, 23, 744-754.

Xia, J., Wang, G., Lin, J., Wang, Y., Chu, J., Zhuang, Y. and Zhang, S., 2015. Advances and practices of bioprocess scale-up. *Bioreactor Engineering Research and Industrial Applications II*, pp.137-151.

CHAPTER 2

Literature review

2.1 Introduction

Rising global population has led to an increase in the demand for food supply, resulting in the use of rigorous agricultural practices (Borriss, 2011; Aloo *et al*., 2018). Modern agricultural practices are aimed at salvaging the massive loss due to various diseases that are caused by insects, pathogenic fungi, bacteria, and nematodes. The most common strategies for improved yields from modern agricultural farming includes the extended use of pesticides and chemical fertilizer. Despite the successes of these intensive practices, the soil and food accumulation of toxic agents derived from chemical pesticides poses a negative threat. The threat of extended use of pesticides includes reduction in effectiveness as a result of time-dependent increase in disease resistance (Mardanova *et al*., 2017; Borriss, 2011). However, understanding the mechanism of infection of plant pathogen can facilitate in the development of eco-friendly and efficient methods capable of tackling this menace. Generally, extremely problematic plant pathogens belong to bacterial and fungal species, each with a unique mode of infection. The fungi penetrates through hyphae and haustoria, where it accumulates in the plant and secretes various cell wall degrading enzyme to break down the cuticle and cell wall of the plant (Abdulkhair and Alghuthaymi, 2016; Mendgen, 1996). On the other hand, bacterial species penetrate through wounds or natural openings (Abdulkhair and Alghuthaymi, 2016; Agrios, 2005). Specie that penetrates through the root region commonly trigger the induced systematic resistance (IRS), a mechanism of plant defense that uses physical and chemical barriers to stop further infection (Borriss, 2011). Nevertheless, plant pathogens gain resistance to these barriers and therefore, a need for effective biocontrol agents such as microbial pesticides.

The use of microbial pesticides is a sustainable and environmentally friendly method of controlling plant diseases compared to the use of chemical agents (Mardanova *et al*., 2017).

Bacterial species are known to assist in promoting plant growth through several mechanisms such as, toxicity to pest antagonism, pathogens, and improvement of plant nutrition uptake. One of the bacterial species efficient in this regard is the *Bacillus* specie (Ongena *et al*., 2005; Bottone and Peluso, 2003). *Bacillus* isolates have been known to exhibit both antifungal and antibacterial activities. These antimicrobial potentials are associated with the production of various antimicrobial compounds, possessed by a good biocontrol agent (Ongena *et al*., 2005). However, efforts into high quality and quantity production have encounter significant problem thus probing the need for novel strategies to enhance their production. Generally, microbial bioprocessing is influenced by various biological and physico-chemical parameters such as microbial strain, media composition, temperature, pH, substrate and oxygen availability, among others (Lalloo, 2010; Moodley *et al*., 2014). Optimization of these parameters using mathematical models such as response surface methodology (RSM) and artificial neuron network (ANN) can be employed for proper understanding of parameter interaction during bioprocessing to enhance product formation. This subsequently could aid in the process scaling up and downstream processing (Nwabueze, 2010; Granato and de Araújo Calado, 2014).

The downstream bioprocessing includes product harvesting, extraction and purification leading to the end-product (Brar *et al*., 2006). It therefore has a considerable influence on the development and commercialization of biological-based products (Lalloo, 2010). The biological agent produced needs to meet requirements such as effective, stability, reliability, easy to handle and affordable (Moodley *et al*., 2014; Schisler *et al*., 2004; Brar *et al*., 2006). Even though there are commercial biological products in use, there is limited information on the recovery and formulation of these products (Schisler *et al*., 2004; Brar *et al.,* 2006).

2.2 Plant pathogens

Plant pathogens range from insects and nematodes to bacteria and fungi. These pathogens cause serious plant diseases because they could penetrate plant tissues to feed and proliferate within the plant tissue. A substantial number of crops, fruits and vegetables are destroyed per annum due to diseases caused by these plant pathogens. Report has shown that 14.1% of crops are lost to pathogenic plant diseases alone. The total annual global crop loss from pathogenic plant diseases is approximately $220 billion. Thus, the control of plant pathogen is essential to prevent the damage to food crops and ensure food security. Developing effective biocontrol agents is an efficient strategy to ensure plant pathogen control, and this can be achieved through a comprehensive understanding of the underlying mechanisms involved in the plant pathogen infection especially fungi and bacteria pathogens.

2.3 Plant pathogenic fungi

Fungal species are the most challenging plant pathogens, this is attributed to the their co-existence with plants (Mendgen *et al*., 1996). Fungi mode of infection can be divided into three classes due to their infection mechanism: biotrophs, necrotrophs and hemibiotrophs (Pawlowski and Hartman, 2016). The biotrophic fungi penetrate the plant without causing immediate death, while the necrotrophic fungi kills plant cells as they penetrate and the hemibiotrophic fungi exhibit both the biotrophic and necrotrophic fungi characteristics (Pawlowski and Hartman, 2016). In a more descriptive detail, necrotrophic infection begins in one or two ways: firstly, the germination of the conidia that directly infects the plant; alternatively, the fungi develop penetration hyphae that penetrate and form an appressoria (develop to penetration pegs) to penetrate the epidermis. The appressoria then secretes several lytic enzymes that help degrade the plants cuticle and wax layers (Pawlowski and Hartman, 2016). The penetration pegs continue growing through the damaged epidermal tissue and excretes cell wall degrading enzymes that break down the cell wall and progresses through the

plant, eventually causing plant death. Cell wall degrading enzymes allow fungi to break down cells and get a hold of host nutrients (Mendgen, 1996). The lipopeptidic moiety reduces plant growth by affecting the cell wall and cell membrane causing leakage of cell material and consequently the death of the host cell. Similarly, certain penicillium species such as *Penicillium digitatum* are classified as necrotrophs, they are known to affect citrus fruits. *P. digitatum* infects through surface injuries of the peels of the fruit. The spores enter, germinate and inhabit the tissue, resulting in the plant disease (Julca *et al*., 2015).

On the other hand, biotrophs do not damage cells as they penetrate the plant tissue (Pawlowski and Hartman, 2016). Fungal biotrophic infection is initiated by spore germination and formation of a germ tube of varying length. This transforms to an appressorium that attaches at the site of penetration. After attachment, an appressorial cone develops that instigates penetration of the epidermal cell by turgor pressure (Abdulkhair and Alghuthaymi, 2016; Keen, 2000). The epidermal cells and the intercellular spaces are breached by the invasion hyphae resulting in primary and secondary invading hyphae that populate the plant. Through this, the plant defense mechanism could be by-passed without being triggered leading to primary and secondary haustoria formation in the mesophyll cells which then become necrotic (Pawlowski and Hartman, 2016). A typical example is the *Puccinia* species. *Puccinia* species have been reported as obligate biotrophs with distinguishing infection structures (haustoria) capable of by-passing their host defense mechanism to obtain nutrients. Other plant pathogenic biotrophs include *Blumeria graminis* and *Sphaerotheca panmosa* which infect barley and roses respectively.

Finally, the hemibiotrophs (best-armed phytopathogens) attack by employing both biotrophic and necrotrophic modes of infection (Pawlowski and Hartman, 2016). The proliferation of this fungus starts with a biotrophic phase where it forms a relationship with the host and then a change occurs to the necrotrophic phase in order to obtain nutrients and inhabit the host plant

tissue (Pawlowski and Hartman, 2016). Hemibiotrophic plant pathogens include *Mangnathorpe oryzae, Fusarium oxysporum* and some *Colletotrichum* species. *M. oryzae* has been reported as the most virulent plant pathogen, followed by *F. oxysporum* at close range. *F. oxysporum* infects a wide variety of plants, passively and actively, through openings, stomata and direct penetration, respectively (Dean et al., 2012).

2.4 Plant pathogenic bacteria

Bacterial species require contact with their hosts to cause infection. The distribution of plant pathogenic bacteria is achieved by passive agents. Most pathogenic bacteria penetrate their plant-hosts via a wound or opening, this is often achieved under warm and moist conditions (Quadt-Hallmann *et al*., 1997). Bacteria colonize the host plant by multiplying in the intercellular spaces of the cells and absorbing the nutrient leaked into the spaces. Depending on the plant environment, the bacterial species alter the normal plant growth by secreting growth regulators; break down cell wall and cell membranes using cell degrading enzymes and toxins, respectively. (Abdulkhair and Alghuthaymi, 2016). Bacterial species such as *Pseudomonas* and *Xanthamonas* cause plant disease such as bacterial cankers, spot, and blight while *Erwinia* spp. are known for causing vascular wilt and soft rot. (Schaechter, 2009).

2.5 Bacteria as biocontrol agents

The lethal effects of chemical pesticides on beneficial microbes and the deterioration of the environment as well as toxic accumulation in food has encouraged the exploration of alternative control measures. (Yu *et al*., 2002; Aloo *et al*., 2018). Plant-microbe interactions are being studied to develop sustainable crop-pathogen management techniques (Borriss, 2011). Microorganisms with desirable antagonist activity against plant pathogens have been studied, these microbes have several advantages against synthetic pesticides. Microbial products are environmentally friendly because they are degradable and can be easily recycled as part of the geo-biochemical cycle in the soil (Radovanović *et al*., 2018). *Bacillus* species,

Psuedomonas and *Agrobacteruim*, are the most studied due to their effectiveness against a broad range of phytopathogens (Fira *et al*., 2018; Radovanović *et al*., 2018).

The *Bacillus* genus are rod-shaped, gram positive, endospores forming bacteria. The *Bacilli* spp. are ubiquitous, they colonize the soil, water and air among other extreme niches (Al-Thubiani *et al*., 2018). These spore formers are able to survive at extreme temperatures, low pH, and under unfavorable nutrient conditions (Al-Thubiani *et al*., 2018; Fira *et al*., 2018). Additionally, *Bacillus* spp. exhibit many desirable properties such as antimicrobial activity against phytopathogens (Fira *et al*., 2018). Consequently, with *Bacillus* spp. antagonistic activity potential against plant pathogens as well as their presence in the environments, they are considered to be attractive as biocontrol agents (Lalloo *et al*., 2010). Moreover, *Bacillus* produce various antimicrobial metabolites: structural microbial-antagonist compounds (Fira *et al*., 2018).

Furthermore, different *Bacillus* based biocontrol agents are already being used in countries like the United States of America, South Africa, Canada, Switzerland and Germany. Table 2.1 shows different bacillus-based biocontrol agents currently in the market. The most commonly studied *Bacillus* spp. for biocontrol are *Bacillus subtilis* and *Bacillus amyloliquefaciens* for formulation, commercialization and application. Other species of the *Bacillus* genus also show potential of antagonistic activity; however, they have not been fully exploited for biocontrol activity. Other *Bacillus* species in the market as biofungicides are *B. licheniformis* and *B. pumulis*. Hence, exploiting of other bacilli rhizobacteria for biocontrol activities and commercialization potential is desirable.

Table 2.1: *Bacillus* based biocontrol agents (Modified from Cawoy *et al*., 2004)

Product	Bioagent	Company
AmyProtec 42	*Bacillus amyloliquefaciens FZB42*	Andermatt biocontrol, Switzerland
RhizoVital	*B. amyloliquefaciens FZB42*	ABiTEP GmbH, Germany
BioYield	*B. amyloliquefaciens GB99 + B. subtilis GB122*	Gustafson, United States of America (USA)
Rhizocell GC	*B. subtilis sp*	Lallamand Inc., Canada
Avogreen	*B. subtilis B246*	Ocean Agriculture Pty (Ltd), SA
GB34	*Bacillus subtilis* GB34	Gustafson, USA
Serenade	*Bacillus subtilis QST 713*	AgraQuest, USA
Kodiak	*Bacillus subtilis GBO3*	Gustafson, USA
Yield shield Bio-fungicide	*Bacillus Pumilus GB34*	Bayer crop science, USA
EcoGuard	*B. Lichenformis SB3086*	Novozyme Biologics, USA

Similarly, *Bacillus* produce numerous ribosomal and non-ribosomal secondary metabolites. The secondary metabolites (biocontrol agents) are lipopeptides which include surfactin, fengycin, iturin, polymixins and bacteriocin (Wang *et al*., 2015). Theses cyclic lipopeptides have several mechanisms of action that enables them to achieve a broad spectrum of effectiveness.

2.5.1 Mechanism of action of bacteria - (bacillus) based biocontrol agents

The development of an effective biocontrol agent is dependent on a few things including the pathogen strain, host vulnerability and the environment (Hashem *et al*., 2019). *Bacillus* species are the most studied microorganism that exhibit biocontrol. The mechanism of action of bacillus based biocontrol agents include competition for nutrients (competitive exclusion) and space, production of lytic enzymes, siderophores and antibiotics as well as induced systematic resistance in plants (Lalloo *et al*., 2010). Additionally, they can act as biostimulator or biofertilizer, by synthesizing plant hormones that aid in plant growth or by assisting in the uptake of nutrients in the environment. *Bacillus* spp. found in soil can occur as endophyte that protects plants. Moreover, biocontrol activity mechanism against plant pathogens includes,

biofilm formation, plant growth promotion (PGP), competition for nutrients and colonization sites, ability to induce cell lysis, and induced systemic resistance (ISR) (Wang *et al*., 2018). To manage plant diseases, *Bacillus* species produce antimicrobial metabolites that can be employed in addition to or as a replacement for synthetic chemicals, bio-pesticides, and biofertilizers (Ongena *et al*., 2005). Additionally, bacteria create a variety of metabolites, including enzymes that break down cell walls, which might inhibit the development or activity of other microbes (Shoda, 2000). Particularly, *B. subtilis* strains are capable of producing antimicrobial lipopeptides like fengycin, surfactin, and iturin. Additionally, *Bacillus* spp. also create endospores, which aid in the bacteria's ability to endure challenging environmental conditions, permit the long-term storage of the biocontrol agent, and simplify the formulation process (Collins and Jacobsen, 2003). *Bacillus* spp. also secrete exopolysaccharides and siderophores that inhibit the movement of toxic ions and help to maintain the ionic balance, promote the movement of water in plant tissues, and inhibit the growth of pathogenic microbes.

Moreover, bacteria-based secondary antimicrobial metabolites (ribosomal and non-ribosomal) are used in the development of biocontrol agents for pre- and post-harvest of crop diseases (Wang *et al*., 2015). *Bacillus* non-ribosomal synthesis involves large multi-enzyme complexes for catalyzation of important steps in peptide synthesis. This group comprises of iturin, fengycin, bacilysin, rhizocticin, amicoumacin and surfactin (Fira *et al*., 2018; Wang *et al*., 2015). Surfactin and fengycin interferes with the cell membrane of the cell causing destabilization of the structure and permeability while iturin affects the ion pore channels of the cell (Fira *et al*., 2018). The success of biocontrol approaches depends on the proper selection of effective biocontrol agents and their ability to provide protection against specific target pathogens in specific crops (Hashem *et al*., 2019).

2.6 Lipopeptides

Lipopeptides are produced as antifungal and antibacterial agents, which are useful for plant development (Borriss, 2011). Lipopeptides are low molecular weight compounds with amphiphilic features. Various wild type *B. subtilis* strain have shown the ability to produce antimicrobial lipopeptides of the families: surfactin, fengycin and iturin (Stein, 2005). These are structurally cyclic in nature and composed of seven to ten amino acid residues associated with fatty acid derivatives. Due to the length of the fatty acid hydrocarbon chain or the variation in amino acid arrangement, they exist in many isomeric forms (Ongena and Jacques, 2008). Among these antimicrobial compounds, cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin (or plipastatin) families have biotechnological and biopharmaceutical applications because of their surfactant properties.

2.6.1 Description of lipopeptides from *Bacillus* species

The surfactin family (Fig. 2.1A) comprises of peptides with seven amino acids bonded to the beta-hydroxyl fatty acid hydrocarbon chain with 13-15 carbon atoms (Fira *et al*., 2018; Ongena and Jacques, 2008). The production of surfactin promotes the motility and biofilm formation characteristics of *Bacillus* surfactin producing strain. Surfactin exhibit haemolytic, antiviral, antimycoplasma and antibacterial activity but no antifungal activities. The mode of action of surfactin is membrane destabilization. The susceptibility of the lipid bilayer may differ depending on the sterol content of the target organism. The 3-D representation of the surfactin molecule shows the charged side chain facing the aqueous phase and the polar moieties facing the hydrophobic core of the phospholipid membrane (Ongena and Jacques, 2008). This family also incorporates variants such as pumilacidin, lychenisin and halobacilin.

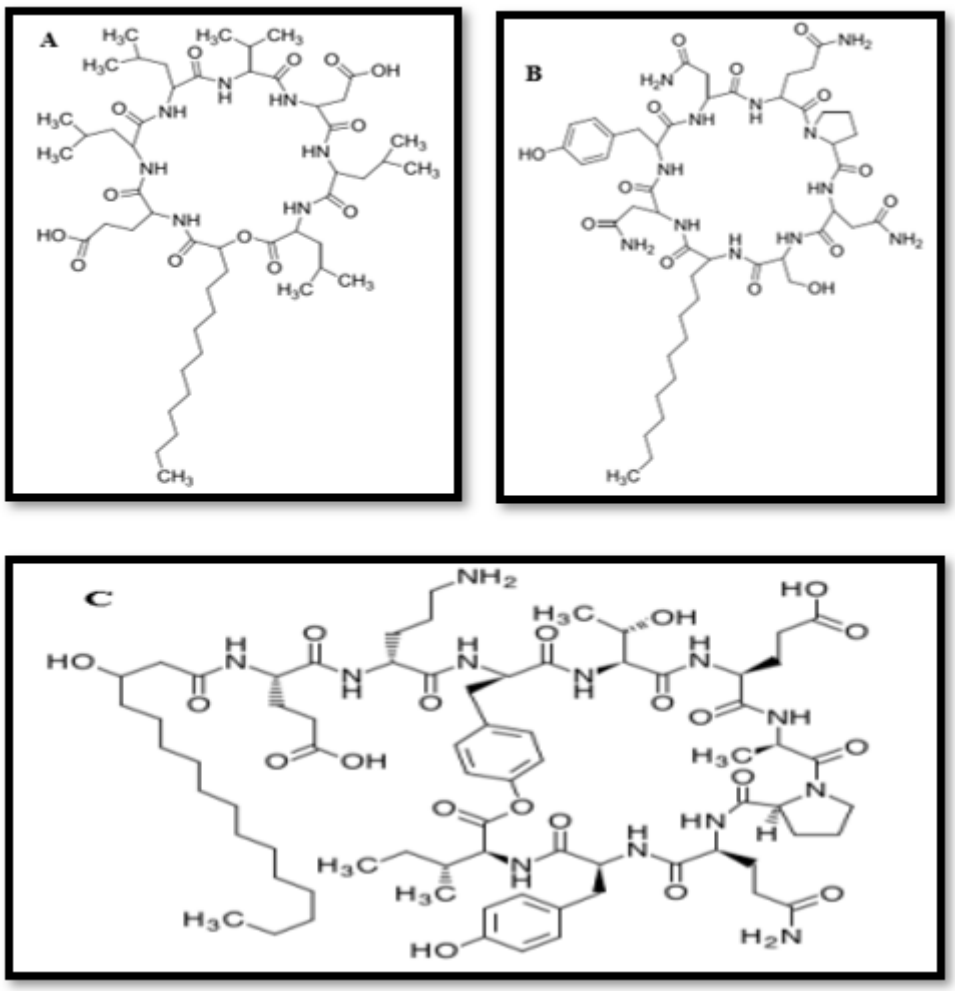

Fig. 2.1: Chemical structure of lipopeptide family (A) Surfactin, (B) Iturin and (C) Fengycin. Modified from Pretorius (2014)

Another lipopeptide group is the iturin (Fira *et al*., 2018). The Iturin family of lipopeptides are characterized by peptides with seven amino acids joined to a beta-amino fatty acid chain of different length (C14-C17) (Fig. 2.1B) (Fira *et al.*, 2018). The most fundamental representatives of this family are iturin A, C, D and E, also variants of mycosubtulin and bacillomycin. Iturin and surfactin are similar as they both exhibit haemolytic activity and membrane integrity disruption. The mode of action of iturin is the formation of ion conducting pores, while surfactin connects to the membrane bilipid layer for destabilization. This family has shown strong in-vitro antifungal activity against various fungi and yeast. However, they

possess partial antibacterial and no antiviral activity. Antifungal activities of iturin are dependent on their membrane permeability characteristics (Ongena and Jacques, 2008).

The next known lipopeptide family is the fengycin. The fengycin family (Fig. 2.1C) of lipopeptides comprises of numerous isomers of fengycin, plipastatin and malticin. They are composed of 10 amino acids connected to the beta hydroxyl fatty acid chain with 18 carbon atoms (Fira *et al*., 2018). Fengycin is known to possess strong fungitoxicity against filamentous fungi. Contrary to iturin and surfactin, fengycin displays less haemolytic activity. Antagonistic mode of action of fengycin is not entirely known, however, it is speculated that it functions by disrupting cell membrane structure and permeability. Research has suggested that these lipopeptides (Surfactin, iturin, fengycin) attack in a synergistic manner to effectively control plant pathogens (Wang *et al*., 2015; Ongena and Jacques, 2008). These cyclic lipopeptides are amphiphilic molecules with peptide and fatty acid chains that alter, resulting in diverse infection mechanisms. Summarily, Surfactin possess strong haemolytic and surfactant properties but weak anti-infection action. On the other hand, the fengycin family are strong antifungal agents and the iturin family, display various degrees of antibacterial, haemolytic, and antifungal activity. Biosynthesis of lipopeptides comprises of monomeric building blocks such as amino acids which are serially connected via non-ribosomal peptide synthetase (NRPS). The biosynthetic mechanisms and gene regulation systems of lipopeptide surfactants have been extensively analyzed over the last decade. Other antimicrobial compounds produced for antagonistic effect on plant pathogens are compounds such lantibioics, hydrogen cyanide, siderophores and some volatile compounds.

2.6.2 Biosynthesis of lipopeptides

Lipopeptides are amphiphilic with change in their peptide and unsaturated fat moieties (Stein, 2005). Non-ribosomally synthesized lipopeptides are composed of amino acids, amino- or Hydroxyl- fatty acids with different lengths of hydrocarbon chains that can undergo post-

translation modifications such as acylation, methylation, and glycosylation. *Bacillus* species' peptide synthesis involves the non-ribosomal peptide synthetase (NRPS). These are responsible for synthesis of bioactive compounds. NRPSs are mega-enzymes that consists of large domain enzymes complexes that catalyze the reaction leading to peptide formation (Chen *et al*., 2009).

These modular proteins lead to heterogeneity of the lipopeptides biosynthesized by *Bacillus.* These peptides differ by the type and amino acid monomers, peptide cyclization, length and branching of fatty acid chain. Each module of the mega-enzymes is divided into catalytic domains that catalyze a reaction in the biosynthesis process. Throughout the process, each module is responsible for the merging of a particular amino acid. Each reaction step involves three domains; A (Adenylation) domain, PCP (Peptidyl-Carrier-Protein) domain, and the C (Condensation) domain (Chen *et al*., 2009; Ines and Dhouha, 2015).

Each elongation cycle requires the participation of three domains. The A domain chooses its related/cognate amino acid and produces an enzymatically stable aminoacyl adenylated compound. This process is similar to the aminoacylation of tRNA synthetase during ribosomal peptide synthesis. The PCP domain has a 40 Phosphopantethiene (PPan) prosthetic group which the adenylated amino acid is transferred and bound as thioester. The PPan cofactor acts as a thiotemplate and a swinging arm to transport intermediates between the catalytic domains. The PPan transferase catalyzed the post-translational conversion of the inactive apoforms of the PCP into an active holoforms. The C domain catalyzes the formation of a new peptide bond between adenylated and peptidyl carrier domains. The linear arrangement of the core domains facilitates the co-ordination of elongation cycle of peptide products. The assembly of the multifunctional proteins of the peptide synthetases is reflected in its genetic organization following the collinearity rule (Stein, 2005; Chen *et al*., 2009; Ongena and Jacques, 2008).

Lipopeptide display various advantages over synthetic surfactants; thus, a lot of interest towards microbial-based surfactant production. The enhancement of lipopeptide production is desirable and can be achieved by the use of low-cost media, choice of strain, optimization of lipopeptide production process parameters using optimization tools such as response surface methodology (RSM).

2.6.3 Lipopeptide production: fermentation process

The production of lipopeptide can be carried out in two ways, submerged or solid-state fermentation processes. Solid-state fermentation (SSF) is a process in which microorganisms grow on or within solid substrates or supports in the absence of free water (Pandey *et al*., 2000; Nalini *et al*., 2016). SSF has been reported to utilize renewable resources like cassava wastewater (Barros *et al*., 2008), potato and soybean residues. However, this fermentation method is slow resulting in longer fermentation times, and the SSF process cannot be used for microorganisms that need a high-water activity.

Submerged fermentation (SmF) is another approach used in the production of lipopeptides. The SmF is a process involving the development of microorganisms in a liquid media (Subramaniyam and Vimala, 2012). Substrates usually used for SmF include molasses and broths. Unlike SSF, SmF occurs rapidly, and nutrients need to be replaced, also, the process supports microorganism that require a high moisture content. During fermentation of SmF process, the product, secondary metabolites, is released into the fermentation broth. SmF is preferred when end-product is required in liquid form, it offers an advantage, making the purification step easier.

Although, SmF offer an ease downstream processing, SSF is commonly used due to high similarities between the fermentation conditions and the natural microorganism environment. SSF is advantageous compared to SmF as it uses low-cost raw materials, reduces cost of fermentation process, low energy consumption, carried out in smaller volumes, no foam is

generated, and higher yields of secondary metabolites and enzyme are achieved (Subramaniyam and Vimala, 2012; Piedrahíta-Aguirre and Alegre, 2014). However, the fermentation processes are influenced by various physical and chemical parameters such as temperature, pH, oxygen availability, moisture content, fermentation time, substrate concentration and the addition of mineral salts (Fe^{2+}, Mn^{2+}, etc.), etc. (Wei *et al*., 2007). For increased biosurfactant yields, optimal addition of media components and selection of the optimal process conditions will induce the maximum or optimum biosurfactant productivity.

2.6.4 Factors affecting lipopeptide fermentation

Lipopeptides productivity and yield is influenced by many fermentation operation factors as listed above (Bocchini *et al*., 2002; Guo *et al*., 2018). Lipopeptides have been reported to grow on both complex and defined media (Wei *et al*., 2005). Lipopeptides can be produced on media containing carbon sources such as carbohydrates, vegetable oils, hydrocarbons, and renewable resources. Aside from carbon sources, trace elements have been found to be significant in the fermentation of biosurfactant. Trace elements and their concentration are known to significantly affect the biosynthesis of lipopeptides as major co-factors in the mega enzyme complex system. Metal cations such as Mn^{2+}, K^{+}, Zn^{2+}, Mg^{2+} and Fe^{2+} have shown influence on *B. subtilis* based lipopeptide production (Rangarajan and Clarke., 2015). Wei *et al*. (2007) investigated the optimized trace element composition for surfactin production (3.34g/L) using *B. subtilis* ATCC 21332 and revealed optimized concentration of 2.24 nM, 10 nM, 0.01 nM, 0.008 nM and 7 µM of $Mg^{2+,}$ K^{+}, Fe^{2+} and Ca^{2+}, respectively.

Similarly, the growth and activity of *Bacillus* species is greatly influenced by the pH of the growth media (Nafaji *et al*., 2010). This influences factors such as growth, transport of various nutrients as well as production of secondary metabolites. Nafaji *et al*. (2010) observed high biosurfactant production for *Bacillus mycoides* when the medium pH was 10.19. However, Nalini and Parthasarathi, (2018) observed high biosurfactant production at pH range of 6-9

using *Serratia rubidaea*. Similarly, Chen *et al*. (2015) reported that pH should be maintained in the range of 6-9 as lower pH result in precipitation and higher pH results in lower yields. Therefore, understanding the effect of pH for each microorganism used in a lipopeptides bioprocessing is essential.

Another critical parameter that needs to be controlled in any bioprocess is temperature. Temperature is an important factor as it affects the nature of the homologue and isomer produced (Kumar *et al*., 2017; Chen *et al*., 2015). Generally, mesophilic temperatures (25^{o}C to 45^{o}C) favor lipopeptide production. For instance, Kumar *et al*. (2017) observed high iturin A production at temperature (37^{o}C). Likewise, Guo *et al*. (2018) observed that 37^{o}C achieved greater surfactin production using *Bacillus natto*. Similarly, Jha *et al*. (2016) investigated the effect of temperature on the growth and lipopeptide production of *Bacillus subtilis* R1 and observed the growth range of 30 - 45^{o}C

Moreover, environmental factors and growth conditions such as agitation and aeration also affect biosurfactant production through their effect on cellular growth and activity (Ghibli and Ellouze-Chaabouni, 2011). Various studies have also investigated the effect of these parameters. For instance, Ohadi *et al*. (2017) investigated agitation rate (150 – 300 rpm) and observed high Biosurfactant production at 300rpm. Likewise, Wei *et al*. (2005) observed high surfactin production at 300 rpm. On the other hand, Piedrahíta-Aguirre and Alegre (2014) investigated airflow rate (0.4-0.8L/min) on iturin production, high iturin A production was observed at 0.46L/min airflow rate.

2.7 Strategies to enhance *Bacillus* growth and lipopeptide formation

2.7.1 Use of low-cost raw materials

A major bottleneck with all biotechnological process is the need for improved process performance and production yield at a reduced cost. The selection of an appropriate substrate

has a significant effect in the development of a cost-effective process. Raw material account for 10-30% of the total production cost. The type and amount of the crude material considerably contribute to production cost. Application of agricultural-industrial waste in bioprocess provides an alternate way to replace the costly defined raw materials used in the production of bioproducts such as lipopeptides. Moreover, utilization of agro-industrial waste will help to mitigate numerous environmental hazards (Nalini *et al*., 2016). A variety of low-cost material such as coconut oil cake, castor oil, gingelly oil cake, peanut cake oil, palm oil cake and sunflower oil cake have been evaluated for biosurfactant production using *B. cereus* SNAU01 (Nalini *et al*., 2016). Peanut oil cake was found to be the best of substrates with maximum biosurfactant production of $\%EI_{24}$=65 (Nalini *et al*., 2016). On the other hand, Kumar *et al*. (2017) screened 16 agro-industrial waste for iturin A production and only 3 substrates (dry yeast cells, sunflower oil cake and cheese whey permeate) significantly affected fermentative iturin A production. In another related study, Zouari *et al*. (2015) optimized biosurfactant production using olive leaf residues and olive cake fluor; a ratio of olive leaf residue flour/olive cake flour and achieved an optimal yield of 30.67 mg crude lipopeptide, further elucidating the suitability of waste biomass. Other techniques used for enhancing lipopeptide production include process optimization and the development of mutant strain with high product production titres.

2.7.2 Genetically engineered strain

The genetic characteristic of the microorganism has a significant effect on the production yield of the lipopeptide products. The capability of the microbe to produce the desired product is dependent on the existence genetic structure of the microbe. Microbial cell has natural capability to produce metabolites that has antimicrobial properties. However, the yield is generally low, hence, the need for techniques such as genetic manipulation of producing strain for improved lipopeptide production. Various studies have reported on the effectiveness of

lipopeptide production from mutant strains A study by Guo *et al*. (2014) presented work on the ability of *B. subtilis* NCD-2 wild type strain to inhibit activity of *Rhizoctonia solani* in the presence of *fenC* gene. In another instance, Wu *et al*. (2019) developed a systematic engineering approach, improving the biosynthesis of surfactin, where they achieved a final yield of 12.8 g/L of surfactin from an initial yield of 0.4g/l. Similarly, surfactin production by *B. subtilis* ATCC 55,033 was improved four to five fold by the utilization of N-methyl-N′-nitro-N-nitrosoguanidine treatment (Carerra *et al*., 1993). According to Mulligan *et al*. (1989), the UV mutation of *B. subtilis* ATCC 21332 resulted in a stable mutant that produced over three times as much surfactin as the parent strain. Zhao *et al*. (2012) used the genome shuffling approach for improving antimicrobial lipopeptide production by *Bacillus amyloliquefaciens* permitting the generation of mutant strain F2-38 that exhibited 3.5- and 10.3-fold increases in surfactin production in shake flask and fermenter, respectively. Hence, genetically engineering the producing strain is one efficient method to enhance surfactin biosynthesis.

2.7.3 Modelling and optimization techniques

The type, amount and quality of the product formed depends on the operational parameters. Moreover, the parameters may interact to either enhance or negatively influence the production of the desired product. One of the main reasons for the use of optimization strategies is to ensure that a great quality product is developed (Nwabueze, 2010). Conventionally, the method of optimization used includes the one variable at a time (OVAT). In the last few years, statistical approaches such as the response surface methodology (RSM) and the artificial neuron network (ANN) have gain popularity due to the significant advantages over the traditional methods. The one variable at time (OVAT) technique ensures all process parameters are kept constant and varies one parameter over the preferred range. Several research articles have used the OVAT technique to investigate the effect of medium composition, glucose concentration, temperature, pH and salt concentration on biosurfactant production (Avci *et al*.,

2017). *Bacillus* exhibited a high antimicrobial activity in the absence of glucose when glucose concentration was varied. The major limitation of this technique is that the process optimum values may be missed entirely; also, the method does not consider the interactive effect of the process parameters and is very laborious due to large number of experiments required (Nwabueze, 2010).

Alternatively, statistical approaches use smaller set of experimental set ups. A well-known statistical based approach is the RSM method, and several studies have used RSM for the optimization of media composition and physico-chemical parameters for antimicrobial compounds (Grahovac *et al*., 2015). Bocchini *et al*. (2002) use the RSM to optimize Xylanase production from *Bacillus circulans* D1 in submerged fermentation, the model predicted xylanase activity of 19.1 U/ml under the optimum condition of 5 g/L and 48 h for xylan concentration and cultivation time, respectively. Under optimized condition predicted by the RSM, *Bacillus mycoides* SH2 biosurfactant production increased 2-fold (1.7 – 3.3 g/L) (Najafi *et al*., 2010). Piedrahíta-Aguirre and Alegre (2014) used the central composite rotatable design for the optimization of biosurfactant by *Bacillus iso1*, maximum iturin A production (6.88 g/kg of dry substrate) was achieved under optimum conditions of 22.9% rice husks, 0.46 L/min for volumetric air flow rate. More recently, the artificial neuron network (ANN) computational models have attracted more attention. ANN is a computational model inspired by the central nervous system; it has interconnected group of artificial neurons. The use of ANN is advantageous as it reduces costs; explains interactions and is able to learn from process data and can predict responses from new process variables. Peng *et al*. (2014) used the ANN model to optimize Amino acid (Asparagine, Glutamic acid, proline) concentration for Iturin A production; However, most biosurfactant production and optimization have only been implemented in shake flask. Thus, there is a need to gain knowledge on the scale up of

biosurfactant production in a large bioreactor if the industrial scale production of biosurfactant is to be achieved.

2.8 Scale up strategies

Biosurfactant fermentation experiments are frequently carried out with shake flash while, data on scale up biosurfactant production studies are scantily reported literature (Ghimire *et al*., 2015). Four scaling up bioprocess techniques, specifically, fundamental methods, semi fundamental methods, dimensional analysis, and the rule-of-thumb are widely used. Different parameters are usually correlated to reactor geometry, rheology, mixing activity, pumping capacity, power consumption, cell viability, substrate, and products concentration in the bioreactor (Deniz *et al*., 2015; Qazizada, 2016). The design of a commercial scale biosurfactant production depends on growth conditions, strain type, nutrient formulation, targeted biosurfactant product, bioreactor geometry and fluid rheology. Therefore, for a certain biosurfactant production, suitable and comprehensive process conditions which are directly linked to enhanced yield and scaling up potential must be determined.

2.9 Downstream processing

2.9.1 Recovery and purification

A major contributor to product commercialization is the downstream process. The downstream process involves the recovery and preservation of the biological product. Downstream operations are necessary in ensuring that the biological product is safe to use, stable, consistence, effective and affordable (Moonsamy, 2018; Lalloo, 2010). These operations are known to account for approximately 60% of production cost (Inès and Dhouha, 2015). The selection of the downstream operational unit is dependent on the location of the product (intra- or extra-cellular), charge, solubility and the physico-chemical parameter that effects the desired end-product purity (Satpute *et al*., 2010; Inès and Dhouha, 2015). Numerous downstream techniques have been developed for the extraction and purification of product from the

fermentation broth. Table 2.2 shows the mechanism of the different extraction and purification techniques. The commonly used techniques include centrifugation, acid precipitation, ammonium sulfate precipitation, solvent extraction and foam fractionation. Centrifugation is the first step prior to any selected lipopeptide purification technique. It is used to separate the supernatant and bacterial cells and solid constituents under a centrifugal force (Mukherjee *et al*., 2006; Lalloo, 2010). The resulting cell-free supernatant contains the product which can then be purified by applying other unit operations such as filtration, acid precipitation and chromatography (Table 2.2). The acid precipitation method is the most used of the four techniques. For instance, Zouari *et al*. (2014) used acid precipitation for the extraction of the biosurfactant and successfully extracted 30.67mg/L of lipopeptide surfactant. Likewise, Kumar *et al*. (2017) obtained Iturin A production in the range of 480 – 819 mg/L, when extracted using acid precipitation. Similarly, Nalini *et al*. (2018) used acid precipitation followed by solvent extraction. Another technique is the foam fractionation technique. This is an emerging technique for in situ removal of product. Foam fractionation is particularly valuable when using an integrated system to produce lipopeptides (Chen *et al*., 2015). Foam fractionation was used as a recovery method in the production of biosurfactant using *Bacillus* sp. GY19. It was observed that high aeration and agitation rates are required to increase the recovery and characterization of a foamate with less impurities (Khondee *et al*. 2015).

2.9.2 Detection and characterization

Developed techniques such as chromatographic methods have been employed for detection and characterization of biosurfactant. High performance liquid chromatography (HPLC), hydrophobic interaction chromatography and gel filtration methods are used in the purification of lipopeptide compounds (Kumar *et al*., 2017). The reverse phase high performance liquid chromatography (RP-HPLC) is highly used as it is able to separate each peptide based on the polarity, subsequently ultraviolet absorbance is used for detection and each peak is collected a

fraction collector (Ines and Dhouha, 2015). Other methods of characterization include Fourier Transform Infrared spectroscopy (FTIR) and gas chromatography mass spectrometry (GC-MS) (Kumar *et al*., 2017; Nalini and Parthasarathi, 2018). Najafi *et al*. (2010) used thin layer chromatography (TLC) followed by FTIR for characterization of biosurfactant with similar Retention factors (Rf) and peaks values as surfactin. Extraction of products is not the final step of downstream bioprocessing, formulation and packaging are required and these has to meet marketable standard.

Table 2.2: Common methods of extraction and purification of biosurfactant lipopeptides (Modified from Mukherjee *et al*., 2009)

Process	Mechanism	Product	Reference
Centrifugation	Separates insoluble substances by centrifugal force.	Iturin, *Bacillus cereus* NRRL 100132(Active agent)	Yu *et al*., 2002; Lalloo *et al*., 2009
Acid precipitation	Precipitation at low pH.	Lipopeptide Biosurfactant	Nalini *et al*., 2016; Zouari *et al.,* 2014
Ammonium sulphate precipitation	Uses salt concentrations to salt out the polymeric and protein rich lipopeptide.	Bacteriocin	Avci *et al*., 2017
Solvent system	Biosurfactants lipopeptide are soluble in solvents due to the hydrophobic end of the molecule.	Surfactin, Iturin and Fengycin	Arroyave-Toro *et al*., 2017; Piedrahíta-Aguirre and Alegre, 2014
Foam fractionation	When lipopeptides form, they partition into the foam due to the surface activity.	Surfactin	Davis *et al*., 2001; Rangarajan and Clarke, 2016
Adsorption	The biosurfactant lipopeptide adsorbs to the resin/ activated carbon and desorbed using an organic solvent.	Biosurfactant	Dubey *et al*., 2005; Mazibuko, 2018
Ultra-Filtration	Biosurfactant lipopeptide form micelles and get trapped into the polymeric membrane.	Surfactin	Yu *et al*., 2002; Coutte *et al*., 2013; Sen and Swaminathan, 2005
Chromatography	Biosurfactant is separated by size and/or charge and eluted using a buffer.	Iturin (HPLC)	Kumar *et al*., 2017; Mukherjee *et al*., 2009

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2.9.3 Bioproduct marketability package strategies

The agricultural industry future is reliant on the use of microbial control for environmental sustainability, and for this to be achieved microbial control must meet appropriate formulation requirements. Formulation development plays a major role in addressing the following requirements for successful commercialization: (1) ensures stabilization during distribution and storage; (2) allows for ease handling and application of product; (3) provides protection against environmental factors; (4) Enhance activity of biocontrol agent (Brar *et al*., 2006; Schisler *et al*., 2014).

Bacillus spp. strain formulation of microbial control is more successful as the organism has a resistant stage. Formulations to be used against plant pathogens is readily achievable when the microbial biomass possess a resistant life stage. *Bacillus* spp. have an advantage as they possess this stage (endospore stage). Therefore, the robustness of *Bacillus* spores allows for the formulation process that is more severe and would not be viable for microbial biomass without spore forming ability. Biocontrol agent formulation characteristics and constituents vary depending on the type of habitat, pathogen, rheology of material, host-pathogen environment interactions, mode of application and rate of application. The type of media in fermentation, fermentation conditions, and downstream processing influence the final product outcome. In spite of the apparent advantage, the difficulty of formulation research is the scarcity/rarity of information available.

In general formulations are classified into dry solid and liquid formulations (Table 2.3). Dry solid formulations comprise of dusts, granules, powders, while the liquid formulations are suspensions of oil, water based or emulsions (Brar *et al*., 2006; Bashan *et al*., 2014).

2.9.4 Dry solid formulations

Dry formulation products include wettable powders, dusts, and granules. Wettable powders comprise of dry inactive and active ingredients (biomass) intended to be applied as a suspension in liquid. Dusts are powder-like and consist of dry inactive and active ingredients to be applied dry, generally to seeds or foliage. Dusts are formulated by the sorption of an active agent onto a finely ground, solid inert material such as talc, clay, or chalk, with particle size ranging from 50–100 mm. Although, finer particles adhere better, they pose serious inhalation hazard for the user and drift hazard for the sprayer. Granules can be described as a free flowing, aggregated product composed of dry inactive and active ingredients. They can be applied directly to the target plant, in furrow, or in the case of water dispersible granules, mixed into water where the suspension of biomass and inactive ingredients are applied to targets as a spray (Brar *et al*., 2006). Current formulations of *Bacillus* species are mostly aqueous solutions or wettable powders. Although they have exhibited promising bio-logical control activity, these formulations are disadvantaged by their short shelf life, lower stability and difficulty in transport. It remains a major challenge to successfully develop effective formulations and scale-up productions of the organisms.

Dry flowable formulations contain inert ingredients, which pro-long the shelf life and increase the efficacy of the products. It has been considered as an important development direction of pesticide formulations. Spray drying has been commonly used in pharmaceutical industries, and dry flowable formulations could be synthesized by spray drying because of its lower cost and higher energy efficiency (Meng *et al*., 2015). Spray-drying is drying method that can be used to preserve biocontrol agents in a dry state and has the advantage of being able to dry large quantities of cultures in a short time and at low cost. Only a small population of microorganisms, are able to survive the high temperatures used in this drying process. Biocontrol agents that are able to produce heat-resistant endospores, such as *Bacillus*, are

suitable for spray drying. In contrast, liquid formulations are the simplest way to stabilize the viability of microbial cells (Schisler *et al*., 2004).

2.9.5 Liquid formulation

This formulation method involves storing cells in a water- or oil-based solution with different protectants and additives, typically at low temperatures. Liquid formulation products are also known as flowable or aqueous suspensions and consist of biomass suspensions in water, oils, or combinations of both. Flowable are liquid suspensions that contain particulates at different concentration; 10 – 40 % microorganisms, 1 – 3 % suspender ingredients, 1 – 5 % dispersant, 3 – 8 %, and 35 – 65 % carrier liquid (oil or water). The particulates are prevented from settling due to the reversible accumulation by dispersants; surfactants act as wetting agents and spreaders, generally, non-ionic ones are favored with water soluble sunscreens. Emulsions comprise of liquid droplets dispersed in another immiscible liquid, e.g., oil – in – water (normal emulsions) and water – in – oil (invert emulsions). The emulsions do not have sedimentation problems, however creaming and layer separation occurs. Losses due to evaporation and spray drifting are limited due to the oil being an external phase in the invert emulsions. Yet, overall performance is affected by the phytotoxicity and lower shelf-life stability. Encapsulations are recent developments in bioproduct formulations and protect from extreme environmental conditions and improved residual stability due to slow release of formulations. They are liquid suspensions with the option of powders or granules as well. Microbial propagules are encapsulated in a capsule made of gelatin, starch, cellulose and other polymers and sometimes microbial cells (also referred to as ghost encapsulations). Originally encapsulation was used as an addition to chemical pesticides. These encapsulations involved addition of clays and matrices such as polyvinyl propylene and polyvinyl alcohol, which led to the exploitation of biopolymers to ensure eco-friendly products. Fine encapsulated bioproducts can be sprayed in any volume as the pathogen is held tightly by the additives.

The development of liquid formulation has several advantages including high cell count, zero contamination, greater protection against environmental stresses and increased field efficacy (Brar *et al*., 2006). In liquid formulation, the microbial organisms are present in a dormant cyst form and after application in the field, the dormant form gives rise to active cells. The dormant stage helps increase the shelf life of liquid formulations for roughly twelve (12) months. Formulation information has been kept as propriety of biocontrol agent companies. Also, the adoption of biological agents is lacking in commercial applications due to limitations in product development that address key end user product requirements. Such requirement include cost, efficacy, shelf life and convenience. Hence, research on the appropriate formulation that meet these requirements are to ensure the commercialization of the product.

Table 2.3: Selected *Bacillus*-based plant disease biocontrol products and their formulation types (Modified from Schisler *et al*., 2004)

Product name	Formulation types
Serenade	Wettable powder (Dry)
EcoGaurd	Flowable (Liquid)
Kodiak	Wettable powder (Dry), flowable (Liquid)
Yield Shield	Wettable powder concentrate (Dry)
BioYield	Dry flake (Dry)

2.10 Challenges and prospects of *Bacillus* based biocontrol agents

Bacillus species have proven to be effective in control of plant pathogen when used in antifungal assays. However, production and processing have not reached industrial scale due to low titre and complexity of selecting efficient unit operation for post fermentation recovery. The scarcity of formulation research has made it even more difficult to achieve commercialization of product that meets all the requirements. Therefore, bioprocess

development needs to ensure that all process parameters affecting production need to be improved to maximize lipopeptide production. Hence, process kinetics, modelling and optimization need to be explored for better product yields. For product commercialization, the potential scale up approaches need also to be explored. Similarly, maximizing product recovery and viability during downstream processing are imperative.

References

Abdulkhair, W.M. and Alghuthaymi, M.A., 2016. Plant pathogens. *Plant growth*, *49*.

Agrios, G.N., 2005. Sclerotinia diseases In. *Plant pathology. 5th ed. Elsevier Academic Press, New York, NY*, pp.546-550.

Ahemad, M. and Kibret, M., 2014. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *Journal of King saud University-science*, *26*(1), pp.1-20.

Al-Thubiani, A.S., Maher, Y.A., Fathi, A., Abourehab, M.A., Alarjah, M., Khan, M.S. and Al-Ghamdi, S.B., 2018. Identification and characterization of a novel antimicrobial peptide compound produced by *Bacillus megaterium* strain isolated from oral microflora. *Saudi pharmaceutical journal*, *26*(8), pp.1089-1097.

Aloo, B.N., Makumba, B.A. and Mbega, E.R., 2019. The potential of *Bacilli* rhizobacteria for sustainable crop production and environmental sustainability. *Microbiological Research*, *219*, pp.26-39.

Amer, G.A. and Utkhede, R.S., 2000. Development of formulations of biological agents for management of root rot of lettuce and cucumber. *Canadian journal of microbiology*, *46*(9), pp.809-816.

Arroyave-Toro, J.J., Mosquera, S. And Villegas-Escobar, V., 2017. Biocontrol activity of *Bacillus subtilis* EA-CB0015 cells and lipopeptides against postharvest fungal pathogens. *Biological control*, *114*, pp.195-200.

Avcı, A., Çağrı-Mehmetoğlu, A. and Arslan, D., 2017. Production of antimicrobial substances by a novel *Bacillus* strain inhibiting *Salmonella Typhimurium*. *LWT*, *80*, pp.265-270.

Bashan, Y., De-Bashan, L.E., Prabhu, S.R. And Hernandez, J.P., 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil*, *378*(1-2), pp.1-33.

Barros, F.F.C., Ponezi, A.N. and Pastore, G.M., 2008. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. *Journal of Industrial Microbiology and Biotechnology*, *35*(9), pp.1071-1078.

Bocchini, D.A., Alves-Prado, H.F., Baida, L.C., Roberto, I.C., Gomes, E. and Da Silva, R., 2002. Optimization of xylanase production by *Bacillus circulans* D1 in submerged fermentation using response surface methodology. *Process Biochemistry*, *38*(5), pp.727-731.

Borriss, R., 2011. Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. *Bacteria in agrobiology: Plant growth responses*, pp.41-76.

Bottone, E.J. and Peluso, R.W., 2003. Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against *Mucoraceae* and *Aspergillus* species: preliminary report. *Journal of Medical Microbiology*, *52*(1), pp.69-74.

Brar, S.K., Verma, M., Tyagi, R.D. and Valéro, J.R., 2006. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. *Process biochemistry*, *41*(2), pp.323-342.

Carrera, P., Cosmina, P. and Grandi, G., Eni Tecnologie SpA, 1993. *Method of producing surfactin with the use of mutant of Bacillus subtilis*. U.S. Patent 5,227,294.

Chen, W.C., Juang, R.S. and Wei, Y.H., 2015. Applications of a lipopeptide biosurfactant, surfactin, produced by microorganisms. *Biochemical Engineering Journal*, *103*, pp.158-169.

Coutte, F., Lecouturier, D., Leclère, V., Béchet, M., Jacques, P. and Dhulster, P., 2013. New integrated bioprocess for the continuous production, extraction and purification of lipopeptides produced by *Bacillus subtilis* in membrane bioreactor. *Process Biochemistry*, *48*(1), pp.25-32.

Cawoy, H., Bettiol, W., Fickers, P. and Ongena, M., 2011. Bacillus-based biological control of plant diseases. *Pesticides in the modern world-pesticides use and management*, pp.273-302.

Davis, D.A., Lynch, H.C. and Varley, J., 2001. The application of foaming for the recovery of surfactin from *B. subtilis* ATCC 21332 cultures. *Enzyme and microbial technology*, *28*(4-5), pp.346-354.

Dean, R., Van Kan, J.A., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G.D., 2012. The Top 10 fungal pathogens in molecular plant pathology. *Molecular plant pathology*, *13*(4), pp.414-430.

Dubey, K.V., Juwarkar, A.A. and Singh, S.K., 2005. Adsorption—; Desorption Process Using Wood-Based Activated Carbon for Recovery of Biosurfactant from Fermented Distillery Wastewater. *Biotechnology progress*, *21*(3), pp.860-867.

Deniz, I., Imamoglu, E. and Sukan, F.V., 2015. Evaluation of scale-up parameters of bioethanol production from *Escherichia coli* KO11/[*Escherichia coli* KO11 suşundan biyoetanol üretimi için ölçek büyütme parametrelerinin değerlendirilmesi]. *Turkish Journal of Biochemistry*, *40*(1), pp.74-80.

Fira, D., Dimkić, I., Berić, T., Lozo, J. and Stanković, S., 2018. Biological control of plant pathogens by *Bacillus* species. *Journal of biotechnology*, *285*, pp.44-55.

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N. and Esposito, G., 2015. A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products. *Applied Energy*, *144*, pp.73-95.

Grahovac, J., Grahovac, M., Dodić, J., Bajić, B. and Balaž, J., 2014. Optimization of cultivation medium for enhanced production of antifungal metabolites by *Streptomyces hygroscopicus*. *Crop Protection*, *65*, pp.143-152.

Granato, D. and de Araújo Calado, V.M., 2014. The use and importance of design of experiments (DOE) in process modelling in food science and technology. *Mathematical and statistical methods in food science and technology*, *1*(1), pp.1-18.

Guo, Q., Dong, W., Li, S., Lu, X., Wang, P., Zhang, X., Wang, Y. and Ma, P., 2014. Fengycin produced by *Bacillus subtilis* NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. *Microbiological Research*, *169*(7-8), pp.533-540.

Guo, N., Song, X.R., Kou, P., Zang, Y.P., Jiao, J., Efferth, T., Liu, Z.M. and Fu, Y.J., 2018. Optimization of fermentation parameters with magnetically immobilized *Bacillus natto* on Ginkgo seeds and evaluation of bioactivity and safety. *Lwt*, *97*, pp.172-179.

Inès, M. and Dhouha, G., 2015. Lipopeptide surfactants: production, recovery and pore forming capacity. *Peptides*, *71*, pp.100-112.

Jangir, M., Pathak, R., Sharma, S. and Sharma, S., 2018. Biocontrol mechanisms of *Bacillus* sp., isolated from tomato rhizosphere, against *Fusarium oxysporum f. sp. lycopersici. Biological Control*, *123*, pp.60-70.

Jha, S.S., Joshi, S.J. and SJ, G., 2016. Lipopeptide production by *Bacillus subtilis* R1 and its possible applications. *brazilian journal of microbiology*, *47*, pp.955-964.

Jiao, S., Li, X., Yu, H., Yang, H., Li, X. and Shen, Z., 2017. In situ enhancement of surfactin biosynthesis in *Bacillus subtilis* using novel artificial inducible promoters. *Biotechnology and Bioengineering*, *114*(4), pp.832-842.

Jung, J., Yu, K.O., Ramzi, A.B., Choe, S.H., Kim, S.W. and Han, S.O., 2012. Improvement of surfactin production in *Bacillus subtilis* using synthetic wastewater by overexpression of specific extracellular signaling peptides, comX and phrC. *Biotechnology and Bioengineering*, *109*(9), pp.2349-2356.

Julca, I., Droby, S., Sela, N., Marcet-Houben, M. and Gabaldon, T., 2016. Contrasting genomic diversity in two closely related postharvest pathogens: *Penicillium digitatum* and *Penicillium expansum*. *Genome Biology and Evolution*, *8*(1), pp.218-227.

Keen, N.T., 2000. A century of plant pathology: a retrospective view on understanding host-parasite interactions. *Annual Review of Phytopathology*, *38*(1), pp.31-48.

Keller, K., Friedmann, T. and Boxman, A., 2001. The bioseparation needs for tomorrow. *TRENDS in Biotechnology*, *19*(11), pp.438-441.

Khondee, N., Tathong, S., Pinyakong, O., Müller, R., Soonglerdsongpha, S., Ruangchainikom, C., Tongcumpou, C. and Luepromchai, E., 2015. Lipopeptide biosurfactant production by chitosan-immobilized *Bacillus* sp. GY19 and their recovery by foam fractionation. *Biochemical Engineering Journal*, *93*, pp.47-54.

Krishania, M., Sindhu, R., Binod, P., Ahluwalia, V., Kumar, V., Sangwan, R.S. and Pandey, A., 2018. Design of bioreactors in solid-state fermentation. In *Current Developments in Biotechnology and Bioengineering* (pp. 83-96). Elsevier.

Kumar, P.N., Swapna, T.H., Khan, M.Y., Reddy, G. and Hameeda, B., 2017. Statistical optimization of antifungal iturin A production from *Bacillus amyloliquefaciens* RHNK22 using agro-industrial wastes. *Saudi journal of biological sciences*, *24*(7), pp.1722-1740.

Lalloo, R., 2010. *Development of a bioprocess for the production of an aquaculture biological agent* (Doctoral dissertation, Stellenbosch: University of Stellenbosch).

Lalloo, R., Ramchuran, S., Ramduth, D., Görgens, J. and Gardiner, N., 2007. Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish. *Journal of Applied Microbiology*, *103*(5), pp.1471-1479.

Makovitzki, A., Viterbo, A., Brotman, Y., Chet, I. and Shai, Y., 2007. Inhibition of fungal and bacterial plant pathogens in vitro and in planta with ultrashort cationic lipopeptides. *Applied and environmental microbiology*, *73*(20), pp.6629-6636.

Mardanova, A.M., Hadieva, G.F., Lutfullin, M.T., Khilyas, I.V.E., Minnullina, L.F., Gilyazeva, A.G., Bogomolnaya, L.M. and Sharipova, M.R., 2016. *Bacillus subtilis* strains with antifungal activity against the phytopathogenic fungi. *Agricultural Sciences*, *8*(1), pp.1-20.

Mazibuko, S., 2018. *Purification of Bacillus amyloliquefaciens lipopeptides for postharvest disease control* (Doctoral dissertation, Stellenbosch: Stellenbosch University).

Meng, X., Yu, J., Yu, M., Yin, X. and Liu, Y., 2015. Dry flowable formulations of antagonistic *Bacillus subtilis* strain T429 by spray drying to control rice blast disease. *Biological control*, *85*, pp.46-51.

Mendgen, K., 1996. Fungal attachment and penetration.

Mendgen, K., Hahn, M. and Deising, H., 1996. Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Annual review of phytopathology*, *34*(1), pp.367-386.

Moonsamy, G., 2019. *Development of a bioprocess technology for the production of Vibrio midae, a probiotic for use in abalone aquaculture* (Doctoral dissertation).

Moodley, G., Mashigo, L., Lalloo, R. and Singh, S., 2014. Application of biological agents in abalone aquaculture: a South African perspective. *Sustainable aquaculture techniques*, pp.207-237.

Mukherjee, S., Das, P. and Sen, R., 2006. Towards commercial production of microbial surfactants. *TRENDS in Biotechnology*, *24*(11), pp.509-515.

Mukherjee, S., Das, P., Sivapathasekaran, C. and Sen, R., 2009. Antimicrobial biosurfactants from marine *Bacillus circulans*: extracellular synthesis and purification. *Letters in applied microbiology*, *48*(3), pp.281-288.

Mulligan, C.N., Chow, T.Y.K. and Gibbs, B.F., 1989. Enhanced biosurfactant production by a mutant *Bacillus subtilis* strain. *Applied microbiology and biotechnology*, *31*, pp.486-489.

Myo, E.M., Liu, B., Ma, J., Shi, L., Jiang, M., Zhang, K. and Ge, B., 2019. Evaluation of *Bacillus velezensis* NKG-2 for bio-control activities against fungal diseases and potential plant growth promotion. *Biological Control*, *134*, pp.23-31.

Najafi, A.R., Rahimpour, M.R., Jahanmiri, A.H., Roostaazad, R., Arabian, D. and Ghobadi, Z., 2010. Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. *Chemical Engineering Journal*, *163*(3), pp.188-194.

Nalini, S. and Parthasarathi, R., 2013. Biosurfactant production by *Serratia rubidaea* SNAU02 isolated from hydrocarbon contaminated soil and its physico-chemical characterization. *Bioresource technology*, *147*, pp.619-622.

Nalini, S., Parthasarathi, R. and Prabudoss, V., 2016. Production and characterization of lipopeptide from *Bacillus cereus* SNAU01 under solid state fermentation and its potential application as anti-biofilm agent. *Biocatalysis and Agricultural Biotechnology*, *5*, pp.123-132.

Nanjundan, J., Ramasamy, R., Uthandi, S. and Ponnusamy, M., 2019. Antimicrobial activity and spectroscopic characterization of surfactin class of lipopeptides from *Bacillus amyloliquefaciens* SR1. *Microbial pathogenesis*, *128*, pp.374-380.

Nwabueze, T.U., 2010. Basic steps in adapting response surface methodology as mathematical modelling for bioprocess optimisation in the food systems. *International Journal of Food Science & Technology*, *45*(9), pp.1768-1776.

Ohadi, M., Dehghannoudeh, G., Shakibaie, M., Banat, I.M., Pournamdari, M. and Forootanfar, H., 2017. Isolation, characterization, and optimization of biosurfactant production by

an oil-degrading *Acinetobacter junii* B6 isolated from an Iranian oil excavation site. *Biocatalysis and Agricultural Biotechnology*, *12*, pp.1-9.

Ongena, M. and Jacques, P., 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in microbiology*, *16*(3), pp.115-125.

Ongena, M., Jacques, P., Touré, Y., Destain, J., Jabrane, A. and Thonart, P., 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. *Applied microbiology and biotechnology*, *69*, pp.29-38.

Pandey, A., Soccol, C.R. and Mitchell, D., 2000. New developments in solid state fermentation: I-bioprocesses and products. *Process biochemistry*, *35*(10), pp.1153-1169.

Patel, A.K., Deshattiwar, M.K., Chaudhari, B.L. and Chincholkar, S.B., 2009. Production, purification and chemical characterization of the catecholate siderophore from potent probiotic strains of *Bacillus* spp. *Bioresource technology*, *100*(1), pp.368-373.

Pawlowski, M.L. and Hartman, G.L., 2016. Infection mechanisms and colonization patterns of fungi associated with soybean. *Fungal pathogenicity*, *25*.

Peng, W., Zhong, J., Yang, J., Ren, Y., Xu, T., Xiao, S., Zhou, J. and Tan, H., 2014. The artificial neural network approach based on uniform design to optimize the fed-batch fermentation condition: application to the production of iturin A. *Microbial Cell Factories*, *13*(1), pp.1-10.

Piedrahíta-Aguirre, C.A. and Alegre, R.M., 2014. Production of lipopeptide iturin a using novel strain *Bacillus iso* 1 in a packed bed bioreactor. *Biocatalysis and Agricultural Biotechnology*, *3*(2), pp.154-158.

Pretorius, D., 2014. *Antimicrobial lipopeptide production by Bacillus spp. for post-harvest biocontrol* (Doctoral dissertation, Stellenbosch: Stellenbosch University).

Qazizada, M.E., 2016. Design of a batch stirred fermenter for ethanol production. *Procedia Engineering*, *149*, pp.389-403.

Qazizada, M.E., 2016. Design of a batch stirred fermenter for ethanol production. *Procedia Engineering*, *149*, pp.389-403.

Quadt-Hallmann, A.N.D.R.E.A., Kloepper, J.W. and Benhamou, N., 1997. Bacterial endophytes in cotton: mechanisms of entering the plant. *Canadian journal of microbiology*, *43*(6), pp.577-582.

Radovanović, N., Milutinović, M., Mihajlovski, K., Jović, J., Nastasijević, B., Rajilić-Stojanović, M. and Dimitrijević-Branković, S., 2018. Biocontrol and plant stimulating potential of novel strain *Bacillus* sp. PPM3 isolated from marine sediment. *Microbial pathogenesis*, *120*, pp.71-78.

Satpute, S.K., Banpurkar, A.G., Dhakephalkar, P.K., Banat, I.M. and Chopade, B.A., 2010. Methods for investigating biosurfactants and bioemulsifiers: a review. *Critical reviews in biotechnology*, *30*(2), pp.127-144.

Schaechter, M., 2009. *Encyclopedia of microbiology*. Academic Press.

Schisler, D.A., Slininger, P.J., Behle, R.W. and Jackson, M.A., 2004. Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology*, *94*(11), pp.1267-1271.

Sen, R. and Swaminathan, T., 2005. Characterization of concentration and purification parameters and operating conditions for the small-scale recovery of surfactin. *Process Biochemistry*, *40*(9), pp.2953-2958.

Stein, T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular microbiology*, *56*(4), pp.845-857.

Subramaniyam, R. and Vimala, R., 2012. Solid state and submerged fermentation for the production of bioactive substances: a comparative study. *Int J Sci Nat*, *3*(3), pp.480-486.

Wang, T., Liang, Y., Wu, M., Chen, Z., Lin, J. and Yang, L., 2015. Natural products from *Bacillus subtilis* with antimicrobial properties. *Chinese Journal of Chemical Engineering*, *23*(4), pp.744-754.

Wei, Y.H., Wang, L.F., Changy, J.S. and Kung, S.S., 2003. Identification of induced acidification in iron-enriched cultures of *Bacillus subtilis* during biosurfactant fermentation. *Journal of bioscience and bioengineering*, *96*(2), pp.174-178.

Wei, Y.H., Lai, C.C. and Chang, J.S., 2007. Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. *Process Biochemistry*, *42*(1), pp.40-45.

Wu, Q., Zhi, Y. and Xu, Y., 2019. Systematically engineering the biosynthesis of a green biosurfactant surfactin by *Bacillus subtilis* 168. *Metabolic engineering*, *52*, pp.87-97.

Yu, G.Y., Sinclair, J.B., Hartman, G.L. and Bertagnolli, B.L., 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. *Soil Biology and Biochemistry*, *34*(7), pp.955-963.

Zouari, R., Ellouze-Chaabouni, S. and Ghribi-Aydi, D., 2014. Optimization of *Bacillus subtilis* SPB1 biosurfactant production under solid-state fermentation using by-products of a traditional olive mill factory. *Achievements in the Life Sciences*, *8*(2), pp.162-169.

Zhao, J., Li, Y., Zhang, C., Yao, Z., Zhang, L., Bie, X., Lu, F. and Lu, Z., 2012. Genome shuffling of *Bacillus amyloliquefaciens* for improving antimicrobial lipopeptide production and an analysis of relative gene expression using FQ RT-PCR. *Journal of Industrial Microbiology and Biotechnology*, *39*(6), pp.889-896.

CHAPTER 3

Process development for antifungal production by *Bacillus subtilis* BS20: Optimization and nanoparticle supplementation

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This chapter has been prepared to be submitted for publication in a peer review journal-*Process Biochemistry* with the title: Process development for antifungal production by *Bacillus subtilis* BS20: Optimization and nanoparticle supplementation. The manuscript is presented in the following pages.

Process development for antifungal production by *Bacillus subtilis* BS20: Optimization and nanoparticle supplementation

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Abstract

This study investigated the effect of different process parameters on *Bacillus subtilis* BS20 biosurfactant production, as well as the impact of nano-biocatalyst on biosurfactant production. Furthermore, the logistic, kinetic model was used to elucidate the dynamics of *B. subtili*s BS20 growth during the biosurfactant production. The response surface methodology model was used to optimize the effect of glucose concentration (10 – 30 g/L), incubation temperature (25 – 45°C) and incubation time (24 – 96h) on biosurfactant (antifungal metabolite) production. The results showed that there was a positive relationship between the input parameters and the biosurfactant production with high coefficient of determination (R^2) > 0.86. The optimized conditions (glucose concentration-11.5 g/L, incubation temperature-41°C and incubation time-24 h) resulted in maximum antifungal (biosurfactant) activity of 68 mm. This was 1.13-fold high than the control experiment (60 mm). Moreover, supplementary inclusion of nanoparticles (NP) significantly improved biomass concentration (10.28 g/L) compared to the control experiment (1.90 g/L). The study demonstrates the potential of improving biosurfactant production with high antifungal activity representing a prospective transition towards large scale production.

Keywords: Antifungal activity; Response surface methodology; Nano biocatalyst; Kinetic logistic model

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3. Introduction

In recent times, the rising concerns of environmental health has directed farmers towards biobased agricultural practices and products such as biosurfactants. The cost and environmental concerns of using conventional chemical fertilizers are problems that affect crop productivity and accessibility (Tredgold, 2021). Hence, eco-friendly practices are crucial for the attainment of sustainable commercial scale crop production. Additionally, there is global increase in food demand due to overall growth in world population. The world's population has increased to over 7 billion, and currently the United Nations estimates that the world population will increase to ~10 billion people by the year 2050 (United Nations, 2022). This has made the development of a biobased food loss control such as use of biosurfactant an imperative approach in mitigating the negative impact of synthetic pesticides (Faloye, 2015). These synthetic chemicals have negative side effects towards both the environment and human. Synthetic fungicides cause environmental pollution due to their slow degradation leading to toxic residue (Wu *et al*., 2018). Furthermore, synthetic-based/chemically derived products require strict regulation regarding maximum limits which is not required in the use of bio-based products such as biosurfactant (Pretoruis *et al*., 2014).

Biosurfactants are one of the most important materials used in various industries for their benefits. These can be used in the agricultural industry, hair conditioning industry, and in the improvements of oil recovery (Heryani and Putra, 2017). They are natural surfactant biomolecules produced by microorganism such as *Bacillus* spp. which have gained interest from various industries for their eco-friendliness (Heryani and Putra, 2017; Kim *et al*., 2004). Biosurfactant consists of hydrophilic and lipophilic moieties. The hydrophilic component of the biosurfactant is made up of the hydrocarbon chain of fatty acid or sterol ring while the lipophilic component consists of the carboxyl group of fatty acids or amino acids, the phosphoryl group of phospholipids and the hydroxyl group of the saccharides, and peptides

(Kim *et al*., 2004). These biosurfactants are classified into groups according to their chemical structure: lipopeptides, glycolipids polysaccharides and lipopolysaccharides (Zouari *et al.*, 2014; Gürkök and Özdal, 2021). Lipopeptides are a group of biosurfactants that are known to play a major role in biological activities such as antibacterial, antifungal, antiviral and cytolytic activities (Kim *et al*., 2004; Heryani and Putra, 2017). These have become attractive globally for biological control of microbial food spoilage since chemical pesticides are toxic and non-biodegradable (Borriss, 2011). The global issue of microbial food spoilage has resulted in major effects such as crop spoilage and foodborne illnesses (Alegbeleye, 2022). Recent report by the FAO show that approximately 600 million cases of foodborne illness and 40 000 deaths occur annually across the globe (FAO, 2022). Moreover, the annual FAO recent document reported that the global application of pesticides went up 36%. Thus, to achieve sustainable environmental health and alleviate the threat to food security the development of alternative methods in food pathogen control is imperative (FAO, 2021). Hence, biological based food pathogen control such as the use of surface active lipopeptides has become indispensable.

Lipopeptides are non-ribosomal enzyme complexes that represent the most common class of antifungal compounds produced by *Bacillus* sp. These amphiphilic lipopeptides have a common cyclic structure, however, they are further classified into three different families based on their amino acid sequence: surfactin, iturin and fengycin families (Hmidet *et al*., 2017). These lipopeptide biosurfactants act synergistically to effectively reduce and hinder plant pathogens (Wang *et al*., 2015; Ongena and Jacques, 2008). The production of lipopeptide biosurfactants is sustainable and eco-friendly compared to synthetic chemical pesticides. Although, pesticide is efficient in food pathogen control but are not sustainable due to environmental, and health concerns (Mardanova *et al*., 2017). Traditionally the agricultural sector uses chemically synthesised compounds to control pathogens. Synthetic fungicides are extensively used to control plant phytopathogens in pre-and post-harvest plants, and these are

considered one of the cheapest and most effective controls of these plant diseases (Bocchini *et al*., 2002). However, lipopeptide biosurfactants offer great advantage over synthetic compounds because they are highly biodegradable, less toxic, and environmentally friendly (Lim *et al*., 2017). Presently microorganisms produce lipopeptides as a protective agent for themselves against other microorganisms for competition of nutrients among other things (Rebib *et al*., 2012; Kim *et al*., 2004). One of the most effective and powerful lipopeptides is surfactin produced by *Bacillus* (Heryani and Putra, 2017). Similarly, *Bacillus* species are known for their ability to synthesize a variety of antimicrobial compounds that have shown variable antagonistic effects against bacterial and fungal phytopathogens (Kespar *et al*., 2019). The cyclic lipopeptide family are the most important of these antimicrobial as they are responsible for *Bacillus*-plant root associations and are known to demonstrate efficient pathogen control. *Bacillus* spp., such as *B. subtilis* and *B. amyloliquefaciens*, were reported to produce various plant bactericides and fungicides (Rebib *et al*., 2012). However, production processes of these antimicrobial compounds have not reached the desirable yields. There is a death of study on improving biosurfactant yield. Presently, *Bacillus* spp naturally produce antimicrobial compounds at low quantities which has driven researcher to make notable efforts to enhance their antimicrobial production quantitatively and qualitatively.

These biosurfactant production are influenced by operational parameters such as carbon source, pH, incubation time and temperature (Ghribi and Ellouze-Chaabouni, 2011; Mouafi *et al*., 2016; Ebadipour *et al*., 2016; Heryani and Putra, 2017), Despite the abundance of previous studies on key input parameters, determining optimum parameter conditions that effectively facilitates microbial growth and metabolism for biosurfactant production is essential for improving biosurfactant yield. Therefore, the application of optimization tools to ensure an improved overall process performance and high biosurfactant yield as well as high product quality at lower process cost is desirable. The traditional used of one variable at a time (OVAT)

methods for process optimization have been found to be limited, hence, the use of modelling tools such as response surface methodology (RSM), genetic algorithm (GA) and artificial neuron network (ANN). These are mathematical models that help understand the effect of several independent variables on dependent variables or a response. Moreover, parameter interaction during the production process to enhance product formation are analyzed (Nwabueze, 2010). Employment of such models stipulate/specify set points that drive bioprocesses to their ideal course of optimization and allow for better process design and regulation. Response surface methodology is a step by step mathematical, statistical, and empirical techniques developed for process optimization. The merits of this technique include, least experimental runs, less process time, flexibility of variable assigning and closer confirmation of the output response to the target requirements (Talasila and Vechalapu, 2015).

Aside the use of process optimization tools, biocatalytic materials such as nanoparticles have recently been employed to improve bioprocess productivity. Nanoparticles have gained much attention due to their unmatched physical and chemical properties compared to their macro counterparts. These are gaining prominence in industries such as biomedical science, environmental science biotechnology, optics, magnetics, catalysis, and energy science. The application of nano biocatalyst agents in bioprocessing is to enhance process productivity through increased mass and heat transfer, enzymatic and cell metabolic activities arising from their large surface areas, catalytic properties, growth, and enzyme cofactor functionality. Furthermore, nanoparticles are known to improve the capacity of electron donor reactions and enhance biological activity of microbes, thus improving kinetic bioprocess (Yang *et al*., 2020; Sanusi *et al*., 2021). Yet, there exist a dearth of knowledge in nano supplementation application in lipopeptide biosurfactant production due to poor understanding of such processes. Therefore, this study is aimed at optimal biosurfactant production by *B. subtilis* BS20. The individual and interactive effects of glucose concentration, process temperature and incubation time on

biosurfactant production was investigated using response surface methodology. Additionally, the evaluation of nano biocatalyst to improve *B. subtilis* BS20 growth and biosurfactant production was undertaken.

3.1 Materials and Methods

3.1.1 Microorganisms and inoculum preparation

Bacillus subtilis BS20 obtained from Dr S. Ramchuran, the Discipline of Microbiology, University of KwaZulu-Natal, Durban, South Africa, was used in this study. A single flask containing 100 mL Luria Bertani (LB) medium was inoculated with a single colony of the respective *B. subtilis* for inoculum development and grown at 120 rpm, 30^{o}C for 24 h. The culture was then used as the inoculum for subsequent fermentation processes in the biosurfactant production.

3.1.2 Modelling and optimization

3.1.3 Biosurfactant production

Biosurfactant production was carried out using a working volume of 50 ml. Ten percent (5 ml) of the seed culture (10% v/v; ~1×10^{6} cells/mL), was fed into 45 mL of Luria Broth supplemented with varying glucose concentration (10 – 30 g/L), at varying temperature (25 – 45°C), and incubation time (24 – 96 h) according to experimental design on Tables 3.1 and 3.2). Samples for further analysis were taken and bacterial cells were separated using centrifugation (10 000 rpm, 20 min, 4°C). The supernatant that contained the biosurfactant was then used for antifungal activity assay against *Rhizoctonia solani.*

Table 3.1: Modelled parameter operational range

Variables	Ranges			References
	-1	0	+1	
X_1 Glucose (g/L)	10	20	30	Ghribi and Ellouze-Chaabouni, 2011; Mosquera *et al*., 2014
X_2 Temperature (ºC)	25	35	45	Ohno *et al*., 1995; Mouafi *et al*., 2016
X_3 Incubation time (h)	24	48	72	Puri *et al*., 2001; Bocchini *et al*., 2007; Mouafi *et al*., 2016

3.1.4 Nanoparticle preparation

Seven nanoparticles used in this study were prepared using the co-precipitation method (Sanusi *et al*., 2019). Nickel oxide (NiO) nanoparticles (NPs) were synthesized by dissolving an appropriate amount of $NiCl_2.6H_2O$ in distilled water. Then NH_3 solution was added dropwise to reach a pH of 10. The solution was treated with microwave irradiation operated at a power of 700 W for 180 s, and the culmination of the reaction was signaled by the precipitation of light green NiO NPs. The NiO nanoparticles obtained were washed a three times with deionized water and oven dried for six hours.

Iron (III) oxide (Fe_3O_4) NPs were synthesized by dissolving 1.0 g of $FeSO_4.7H_2O$ in distilled water, and the pH was adjusted to 12, then the volume was made up to 200 mL. The solution was heated in a microwave oven at 700 W for 600 s. The obtained black magnetic Fe_3O_4 NPs precipitate was rinsed and dried at 70°C for a 12 h.

Iron (II) Oxide (Fe_2O_3) NPs were synthesized by dissolving 3.24 g of $FeCl_3$ in 20 mL sterile distilled, thereafter ammonium solution was slowly added to the mixture. The pH of the solution was adjusted to 12 prior to microwave radiation treatment (700 W, 30 s on, 60 s off) for 4 min. The precipitate, red brown, was then centrifuged at 1000 rpm for 10 min and rinsed with distilled water, the Fe_2O_3 NP obtained was dried at 70°C for 12 h.

Zinc oxide (ZnO)NPs were prepared by completely dissolving 15 g of starch in 65 wt% $ZnCl_2$ aqueous solution at 80 °C, constantly stirring at 500 r/min. Next, 15 wt% NaOH aqueous solution was added drop wise to the solution at constant stirring of 5000 r/min to achieve a final pH of 8.4. The nano composite obtained was allowed to mature for 30 min at 80°C at constant stirring. To obtain the ZnO NPs the ZnO-starch nanocomposite was calcinated at 575°C for 1 h and subsequently grounded.

The manganese oxide (MnO_2) NPs were prepared using the co-precipitation method, where 6.76 g of $MnSO_4 \cdot H2O$ was dissolved in 40 mL distilled water, followed by the drop wise addition of ammonia to achieve pH 11, with continuous stirring at 60°C for 2 h to precipitate the MnO_2 NPs. The resulting brownish precipitated particles were then washed thrice with distilled water and dried in a hot air oven at 70°C for 12 h.

Cobalt Oxide (CoO) NPs were prepared using a five-step preparation scheme. First dissolve 4.76 g of cobalt (II) chloride hexahydrate ($CoCl \cdot 6H_2O$), in 20 mL distilled water to obtain a greenish solution. This was followed by the addition of ammonia to a pH of 11.3. Thereafter, the mixture was subjected to a microwave irradiation for 3 min at 700 W. In the fourth step, the obtained precipitate was washed three times and lastly, the deep dull green CoO NPs was dried in an oven at 100°C for 6 h.

The copper oxide (CuO) NPs were prepared using copper salt and a reducing agent. 0.04 M of copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) was mixed with 1 M NaOH drop-wisely with continuous stirring to achieve pH 12.7. The mixture was then subjected to microwave irradiation (700 W, 2.5 min). A black-grey precipitate was formed, which was then washed with distilled water and dried overnight at 70°C.

3.1.5 Nanoparticle supplementation set up

Seven nanoparticles (Fe_2O_3, Fe_3O_4, CoO, CuO ZnO, NiO and MnO_2) were assessed for their potential to enhance the growth and antifungal production of *B. subtilis* BS20. Each nanoparticle type was added in an independent set up before incubation at concentrations of 0.01 and 0.05 g/L for the batch fermentation. This was undertaken using a working volume of 50 mL, with process conditions of 11.5 g/L, 41°C and 24 h for glucose concentration, process temperature and incubation time, respectively.

3.2 Analytical methods

3.2.1 Determination of biomass concentration

An exponentially growing (18-24 h) *B. subtilis* BS20 culture grown in LB broth was used. The cell biomass concentration (g/L) was evaluated using the bacterial cell count as a function of concentration of cells. A standard curve was prepared by determining the dry weight and corresponding cell counts at varied dilutions (1, 1/2, 1/4, 1/8 and 1/16). The biomass dry weights were obtained by centrifuging 10 mL of each dilution at 5000 rpm for 10 min. The supernatant was removed, and the biomass cell pellet was dried at 90 °C until a constant mass was obtained.

3.2.2 Antifungal activity –Amended media

The samples collected for each run were centrifuged at 10000 rpm for 10 min to obtain a free-cell supernatant. Thereafter, the supernatant was added to molten Potato Dextrose agar (PDA) at a ratio of 1:10 (v/v) and left to solidify (Goswami and Deka, 2019; Kumar *et al.*, 2017). A mycelial plug was cut out of previously grown fungal isolates and inoculated on the amended media for each strain. The control experiment was a PDA plate inoculated with only the fungal pathogen. All plates were incubated at 30ºC for 3 days. Thereafter, the zone of inhibition was determined.

3.2.3 Nanoparticle characterization

The elemental composition of the NPs was determined by a scanning electron microscope (SEM, ZEISS-EVO/LS15, ZEISS instrument, Germany). Each sample was mounted on an aluminium grid coated with carbon prior to scanning electron microscopy (SEM) analysis. Transmission electron microscopy (TEM) was used to study the shape and the particle size of the NPs. The TEM image was captured on JEM-1400 electron microscope operating at 120 kV. Lastly, to establish the purity and functional nature of the nanoparticles, the Fourier transform infra-red spectra of the NPs was obtained using an FT-IR spectrometer (Spectrum 100, PerkinElmer, USA), recorded from 450 to 4000 cm^{-1}.

3.3 Results and Discussion

3.3.1 Nanoparticles characterization

The SEM Energy dispersive spectrophotometric (EDS) confirmed the incidence of the anticipated elements, the metallic and oxygen unit in each of the seven nanoparticles. The TEM images describe the shape and the particle size of each NPs. The nanoparticles had rough spherical shape, except Co NPs which was irregular in shape (Fig. 3.1). The particle size obtained show an average diameter of 47, 31, 30, 29, 15, 12 and 8 nm was recorded for Fe_2O_3, Fe_3O_4, ZnO, NiO, CuO, CoO, NPs and MnO_2 respectively. The variation in the average diameter may be attributed to differences in microwave treatment, precursors used and precipitation rate. Moreover, Mn-O, Co-O, Cu-O, Ag-O, Zn-O and Fe_3O_4 absorption band were observed at 860, 659, 845, 797, 715 and 664 cm^{-1} respectively. NiO NPs and Fe_2O_3 NPs had an absorption band below 650 cm^{-1}. Oxides and hydroxides of metallic nanoparticles usually give absorption peak below the wavelength of 1000 nm. This arises from inter-atomic vibrations. The other peaks observed suggested the presence of functional groups, with different stretching vibrations of $–CH_3$, $-CH_2$, =C-H, –C-H, C=O, -OH and NH groups (Sanusi *et al*., 2019).

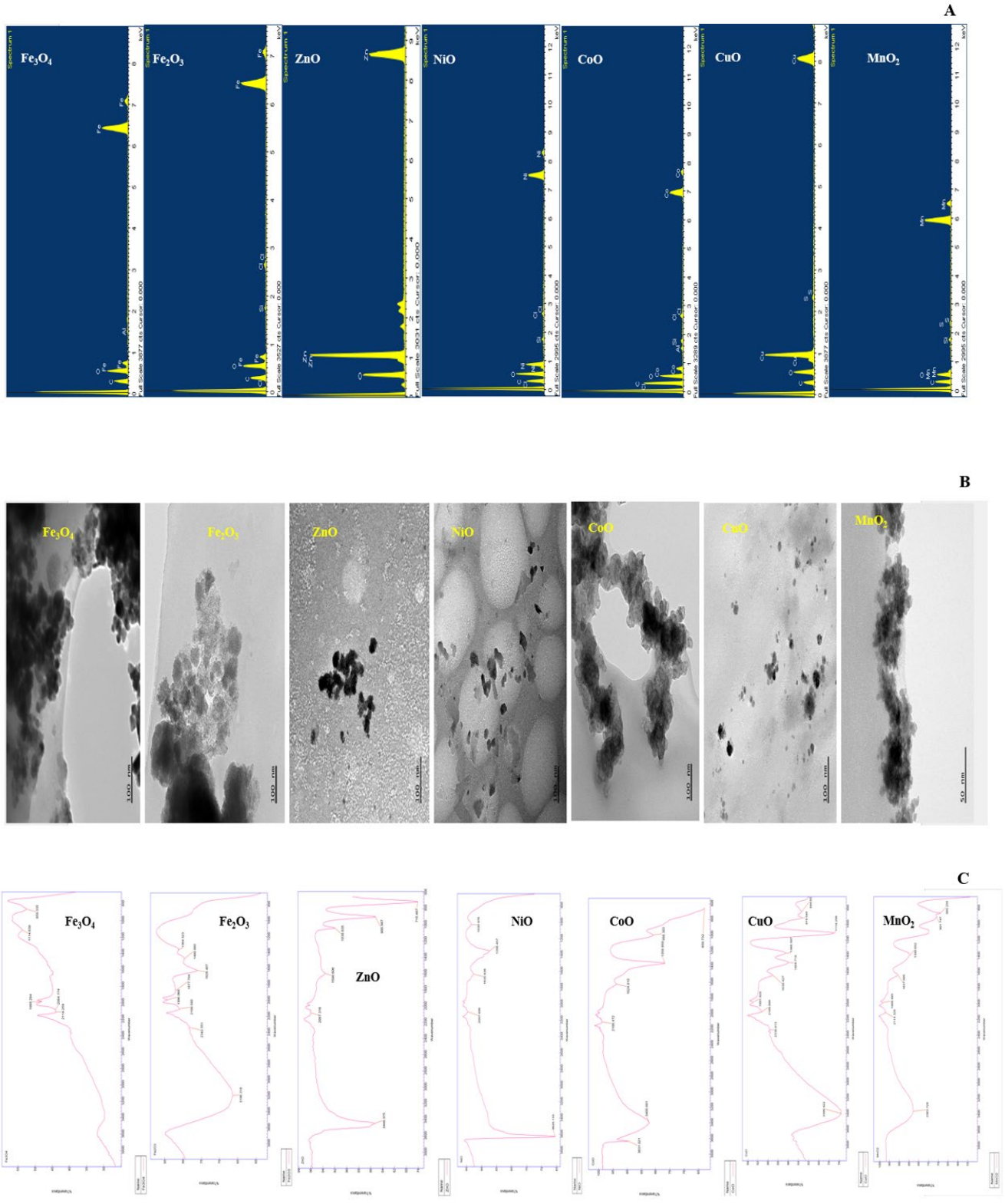

Fig. 3.1: SEM-EDX (A), TEM (B) and FTIR (C) images of the various synthesized nanoparticles

3.3.2 Model development

High coefficient of determination (R^2) of 0.86 was obtained, indicating that the developed model could account for over 86% variability in the observed data. The suitability of the model was further assessed using Analysis of Variance (ANOVA) (Table 3.3). The model had a high F-value of 4.62 and P-value of 0.0279, which indicate model significance (statistically, p-values <0.05 indicate significance) (Mouafi *et al*., 2016). Moreover, the parameters of incubation temperature and time were found to be significant (Table 3.2). The noticeable influence of process temperature on biosurfactant production might be attributed to its impacts on *B. subtilis* BS20 bioactivities and growth kinetics. On the other hand, high temperatures, negatively impact process performance by denaturing the cells' enzymes, shortening the exponential growth phase thus inhibiting biosurfactant production. The incubation time could affect water loss through evaporation, hence, metabolic performance. Water has been identified as an important factor during bioprocessing. This is because water presence influences the dielectric properties of reacting or interacting substances. Also, water improve heating and diffusion of irradiation energy (Aguilar-Reynosa *et al*., 2017).

The model polynomial equation (Eq. 3.1) relates the input parameters and illustrates the linear, interactive, and quadratic effect of the parameters to the antifungal activity (Table 3.1) (Moodley and Kana, 2017).

$$Antifungal\ activity\ (mm) = +67{,}40 - 7{,}62\,A + \mathbf{17,13}\boldsymbol{B} + \mathbf{12,50}\boldsymbol{C} + 14{,}50AB - 1{,}75AC + 14{,}75BC - 0{,}2A^2 - \mathbf{18,20}\boldsymbol{B^2} - 3{,}95C^2 \quad (3.1)$$

Where A = Glucose concentration (g/L), B = Incubation temperature (°C) and C = Incubation time (h).

Table 3.2: Antifungal activity of produced biosurfactant

Run	X_1-Glucose (g/L)	X_2-Temperature (°C)	X_3-Time (h)	Y_1-Antifungal activity (mm)
17	10	25	60	63
16	30	25	60	0
11	10	45	60	69
8	30	45	60	64
2	10	35	24	71
6	30	35	24	78
5	20	35	96	52
1	30	35	96	52
3	20	25	24	57
9	20	45	24	61
7	20	25	96	0
14	20	45	96	68
12	20	35	60	65
15	20	35	60	78
4	20	35	60	63
10	20	35	60	79
13	20	35	60	52

Table 3.3: Model analysis of variance (ANOVA)

		R^2	F-value	p – value	Prob > F
Antifungal activity	**Model**		4.62	0.0279	Significant
	B: Incubation temp.		13.40	0.0081	Significant
	C: Incubation time		7.14	0.0319	Significant
	Lack of fit		1.88	0.2744	Not significant
	Co-efficient of determination	0.86			

3.3.3 Interactive effect of input parameters

Response surface plots were obtained for the interactive effect of each pair of independent variables on the biosurfactant production by *B. subtilis* BS20 (Fig. 3.2). Among the tested

variables the incubation time and the process temperature showed significant effects on the biosurfactant production. The interactive effect of glucose concentration and temperature on biosurfactant production while maintaining the incubation time at its median value is illustrated in Fig. 3.2A. High biosurfactant production was obtained at initial setpoints. Simultaneously increasing the glucose concentration (11 – 20 g/L) and incubation temperature (26 – 45^{o}C) resulted in decreased biosurfactant production. Low glucose concentration correlated with high biosurfactant production. High glucose concentration could result in repression of growth and productivity. Glucose as carbon source is a nutritional requirement for microbial growth and proliferation (Hmidet *et al*., 2017; Ghribi and Ellouze-Chaabouni *et al*., 2011). Contrarily to this study. Hidmet *et al*. (2017) observed the highest lipopeptides production by *Bacillus mojavensis* A21 using a glucose concentration of 30 g/L. Temperature significantly affected biosurfactant production, as any increases in temperature resulted in decreased production. High microbial metabolism has been ascribed to optimal incubating temperature (Nalini *et al*., 2016). In the present study, high biosurfactant production was obtained at 25^{o}C. Ohno *et al*. (1995), evaluated the effect of temperature on iturin and surfactin production and observed that the production of these metabolites had different optimum temperatures of 25^{o}C and 37^{o}C, respectively.

Illustrated in Fig. 3.2B is the interactive effect of process temperature and incubation time. Increasing the incubation temperature (25 – 43^{o}C) while simultaneously varying the incubation time (24 – 60 h) resulted in biosurfactant production with high antifungal activity (62 mm). Further increase in process temperature (>43^{o}C) resulted in slight decrease in biosurfactant production. Temperature is an important parameter for enzyme activity and microbial growth. At extreme temperature, enzymes are inactive, thereby limiting the formation of primary metabolites required for microbial growth (Nafaji *et al*., 2010; Cheng *et al*., 2017). Optimal incubation time depends on the particular species of *Bacillus* because different species have

different metabolic pathways (Demirkan and Usta, 2013). Marajan *et al*. (2018) observed the effect of incubation time and surface tension on the growth of two *Bacillus* spp. (*Bacillus subtilis* and *Bacillus tequilensis*). Surface tension observed for *B. subtilis* reduced after 18h while that of *B. tequilensis* only reduced after 48 h.

Shown in Fig. 3.2C. is the synergetic effect of glucose concentration and incubation time on biosurfactant production. Simultaneous increase in the glucose concentration from 10 to 30 g/L and incubation time from 24 – 60 h resulted in biosurfactant production with high antifungal activity from 0 – 79 mm. Any further increments in the incubation time from 60 – 96 h caused a decrease in the antifungal activity from 79 to 59 mm. Moreover, when the incubation time was maintained at its minimum value (24 h) while varying the glucose concentration (10 – 30 g/L), biosurfactant production with high antifungal activity of 70 mm was obtained. On the other hand, maintaining the incubation time at 96 h while glucose concentration was varied from 10 to 30 g/L resulted in biosurfactant production with low antifungal activity below 40 mm was observed. Furthermore, when the glucose concentration is varied for 10 to 25 g/L while instantaneously varying incubation time from 24 to 60 h occasioned biosurfactant production with high antifungal activity of 65 mm. Further increase in the glucose concentration (>25 g/L) showed a decrease in antifungal activity from 70 mm to 40 mm. Glucose is used as a carbon source for the *Bacillus* sp. growth and is known to be an important component in the production of primary and secondary metabolites. The result in this study shows high sensitivity of biosurfactant production fluctuations to process input parameters, it is vital to ensure that the most favorable conditions are implemented for biosurfactant production with antifungal activity. Bochinni *et al*. (2001) reported on *Bacillus circulans* for Xylanase production. The authors observed that high xylanase production could only be achieved at incubation period of 48 – 72 h. Similarly, Puri *et al*. (2002) observed that *Bacillus* sp. produced alkaline protease that showed decreased protease activity after 96 h of incubation.

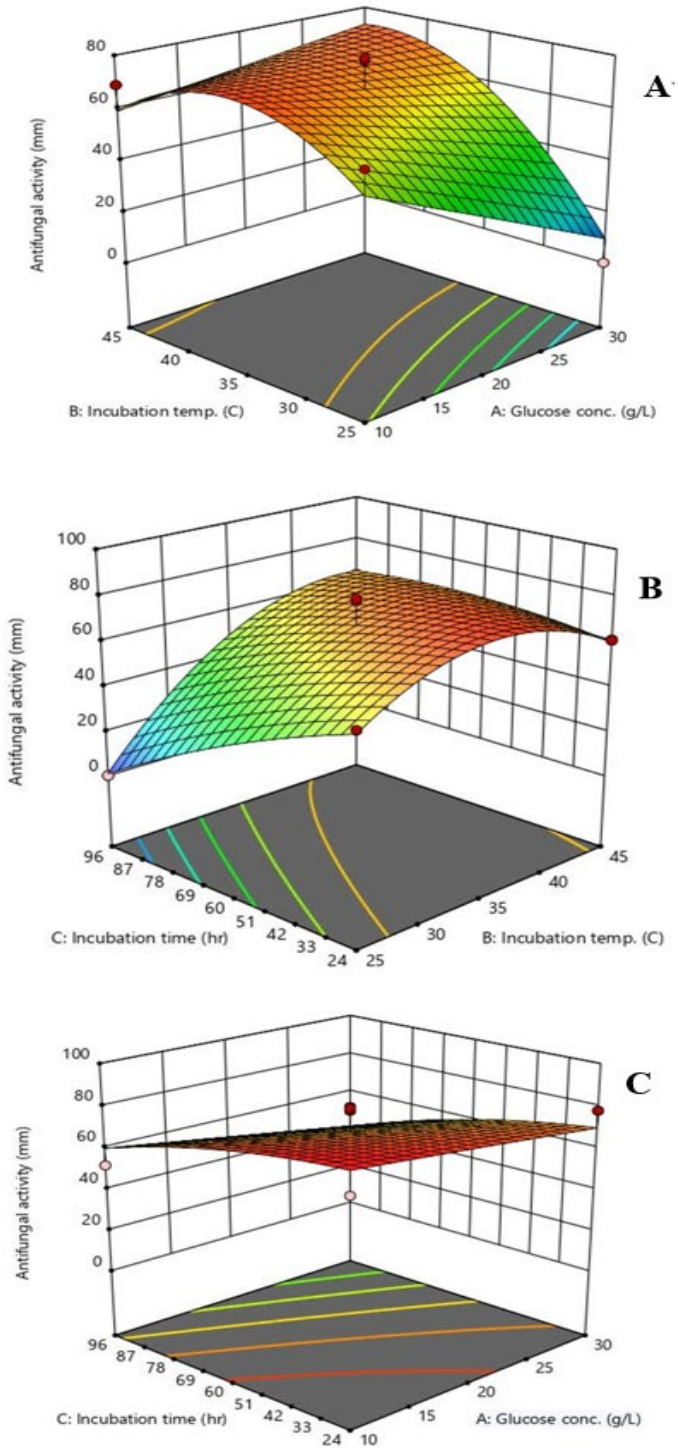

Fig. 3.2: 3-D surface plots showing the interactive effect of glucose concentration and incubation temperature (A), incubation temperature and incubation item (B), and incubation time and glucose concentration (C), on the anti-fungal activity potential of *Bacillus* BS20 against *R. solani* phytopathogen.

3.3.4 Process validation

The model predicted validation parameters of 11.5 g/L (glucose concentration), 41^{o}C (incubation temperature) and 24 h (incubation time) to obtained biosurfactant with antifungal activity of 67 mm. Experimental validation carried out showed the biosurfactant obtained had antifungal activity of 68 mm zone of inhibition, compared to 60 mm observed for the control experiments. Biosurfactant production increased from 0 h till the 12 h (65 mm), after which there was a slight decline in the biosurfactant production and then maximum biosurfactant production was obtained after the 18th hour (Fig. 3.3A). The decline in biosurfactant production could be attributed probably to change in process conditions such as pH change and depleting nutrient in the medium. These results reveal a good correlation between the input parameter and the response parameter. Maximum biosurfactant production was associated with the optimized process conditions employed that provide a relatively favorable metabolic condition for biosurfactant formation. The optimal process conditions favour the production of biosurfactant with high antifungal activity as shown by the range (62 - 68 mm) of antifungal activity observed for the cultivation period. The optimized process condition showed a 13% increment in the biosurfactant production over the control experiment. Biosurfactant production was observed to show no additional increment after >18 h and this can be attributed to glucose and nutrient been used up along with change in pH of the medium. Similar results have been obtained by Mouafi *et al*. (2016), the authors showed that the biosurfactant production obtained has high emulsification index of 71.89% under optimized conditions of 33^{o}C, 8, 10 h and 8.5 g/L for incubation temperature, pH, incubation period and glucose concentration, respectively.

The biomass concentration profiles are shown in Fig 3.3B. Biomass concentration in the control experiment is higher compared to the optimized process. Although, the biomass concentration is lower in the optimized system, biosurfactant with high antifungal activity was obtained. An

indication that the production of biosurfactant with high antifungal activity is inversely proportional to biomass accumulation. Metabolic pathway that favours high cell proliferation do not promote biosurfactant with high antifungal activity.

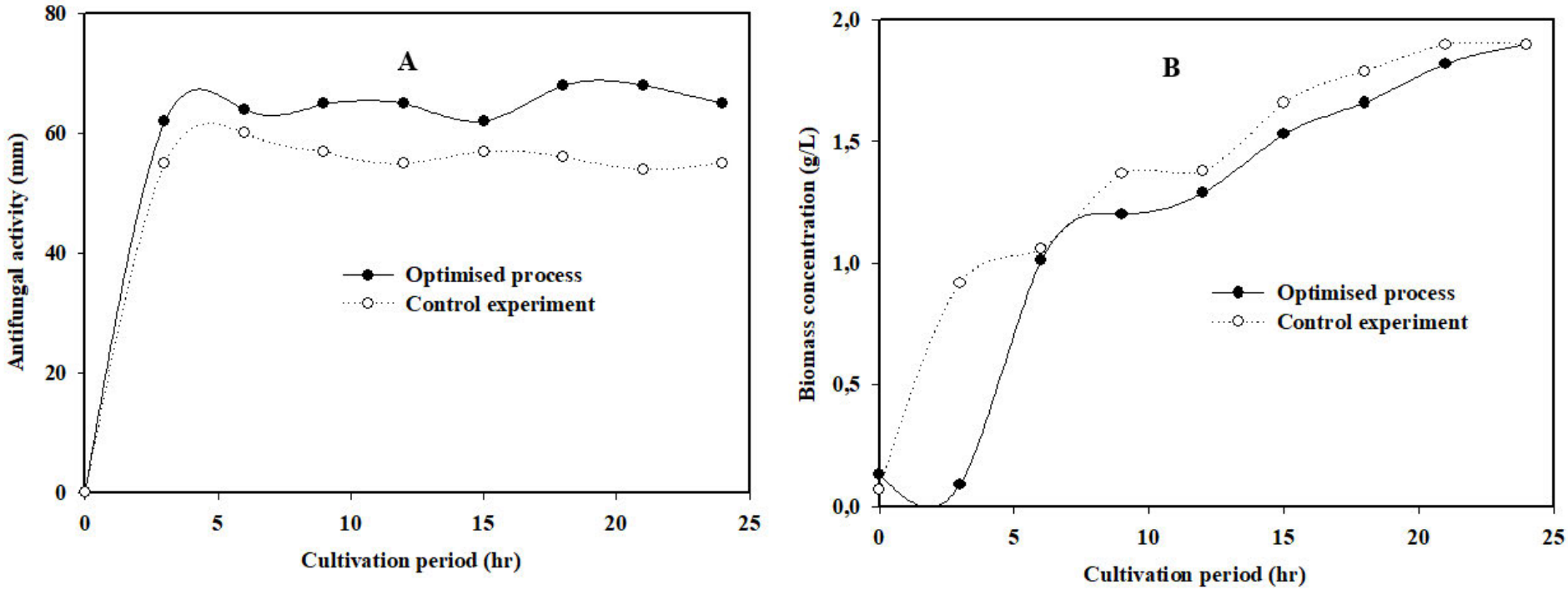

Fig. 3.3: Biosurfactant antifungal activity (A) and biomass concentration (B) of *B. subtilis* obtained under optimized conditions.

3.3.5 Effect of nano-catalyst supplementation

B. subtilis BS20 growth in the nano inclusion process is presented in table 3.4, with high biomass concentration but biosurfactant with high antifungal activity was not obtained. This agrees with the result obtained in section 3.3.3. Higher biomass accumulation in the nano-supplemented processes could be attributed to the impact of nanoparticles on *B. subtilis* BS20 (as microbes require metals like Ni, Fe, Zn, Co, Cu and Mn that are essential for microbial metabolism and growth) and the employed optimal conditions. The influencing effects of the metallic nanoparticles might as well be due to their cellular uptake and incorporation with the metabolic intermediates and key enzyme activities. The higher biomass concentration obtained for the nano-supplemented process in this study could be of interest considering the high cell proliferation in the nano-supplemented system, the cell could be engineered for improve biosurfactant production. In a related study by Yang *et al*. (2020), the authors showed that iron nanoparticle are able to increase surfactin production from 4.93 g/L to 7.15 g/L (Yang *et al*., 2020). The potential correlation between nanoparticles, biosurfactant production, biomass concentration and metabolic activity has been reported in literature. This, however, may depend on the microbial strain, process conditions, biochemical properties of the metal and its interaction with other metal ions in the medium. Moreover, the impact of the nanoparticle on the process could be attributed to nanoparticle biochemical catalysis potentials. Nanoparticles with their high surface area to volume ratio are good catalysts. This catalytic potential probably improved the contact and the interaction between the substrate and *Bacillus* sp. BS20 during the fermentation. The rate of glucose uptake has been indicated as a limiting step that may affect the efficiency of the biosurfactant production. In addition, metallic ions of nanoparticles could act as an enzyme cofactor/enzyme activator, growth factor, enzyme stabilizer and cell growth stimulator to enhance microbial metabolic activities and consequently improved process performance. Furthermore, these metallic ions are important in stimulating the

formation of cytochromes and ferroxins (Fd) which are vital for cell energy metabolism, hence product formation (Sanusi *et al*., 2021).

Table 3.4: Effect of nanoparticle on the antifungal activity and biomass concentration

NPs concentration (0.01g/L)	AA (mm)	Biomass concentration (g/L)	NPs concentration (0.05 g/L)	AA (mm)	Biomass concentration (g/L)
Fe_2O_3	59	1.24	Fe_2O_3	27	2.59
Fe_3O_4	58	1.87	Fe_3O_4	44	2.80
ZnO	51	3.81	ZnO	33	10.28
MnO_2	61	2.94	MnO_2	51	3.41
CuO	0	2.95	CuO	0	0.05
CoO	61	2.89	CoO	0	0.29
NiO	65	5.11	NiO	63	3.43
Control	55		Control	55	

AA=Antifungal activity

3.3.6 Conclusion

In this study, optimization, and nano catalysis for improved biosurfactant antifungal property was achieved. Model developed elucidate functional relationships with maximum biosurfactant antifungal activity of 68 mm obtained with 11.5 g/L glucose concentration at 41^{o}C incubation temperature for 24 h. The most significant parameters in *B. subtilis* BS20 biosurfactant production were incubation time and process temperature (p-values <0.05). Furthermore, inclusion of nanoparticles (NPs) significantly improved biomass concentration compared to the control experiment. Findings from the present work provide insights on the influence of key input parameters for *Bacillus* based antifungal production and nano-catalysis towards bioprocess design and scaling up.

References

Aguilar-Reynosa, A., Romaní, A., Rodríguez-Jasso, R.M., Aguilar, C.N., Garrote, G. and Ruiz, H.A., 2017. Comparison of microwave and conduction-convection heating autohydrolysis pretreatment for bioethanol production. *Bioresource technology*, *243*, pp.273-283.

Bocchini, D.A., Alves-Prado, H.F., Baida, L.C., Roberto, I.C., Gomes, E. and Da Silva, R., 2002. Optimization of xylanase production by *Bacillus circulans* D1 in submerged fermentation using response surface methodology. *Process Biochemistry*, *38*(5), pp.727-731.

Dong, H., Zheng, A., He, Y., Wang, X., Li, Y., Yu, G., Gu, Y., Banat, I.M., Sun, S., She, Y. and Zhang, F., 2022. Optimization and characterization of biosurfactant produced by indigenous *Brevibacillus borstelensis* isolated from a low permeability reservoir for application in MEOR. *RSC advances*, *12*(4), pp.2036-2047.

Ebadipour, N., Lotfabad, T.B., Yaghmaei, S. and RoostaAzad, R., 2016. Optimization of low-cost biosurfactant production from agricultural residues through response surface methodology. *Preparative Biochemistry and Biotechnology*, *46*(1), pp.30-38.

Goswami, M. and Deka, S., 2019. Biosurfactant production by a rhizosphere bacteria *Bacillus altitudinis* MS16 and its promising emulsification and antifungal activity. *Colloids and Surfaces B: Biointerfaces*, *178*, pp.285-296.

Ghribi, D. and Ellouze-Chaabouni, S., 2011. Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnology research international*, *2011*.

Gürkök, S. and Özdal, M., 2021. Microbial Biosurfactants: Properties, Types, and Production. *Anatolian Journal of Biology*, *2*(2), pp.7-12.

Heryani, H. and Putra, M.D., 2017. Kinetic study and modeling of biosurfactant production using *Bacillus* sp. *Electronic Journal of Biotechnology*, *27*, pp.49-54.

Hmidet, N., Ben Ayed, H., Jacques, P. and Nasri, M., 2017. Enhancement of surfactin and fengycin production by *Bacillus mojavensis* A21: application for diesel biodegradation. *BioMed research international*, *2017*.

Ishtar Snoek, I.S. and Yde Steensma, H., 2007. Factors involved in anaerobic growth of Saccharomyces cerevisiae. *Yeast*, *24*(1), pp.1-10.

Kim, P.I., Bai, H., Bai, D., Chae, H., Chung, S., Kim, Y., Park, R. and Chi, Y.T., 2004. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *Journal of applied microbiology*, *97*(5), pp.942-949.

Kumar, P.N., Swapna, T.H., Khan, M.Y., Reddy, G. and Hameeda, B., 2017. Statistical optimization of antifungal Iturin A production from *Bacillus amyloliquefaciens* RHNK22 using agro-industrial wastes. *Saudi journal of biological sciences*, *24*(7), pp.1722-1740.

Lim, S.M., Yoon, M.Y., Choi, G.J., Choi, Y.H., Jang, K.S., Shin, T.S., Park, H.W., Yu, N.H., Kim, Y.H. and Kim, J.C., 2017. Diffusible and volatile antifungal compounds produced by an antagonistic *Bacillus velezensis* G341 against various phytopathogenic fungi. *The plant pathology journal*, *33*(5), p.488.

Marajan, C., Alias, S., Ramasamy, K. and Abdul-Talib, S., 2018, October. The effect of incubation time, temperature and pH variations on the surface tension of biosurfactant produced by *Bacillus* spp. In *AIP Conference Proceedings* (Vol. 2020, No. 1, p. 020047). AIP Publishing LLC.

Moodley, P. and Kana, E.G., 2017. Development of a steam or microwave-assisted sequential salt-alkali pretreatment for lignocellulosic waste: Effect on delignification and enzymatic hydrolysis. Energy Conversion and Management, 148, pp.801-808.

Mosquera, S., González-Jaramillo, L.M., Orduz, S. and Villegas-Escobar, V., 2014. Multiple response optimization of *Bacillus subtilis* EA-CB0015 culture and identification of antifungal metabolites. *Biocatalysis and Agricultural Biotechnology*, *3*(4), pp.378-385.

Mouafi, F.E., Elsoud, M.M.A. and Moharam, M.E., 2016. Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. *Biotechnology Reports*, *9*, pp.31-37.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, *31*(3), pp.426-428.

Najafi, A.R., Rahimpour, M.R., Jahanmiri, A.H., Roostaazad, R., Arabian, D. and Ghobadi, Z., 2010. Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. *Chemical Engineering Journal*, *163*(3), pp.188-194.

Nalini, S., Parthasarathi, R. and Prabudoss, V., 2016. Production and characterization of lipopeptide from *Bacillus cereus* SNAU01 under solid state fermentation and its potential application as anti-biofilm agent. *Biocatalysis and Agricultural Biotechnology*, *5*, pp.123-132.

Nwabueze, T.U., 2010. Basic steps in adapting response surface methodology as mathematical modelling for bioprocess optimisation in the food systems. *International Journal of Food Science & Technology*, *45*(9), pp.1768-1776.

Ohno, A., Ano, T. and Shoda, M., 1995. Effect of temperature on production of lipopeptide antibiotics, iturin A and surfactin by a dual producer, *Bacillus subtilis* RB14, in solid-state fermentation. *Journal of Fermentation and Bioengineering*, *80*(5), pp.517-519.

Ongena, M. and Jacques, P., 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in microbiology*, *16*(3), pp.115-125.

Posada-Uribe, L.F., Romero-Tabarez, M. and Villegas-Escobar, V., 2015. Effect of medium components and culture conditions in *Bacillus subtilis* EA-CB0575 spore production. *Bioprocess and biosystems engineering*, *38*(10), pp.1879-1888.

Puri, S., Beg, Q.K. and Gupta, R., 2002. Optimization of alkaline protease production from *Bacillus* sp. By response surface methodology. *Current microbiology*, *44*(4), pp.286-290.

Rebib, H., Hedi, A., Rousset, M., Boudabous, A., Limam, F. and Sadfi-Zouaoui, N., 2012. Biological control of *Fusarium* foot rot of wheat using fengycin-producing Bacillus subtilis isolated from salty soil. *African Journal of Biotechnology*, *11*(34), pp.8464-8475.

Saito, T., Whatmore, P., Taylor, J.F., Fernandes, J.M., Adam, A.C., Tocher, D.R., Espe, M. and Skjærven, K.H., 2021. Micronutrient supplementation affects transcriptional and epigenetic regulation of lipid metabolism in a dose-dependent manner. *Epigenetics*, *16*(11), pp.1217-1234.

Sanusi, I.A., Faloye, F.D. and Gueguim Kana, E.B., 2019. Impact of various metallic oxide nanoparticles on ethanol production by *Saccharomyces cerevisiae* BY4743: screening, kinetic study and validation on potato waste. *Catalysis Letters*, *149*(7), pp.2015-2031.

Sanusi, I.A., Suinyuy, T.N. and Kana, G.E., 2021. Impact of nanoparticle inclusion on bioethanol production process kinetic and inhibitor profile. *Biotechnology Reports*, *29*, p.e00585.

Talasila, U. and Vechalapu, R.R., 2015. Optimization of medium constituents for the production of bioethanol from cashew apple juice using Doehlert experimental design. *International Journal of Fruit Science*, *15*(2), pp.161-172.

Wang, L., Luo, Z. and Shahbazi, A., 2013. Optimization of simultaneous saccharification and fermentation for the production of ethanol from sweet sorghum (Sorghum bicolor)

bagasse using response surface methodology. *Industrial crops and products*, *42*, pp.280-291.

Wu, J.Y., Liao, J.H., Shieh, C.J., Hsieh, F.C. and Liu, Y.C., 2018. Kinetic analysis on precursors for 81turin A production from Bacillus amyloliquefaciens BPD1. *Journal of bioscience and bioengineering*, *126*(5), pp.630-635.

Yang, N., Wu, Q. and Xu, Y., 2020. Fe nanoparticles enhanced surfactin production in Bacillus amyloliquefaciens. *ACS omega*, *5*(12), pp.6321-6329.

Zouari, R., Ellouze-Chaabouni, S. and Ghribi-Aydi, D., 2014. Optimization of Bacillus subtilis SPB1 biosurfactant production under solid-state fermentation using by-products of a traditional olive mill factory. *Achievements in the Life Sciences*, *8*(2), pp.162-169.

CHAPTER 4

Preliminary scale up studies of antifungal production by *Bacillus* sp. in a stirred stainless bioreactor

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This chapter has been prepared to be submitted for publication in a peer review journal with the title: Preliminary scale up studies of antifungal production by *Bacillus* sp. in a stirred stainless bioreactor. The manuscript is presented in the following pages.

Preliminary scale up studies of antifungal production by *Bacillus subtilis* BS20 in a stirred stainless bioreactor

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Abstract

This study presents *Bacillus*-based biosurfactant production process scale up based on constant power consumption, Reynold number and impeller tip speed. Wide ranges of process conditions: stirrer speed (n), impeller diameter (di), number of impellers (N), power number (N_p), broth density (ρ), working volume and geometric factor (f_c) were correlated with impeller tip speed (V_{tip}) Reynolds Number (*Re*) and power consumption rate (*P/V*). These were performed in a 10 L bioreactor and compared in terms of biosurfactant production (using antifungal activity) to that obtained in the 1 L bioreactor. The highest antifungal activity of 65 mm zone of inhibition was obtained by maintaining constant impeller tip speed. This was 1.14-fold higher than the 1 L bioreactor (57 mm) as well as 1.38-fold and 1.18-fold better in comparison to the constant Reynolds number (47 mm) and power consumption rate (55 mm). Moreover, shear stress decreased by 0.77-fold from the 1 L bioreactor which resulted in low cell damage and high cell viability for the constant V_{tip}. These findings demonstrate the feasibility of scaling up biosurfactant production by *B. subtilis* BS20 as well as provide insights into industrial biopesticide production towards eco-friendly pest control.

Keywords: Bioreactor; impeller tip speed; biosurfactant; antifungal activity; scale up

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4.1 Introduction

There is a gradual shift towards sustainable microbial crop production, as well as minimizing the use of chemical fertilizers and pesticides. Great efforts have been attempted to enhance the bioprocessing of microbial products such as biosurfactants. These include nutrient formulation, process optimization, scaling up and microbial engineering strategies (Deniz *et al*., 2015; Qazizada, 2016, Sanusi *et al*., 2020; Ganeshan *et al*., 2021). In principle, bioprocess capacity depends primarily on gene functions, enzyme kinetics and fluid dynamics in the bioreactor (Xia *et al*., 2015). In this regard, it is crucial to gain more knowledge of cellular kinetics and bioreactor hydrodynamics to accelerate the transition from the laboratory scale research to its industrial application (Babel *et al*., 1993). A critical concern of bioprocess scale up is its negative impact on the cell kinetic resulting from mixed conditions in the large-scale bioreactor (Xia *et al*., 2015). Large scale bioreactors are challenged with mixing problem, heterogeneous environment, and contamination potential. The cell immediate microenvironment and the cell physiology might be influenced, resulting to critical metabolic variations. Microbial cells have the tendency to transform their genetic make-up due to different environmental conditions which could lead to lose in vital metabolic features required for the bioprocess optimal performance. To achieved relative bioreactor homogenous environment during process, different scale up criteria must be implemented. These scaling up criteria include impeller tip speed, power consumption and mixing time. Impeller system in a stirred tank bioreactors is used to enhance homogeneous mixing of reacting species. The aim of obtaining effective mixing regime from suitable combination of parameters is to achieve optimum mass transfer and temperature gradient homogeneity within the bioreactor in the shortest possible time (Deniz *et al*., 2015; Qazizada, 2016).

This requires appropriate energy being delivered to the bioreactor system through the agitator power input. Impeller tip speed has some advantages in bioprocessing with shear stress

sensitive microorganisms because it determines the optimum shear stress and possible cell damage in the bioreactor (Deniz *et al*., 2015). Usually, the most preferable criteria for scaling up is to maintain the volumetric power input or the volumetric mass transfer coefficient constant. Hence, a vast understand of the interaction between the fluid motion, the impeller speed, and the power consumption will be required to achieve the optimum energy input consequently, improved process performance (Marques *et al*., 2010). Scaling up from laboratory scale to production scale could be very challenging because of the many important but different aspects involved (Qazizada, 2016). The major standpoints which require precise compromise between intrinsically contradictory desirable characteristics are the engineering, metabolic processes and economic implications needed for an industrial scale production at the best economic cost (Qazizada, 2016).

Usually, four techniques are recognized in process scaling up, and these include fundamental methods, semi fundamental methods, dimensional analysis, and rules of thumb (Garcia-Ochoa and Gomez, 2009). The scale up criteria largely used in the bioprocesses are constant power consumption input, constant impeller tip speed and constant mixing time (Garcia-Ochoa and Gomez, 2009). These criteria are directly related to mass transfer, mixing activities, power consumption, bulk rheology, cell viability, shear stress, substrate and products concentration in the bioreactor (Deniz *et al*., 2015; Qazizada, 2016). The design of industrial scale microbial bioprocess depends on the growth conditions, nutrient formulation, target product, microbial strain, reactor geometry and fluid hydrodynamics. Consequently, for a certain product, the adequate and comprehensive process parameters which are directly linked to improved product yield and scaling up potentials must be established. To the best of our knowledge there are dearth of reports on the scaling up of biosurfactant production from *Bacillus* species for antifungal compounds. on scale up studies of this bioprocess. Thus, a suitable scale up technology is imperative.

Moreover, the effect of scale up could significantly impact on the process kinetics and ultimately the process productivity efficiency. Hence, it is necessary to understanding the microbial kinetics that expressed correlations between rates of product formation as well as accurately predicting cell behavior in a dynamic bioreactor fluid environment. Mathematical kinetic models have been employed in relation to understand, predict, and optimize the properties and behavior of cells in bioprocessing (Tyo *et al*., 2010). Currently, there is a scarcity of studies on scaling up and the kinetics of *Bacillus* based antifungal compound production.

Although several technological challenges have been resolved in relatedness to microbial engineering for biosurfactant production, knowledge-based bioreactor design for efficient scale up of biosurfactant production is still underdeveloped. Therefore, the aim of this study was to examine the various scale up criterion at different scales on biosurfactant production as well as the kinetics of biosurfactant production by *B. subtilis* BS20.

4.2 Materials and methods

4.2.1 Inoculum development

Bacillus subtilis BS20 strain was provided courtesy of Dr Santosh Ramchuran from the School of Life Science, Durban Campus, University of KwaZulu-Natal, South Africa. Seed culture of *B. subtilis* BS20 was maintained on Luria-Bertani agar slant containing; Tryptone, yeast extract, sodium chloride kept at 4°C. For inoculum cultivation, a colony of *B. subtilis* BS20 was transferred into 250 mL flask containing 100 mL LB medium. This was incubated under shaking conditions (120 rpm) overnight, at 30°C until the exponential growth phase was reached.

4.2.2 Fermentation conditions

Batch fermentations were carried out in 2 L (Bio/CelliGen 115, New Brunswick, USA) and 10 L (Labfors-INFORS HT, Switzerland) bioreactors under anaerobic conditions with working

volumes of 1 L and 10 L, respectively. Luria Bertani (10g/L tryptone, 5g/L yeast extract, 10g/L sodium chloride) supplemented with 11.5g/L glucose were fed to the sterilized bioreactor and then inoculated with the seed culture (10% v/v). This was followed by fermentation process carried out at 41°C and 120 rpm for 24 h. Samples were taken at regular intervals. Broth sample was centrifuge (5000 rpm, 20 min, 4°C) and the supernatant was used for antifungal activity determination.

4.2.3 Scale up parameter determination

Three scale up parameters namely, constant power consumption per unit volume (*P/V*), Reynolds number (Re) and impeller tip speed (V_{tip}) were used to determine the most suitable operational conditions at a semi-pilot scale production of biosurfactant by *B. subtilis BS20.*

4.2.4 Scale up parameters

4.2.4.1 Constant impeller tip speed

Impeller tip speed (V_{tip}) is used as a parameter for scale up when the relationship between shear stress and microbial cell as well as possible cell damage are to be determined (Bonvillani *et al*., 2006; Marques *et al*., 2010). The V_{tip} is directly proportional to the product of impeller speed and the impeller diameter, the relationship is shown in Eq. 4.1. Constant impeller tip speed as a scale up parameter in this study was obtained also using Eq. 4.1 and 4.2 (Pérez *et al*., 2018).

$$v_{tip} = \pi din \tag{4.1}$$

$$n_{10L} = n_{1L}\,(di_{1L}/di_{10L}) \tag{4.2}$$

4.2.4.2 Constant power consumption

Power consumption per unit volume (*P/V*) is a measure of mixing intensity and mass transfer rate. The constant power consumption per unit of volume was obtained using Eq. 4.3-4.5, while

Eq. 4.6 was employed to compute the stirring speed in the 10 L scale bioreactor (Pérez *et al*., 2018).

$$P/V = Constant \quad (4.3)$$

$$N^3D^2 = Constant \quad (4.4)$$

$$N_1^3D_1^2 = N_2^3D_2^2 \quad (4.5)$$

$$N_2 = N_1 (D_1/D_2)^{2/3} \quad (4.6)$$

where, N is the impeller speed and D the bioreactor diameter.

4.2.4.3 Reynold's number

The Reynold's number was obtained in the bioreactor using Eq. 4.7 and 4.8 (Pérez *et al*., 2018), where ρ represents the broth density, η the viscosity of the broth, n the impeller agitation speed and di the impeller diameter.

$$Re = \frac{\rho ndi^2}{\eta} \quad (4.7)$$

$$n_{10} = n_1 . (d_1/d_{10})^2 \quad (4.8)$$

4.2.4.4 Pumping capacity (V_P)

This is the liquid volume released from the impeller per unit time (m^3/s). The impeller pumping capacity was computed using Eq. 4.9 (Qazizada, 2016). Where N_f is the flow number (N_f = 0.72 for Rushton turbine), di is the impeller diameter and n is the impeller speed.

$$V_p = N_f ndi^3 \quad (4.9)$$

4.2.4.5 Fluid circulation time (t_c)

The fluid circulation time is a function of the volume of the liquid phase (V_L) and pumping capacity (V_P) (Qazizada, 2016). This was computed by Eq. 10 below.

$$t_c = {}^{V_L}/_{V_P} \quad (4.10)$$

4.2.4.6 Scale of turbulence determination

Broth homogeneity and fluid material transfer are proportional to eddies bulk liquid break up and it is a function of input power. The size of eddies formed was computed by Kolmogorov scale of turbulence, λ using Eq. 4.11.

$$\lambda = \left(\frac{V^3}{\varepsilon}\right)^{1/4} \quad (4.11)$$

where λ represents the size of eddies, V the viscosity, ε the turbulence energy per unit mass of liquid ($\varepsilon = N\rho n^3 di^2$).

4.2.4.7 Shear stress

The shear stress relating was obtained by Eq. 4.12, where n is the mixing speed and *k* is the experiential constant for a standard Rushton impeller (*k* = 10 for Rushton turbine) (Deniz *et al*., 2015).

$$\gamma = kn \quad (4.12)$$

4.2.5 Analytical Methods

Biomass dry weight was determined using a calibration standard curve, a correlation of cell dry weight as a function of cell count.

Broth viscosity and density were determined as described by Pérez *et al*. (2018) and Deniz *et al*. (2015) respectively.

Antifungal activity was determined using the amended media protocol. The samples collected for each run was centrifuged at 10000 rpm for 10 min to obtain a free-cell supernatant. Thereafter, the supernatant was added to molten Potato Dextrose agar (PDA) at a ratio of 1:10 (v/v) and left to solidify (Goswami and Deka, 2019; Kumar et al., 2017). A mycelial plug was cut out of previously grown fungal isolates and inoculated on the amended media agar plate. The control experiment was a PDA plate inoculated with only the fungal pathogen. All plates were incubated at 30ºC for 3 days. Thereafter, zone inhibition was determined.

The specific growth rates (μ) of *B. subtilis* BS20 were calculated using Eq. 4.13, where X_2 and X_1 are biomass dry weights (g/L) at t_2 and t_1, respectively.

$$Specfic\ growth\ rate\ (\mu) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \qquad (4.13)$$

Additionally, the integrated logistic model (Eq. 4.14) was used to define the relationship of biomass dry weight (X), at specific times (t) during *B. subtilis* BS20 active growth and stationary phases of cell growth to initial biomass dry weight (X_0), maximum biomass dry weight (X_{max}) and maximum specific growth rate (μ_{max}) during the scale up process.

$$X = \frac{X_0.exp(\mu_{max}.t)}{1-\left(\frac{X_0}{X_{max}}\right).(1-exp(\mu_{max}.t))} \qquad (4.14)$$

4.3 Results and discussion

4.3.1 The effects of scaling up on process performance

The experimental profiles for the biomass concentration and the antifungal activity in the 1 L as well as the 10 L scale bioreactors are presented in Fig. 4.1 – 4.4. The maximum dry-cell mass of 1.49 and 1.35 and 0.65 g/L were obtained for constant *P/V,* V_{tip} and *Re* respectively. Biomass concentration obtained for constant V_{tip} and *Re* were slightly lower than that obtained in the 1 L scale (1.48 g/L). This could probably be ascribed to the variation in the process environment obtained with constant V_{tip} and *Re*, which might be detrimental to cell viability and growth (Deniz *et al*., 2015). Furthermore, the biomass dry weight (g/L) increased in the first few hours (3 h) of the process and extended till the 15th h, this coincided with biosurfactant production during this period (Fig. 4.3 – 4.4). As shown in Fig. 4.3 – 4.4 biosurfactant from *B. subtilis* BS20 effectively inhibits fungal growth for the constant $V_{tip,}$ *Re* and *P/V*. Biosurfactant from impeller tip speed bioreactor had the highest antifungal activity of 65 mm. This was 1.14, 1.18, and 1.38-fold higher than the antifungal activity obtained with biosurfactant from 1 L, power consumption and the Reynold number bioreactors respectively. This result suggests constant V_{tip} process conditions support enzymatic and metabolic activities that favor the production of highly efficacious biosurfactant, consequently, high antifungal activity.

Meanwhile, the antifungal activity indicated by zone of inhibition was lower in the 10 L scale for *P/V* (55 mm) and *Re* (47 mm) compared to the 1 L scale (57 mm). Biosurfactant obtained showed increased antifungal activity from zero hour till the 6th h, afterwards there was a decline in antifungal activity for all the systems at 10L scale (Fig. 4.3 – 4.4). The decline in antifungal activity could be ascribed to the formation of other metabolites that do not work in synergy with the biosurfactant produced. The variation in antifungal activity response under various scales may be ascribed to differences in rheological characteristics and the bioreactor

geometry in the different reactors (Tables 4.1 and 4.2). The rheological characteristics of the nutrient broth changes during the process as biomass and products accumulate (Perez *et al*., 2018). Thus, the velocity and turbulence of fluid leaving the stirrer must be adequate to carry material into the most remote parts of the bioreactor to ensure and maintain effective mixing regime. The performance of a reactor is influenced, to a very significant extent, by mixing effects on metabolic processes and production. Additionally, the liquid volume that was dismissed from the stirrer per unit time (V_p) (Table 4.2) and the circulation time (t_c), another important quantitative mixing characteristics are apparent, sufficient and efficient to obtain good mixing. This must have contributed to the process performance in the bioreactors.

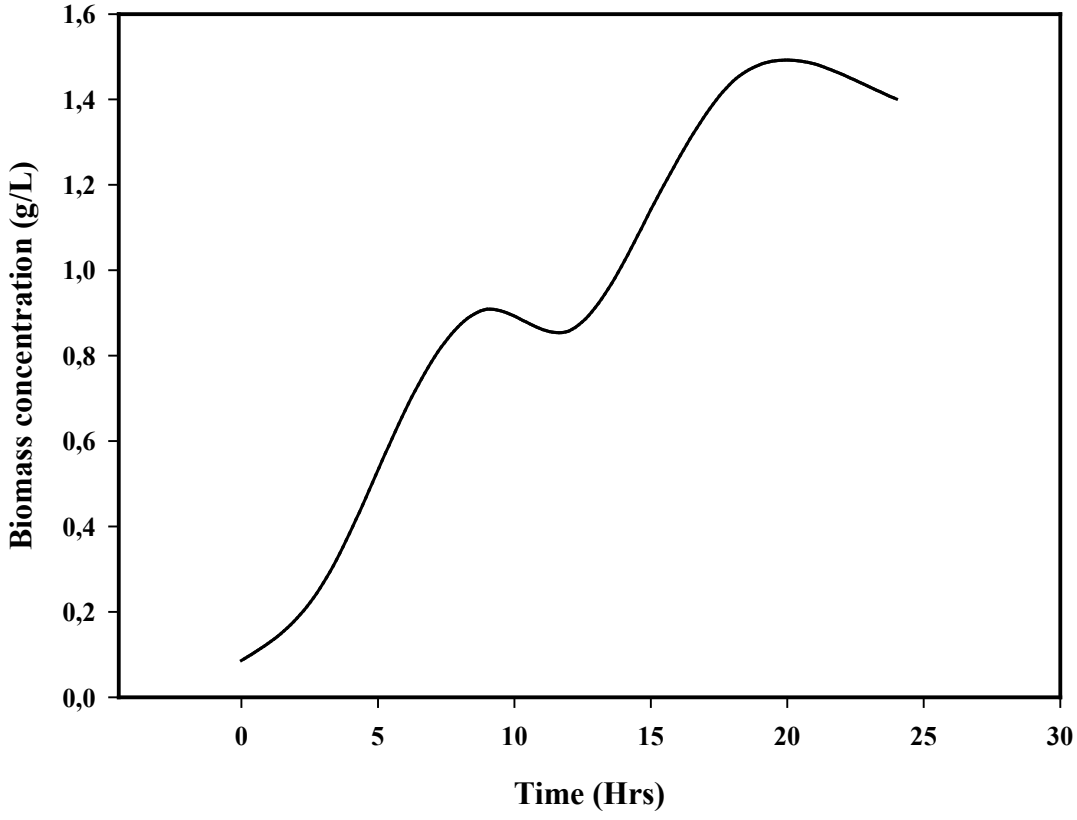

Fig. 4.1: Cell dry weight of *B. subtilis* BS20 in the 1 L working volume bioreactor

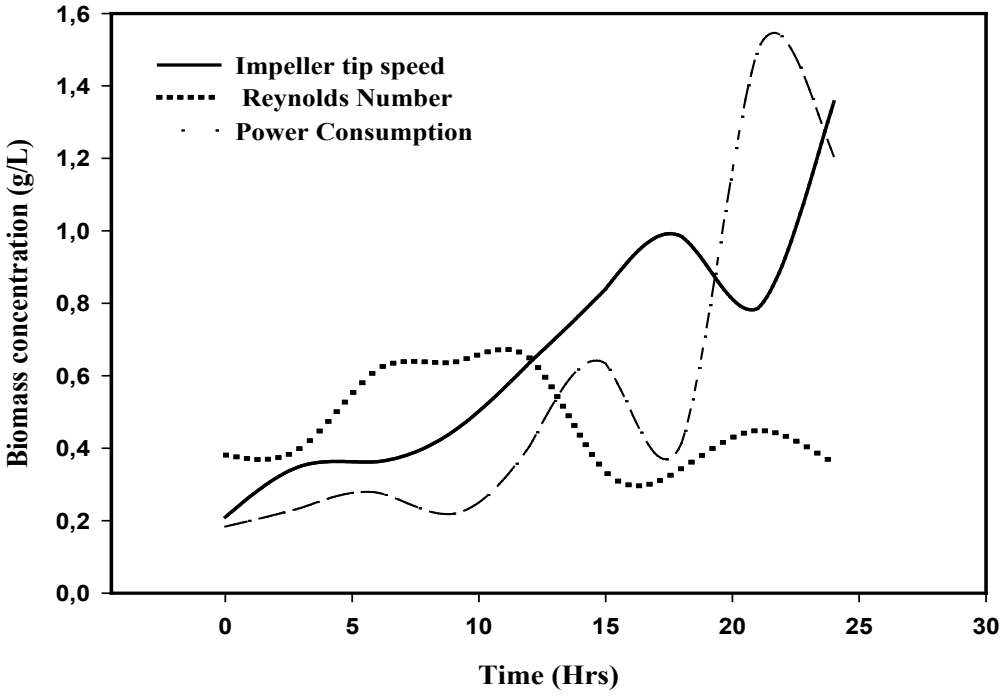

Fig. 4.2: Cell dry weight of *B. subtilis* BS20 obtained from constant V_{tip}, *P/V* and *Re* in the different scale up systems.

Table 4.1: Bioreactor geometry employed in the scale up processes

Parameters	**1 L scale**	**10 L scale**
Total bioreactor volume (m^3)	0.002	0.010
Working volume (m^3)	0.001	0.005
Bioreactor height [h] (m)	0.237	0.427
Bioreactor diameter [D] (m)	0.125	0.200
Static height of broth [H] (m)	0.084	0.162
Number of impellers (N)	1	2
Impeller diameter [di] (m)	0.054	0.070
Impeller thickness (m)	0.001	0.002
Power number (N_p)	5.20	10.40
Broth density [ρ] (kg/m^3)	1013	1013
Broth viscosity [η] (Pa s)	9.173×10^{-5}	9.173×10^{-5}
Impeller type	Rushton turbine	Rushton turbine

Table 4.2: Bioreactor geometry employed in the scale up processes

1 L control bioreactor		10 L bioreactor		
Parameters		**Constant υ_{tip}**	**Constant P/V**	**Constant *Re***
n (rpm/rps)	120/2	93/1.55	88/1.47	71/1.18
υ_{tip} (m/s)	0.34	0.34	0.32	0.26
Re	4.5×10^{-4}	5.9×10^{-4}	5.6×10^{-4}	4.5×10^{-4}
P (W)	0.0156	0.32	0.0156	0.32
P/V_L (W/m^3)	15.62	160	7.8	160
V_P (m^3/s)	2.3×10^{-4}	3.4×10^{-4}	3.6×10^{-4}	2.9×10^{-4}
t_c (s)	4.4	5.2	5.5	6.9
λ (m)	17.5	15.6	16.3	19.2
γ (1/s)	20	15.5	14.7	11.8

Table 4.3: Parameters for scale up studies of cell growth of *B. subtilis* BS20 during biosurfactant production

1 L control bioreactor		10 L bioreactor		
Parameters		**Constant V_{tip}**	**Constant *P/V***	**Constant *Re***
Fermentation performance				
Biomass concentration (g/L)	1.5	3.097	4.129	0.477
Antifungal activity (mm)	57	65	55	47
Kinetic performance				
Logistic function Model				
X_o (g/L)	0.19	0.25	0.11	0.37
X_{max} (g/L)	1.50	3.10	4.13	0.48
μ_{max} (h^{-1})	0.23	0.084	0.12	0.65
R^2	0.95	0.89	0.78	0.064

4.3.2 Scaling up based on constant (v_{tip})

The biosurfactant obtained based on constant V_{tip} experiment had the highest antifungal activity of 65 mm zone of inhibition. This was also slightly higher (12%, 15 % and 28%) when compared to the result obtained in the 1 L scale bioreactor, the constant *P/V* and *Re* experiments, respectively. This result may be ascribed to the lowest mixing rate employed due to constant *P/V* and *Re* at 10 L scale (Table 4.2). Though, stirrer tip speed scale up criterion has some benefits in the instance of bioprocesses with shear sensitive microbes, but in some cases, it is not a good parameter for bioprocess scaling up. This is in disagreement with the current study. For instance, scale up based on maintaining *P/V* resulted in lower shear stress of 11.8 compared to constant impeller tip speed (15.5). This increase in the shear stress may result to probable cell damage as well as affect the cell metabolic physiology and consequently, decrease in biosurfactant production of desired interest. Although, excess shear stress could lead to the loss of cell viability and cell disruption, a certain degree of shear rate is necessary to achieve appropriate transfer of materials and energy within the bioreactor. Impeller tip speed influences impeller shear, which is proportional to the product of impeller tip speed and impeller diameter for turbulent flow conditions (Marques *et al*., 2010). Tip speed is used as a rule for scale up when the relationship between shear stress and cell viability is far from well understood. A rough rule of thumb suggests that cell damage can occur at tip speeds above 3.2 m s^{-1}, but the exact value is influenced by many factors such as broth rheology. Tip speeds are usually greater than 3 m s^{-1} for production scale reactors (Junker, 2004). If scale up is carried out using constant tip speed (with geometric similarity), then the value of P/V is often lowered, which can adversely affect aeration efficiency. It is possible to overcome this drawback by using more impellers in the larger vessel in such a way that both tip speed and *P/V* are kept constant. Hiruta *et al*. (1996) demonstrated that maintaining impeller tip speed of 270 m min^{-1} allowed scaling-up the production of γ - linolenic acid by *Mortierella ramanniana* mutant MM

15-1 from a 30 L to a 1 m^3 reactor. More recently, Dubey *et al*. (2008) scaled up the demethylation of colchicine and their derivatives using *Bacillus megaterium* ACBT03 cells, from a 5 L to a 70 L reactor. Using impeller tip velocity of 4710 cm min^{-1}, the biomass dry weight was 8% lower than the 1 L control reactor experiment. This decrease in *Bacillus megaterium* ACBT03 growth in the 10 L scale reactor based on constant V_{tip} experiment might be ascribed to poor gaseous–liquid diffusion observed at the lower stirrer speed as well as the lower circulation time.

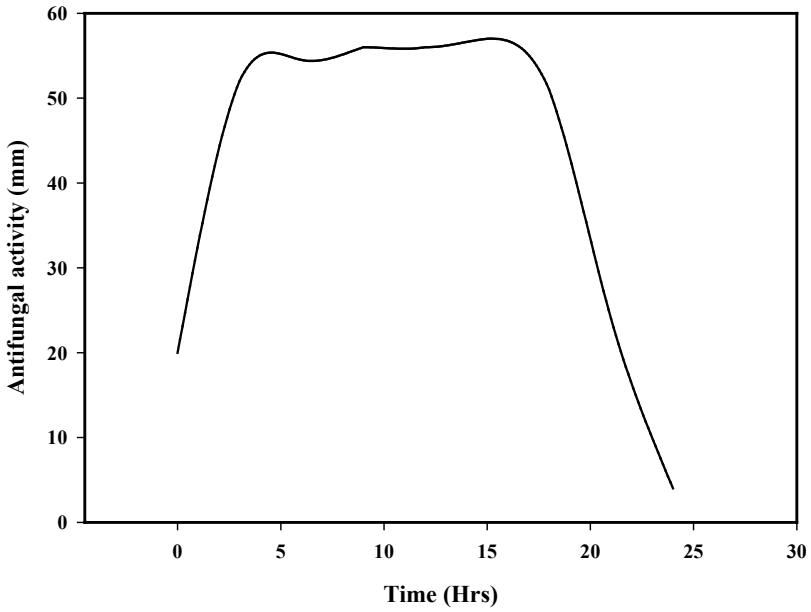

Fig. 4.3: Antifungal activity of biosurfactant obtained in the 1 L working volume bioreactor

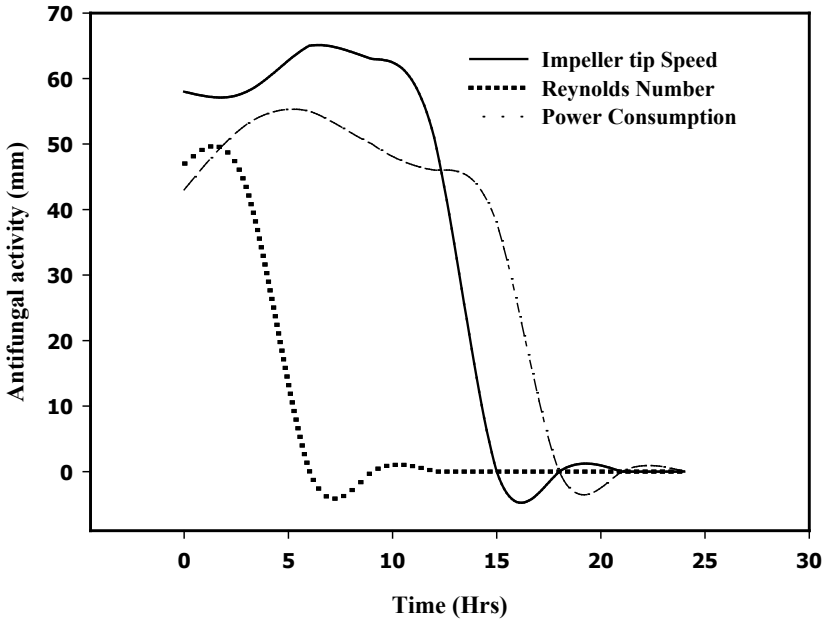

Fig. 4.4: Antifungal activity of biosurfactant obtained from constant V_{tip}, *P/V* and *Re* bioreactor.

4.3.3 Scaling up based on constant power consumption P/V

In the present study, using the constant power consumption criterion, the size of eddies was computed to be 16.3 m which was considerably larger than an average *B. subtilis* BS20 cell size. When the Kolmogorov eddy size equals the cell diameter or gets smaller, the flow lines pattern could shear growing cells (Deniz *et al*., 2015). On the other hand, smaller eddies, facilitates rapid transfer of material, which is proportionate to the power input. The greater the power input (ε) to the fluid, the smaller are the eddies, the better the mixing regime and consequently, the better the system productivity (Qazizada, 2016). Similarly, the power number for the current scale up was 5.20 at fully turbulent flow and 10 for the characteristic experiential constant (*k*) for a standard Rushton turbine impeller (Deniz *et al*., 2015). The increase in impeller tip speed from the 1L scale control reactor to 10 L scale bioreactor was considered negligible since the *P/V* ratio in the constant *P/V* experiment was lower than the constant V_{tip} experiment (Table 4.2). These parameters offer specific information concerning the mixing

system, suggesting the optimum hydrodynamic regime, and predicting the modification of mixing efficiency induced by the scale up strategy employed (Oniscu *et al*., 2002). Furthermore, based on constant *P/V*, biosurfactant produced has antifungal activity of 55 mm zone of inhibition. Antifungal activity obtained with the biosurfactant in the 1 L bioreactor was 1.07-fold higher in comparison with the 10 L scale *P/V* experiment (Fig. 4.3 – 4.4). These can be elucidated from the mixing viewpoint of the fluid homogenization level (Bujalski *et al*., 2002). Regardless of the flow regime achieved in the 10 L scale set up, the flow will remain laminar at micromixing scale, due to its larger surface area and double impeller system employed (Vincent and Meneguzzi, 1994). Moreover, meso and micromixing are known to be important processes for bioprocesses biochemical reactions. Hence, it attained a mixing regime probably lower than the optimal process parameters. Other reasons for biosurfactant production with lower antifungal activity in constant *P/V* experiments could be ascribed to the different geometrical, rheological, and hydrodynamic parameters implemented.

4.3.4 Scaling up based on Reynolds number

Biosurfactant obtained with constant Reynolds number showed antifungal activity of 47 mm zone of inhibition. The antifungal activity based on *Re* was the lowest when compared to the 1 L bioreactor, and to those obtained with constant *P/V* and V_{tip} experiments. Scaling up with the constant Reynold's number criterion, has very low values for impeller agitation speed in the 10 L scale bioreactor. This low impeller speed might provoke an inappropriate mixing, resulting to negative effect on *B. subtilis* BS20 proliferation and consequently lower values for cell dry weight and antifungal activity obtained at 10 L scale bioreactor. This agrees with literature that Reynold's number as scaling up criterion usually result in adverse impact on the process. This is because the degree of agitation decreases very rapidly with the increase of the production scale using Reynold's number as scaling up parameter (Perez *et al*., 2018). Unfortunately, very low impeller speed might physically be impracticable to maintain desirable process conditions

at production scale bioreactors. The reason being that physical processes are dimensional related while metabolic processes are indirectly scale dependent. This could lead to improper mixing regime effecting the growing cell's physiology, metabolic activities, and productivity as it was obtained with constant Reynolds number in the present study. In a related study, Obonna *et al*. (2001), also, reported that implementing the same or lower mixing speed used in a 1 L bioreactor was not appropriate for the 8 L bioreactor. Hence, the cells and the substrate were not homogeneously distributed in the large-scale bioreactor. Mixing rate could influence the mass transfer and temperature gradient homogeneity adversely for viscous fermentation broth of biosurfactant production (Deniz *et al*., 2015). Also, studies have shown that when a scaling up approach resulted in an increased Reynolds number a low *P/V* value is obtained, which is not sufficient for efficient mixing, hence, productivity rate is adversely affected. In other words, a longer mixing time might be obtained with constant *Re* experiment that subsequently affected biosurfactant production unfavorably. This effect might be due to the phenomena that longer mixing time might influenced the mass transfer adversely, leading to probable death regions within the bioreactor (Deniz *et al*., 2015). The probable occurrence of death regions in the 10 L scale bioreactor could have resulted in the lower biomass and product yield upon scaling up based on constant *Re* (Fig. 4.1 – 4.4)

4.3.5 Kinetics of *B. subtilis* BS20 growth using the logistic function

Experimental data from the biomass dry weight over time (Fig. 4.1 and 4.2) for both scales were used to fit the logistic function with correlation coefficients (R^2) of 0.64, 0.781, 0.894 and 0.952 for Reynolds number, power consumption, impeller tip speed and for the 1 L bioreactor, respectively. An indication of the suitability of model to describe *B. subtilis* BS20 growth using the different scale up criteria. Although, lower maximum specific growth rates (μ_{max}) were obtained with V_{tip} and *P/V* experiments, higher maximum biomass dry weight (X_{max}) were observed in these bioreactors when compared to the constant *Re* experiment and the 1 L scale

experiment (Table 4.3). The lower μ_{max} observed in the V_{tip} and P/V bioreactors might be due to high impeller speed implemented in these systems (Table 4.3). The experimental data obtained from keeping V_{tip} and P/V constant suggest the scale up criteria largely provide suitable process conditions that favored *B. subtilis* biosurfactant production (Xia *et al*., 2015).

4.3.6 Conclusion

This study has provided a simple but coherent rheological model for translating an optimized laboratory scale biosurfactant production to a pilot scale successfully. It was demonstrated that the application of constant V_{tip} is a better approach in scaling up the production of biosurfactant by *B. subtilis* BS20 due to the suitable mixing regime, pumping capacity (3.4×10^{-4} m^3/s), circulation time (5.2 s) and turbulence (15.6 m). As evidently presented from the results of maintaining a constant V_{tip} upon scale up from 1 L to 10 L scale, higher antifungal activity (65mm) was achieved. The data obtained in the current study provide valuable sight for potential industrial biosurfactant production using *B. subtilis* BS20.

References

Arapoglou, D., Varzakas, T., Vlyssides, A. and Israilides, C.J.W.M., 2010. Ethanol production from potato peel waste (PPW). *Waste Management*, *30*(10), pp.1898-1902.

Aruwajoye, G.S., Faloye, F.D. and Kana, E.G., 2017. Soaking assisted thermal pretreatment of cassava peels wastes for fermentable sugar production: process modelling and optimization. *Energy Conversion and Management*, *150*, pp.558-566.

Babel, W., Brinkmann, U. and Müller, R.H., 1993. The auxiliary substrate concept—an approach for overcoming limits of microbial performances. *Acta biotechnologica*, *13*(3), pp.211-242.

Ban, D.K. and Paul, S., 2014. Zinc oxide nanoparticles modulates the production of β-glucosidase and protects its functional state under alcoholic condition in *Saccharomyces cerevisiae*. *Applied biochemistry and biotechnology*, *173*, pp.155-166.

Bonvillani, P.M., Ferrari, P., Ducrós, E.M., Orejas, J.A., 2006. Theoretical and Experimental Study of the Effects of Scale-Up on Mixing time for a Stirred-Tank Bioreactor. *Brazilian Journal of Chemical Engineering*, 23 (1), 1-7.

Bujalski, W., Jaworski, Z., Nienow, A.W., 2002. CFD Study of Homogenization with Dual Rushton Turbines—Comparison with Experimental Results: Part II: The Multiple Reference Frame. *Chemical Engineering Research and Design.* 80 (1), 97-104.

Chohan, N.A., Aruwajoye, G.S., Sewsynker-Sukai, Y., Gueguim Kana, E.B., 2020. Valorisation of potato peel wastes for bioethanol production using simultaneous saccharification and fermentation: Process optimization and kinetic assessment. *Renewable Energy,* 146, 1031-1040.

de la Roza, C., Laca, A., García, L.A., Díaz, M., 2003. Ethanol and ethyl acetate production during the cider fermentation from laboratory to industrial scale. *Process Biochemistry*, 38, 1451-6.

Deniz, I., Imamoglu, E., Vardar Sukan, F., 2015. Evaluation of scale-up parameters of bioethanol production from *Escherichia coli* KO11. *Turkish Journal of Biochemistry*, 40 (1).

Dubey, K.K., Ray, A.R. and Behera, B.K., 2008. Production of demethylated colchicine through microbial transformation and scale-up process development. *Process Biochemistry*, *43*(3), pp.251-257.

El-Kemary, M., Nagy, N., El-Mehasseb, I., 2013. Nickel oxide nanoparticles: Synthesis and spectral studies of interactions with glucose. *Materials Science in Semiconductor Processing*. 16, 1747–1752.

Faloye, F.D., Gueguim Kana, E.B., Stefan Schmidt., 2014. Optimization of biohydrogen inoculum development via a hybrid pH and microwave treatment technique - Semi pilot scale production assessment. *International Journal of Hydrogen Energy,* 39, 5607-5616.

Ganeshan, S., Kim, S.H. and Vujanovic, V., 2021. Scaling-up production of plant endophytes in bioreactors: concepts, challenges, and perspectives. *Bioresources and Bioprocessing*, *8*(1), pp.1-16.

Garcia-Ochoa, F., Gomez, E., 2009. Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. *Biotechnology Advances,* 27, 153–176

Han, H., Cui, M., Wei, L., Yang, H. and Shen, J., 2011. Enhancement effect of hematite nanoparticles on fermentative hydrogen production. *Bioresource technology*, *102*(17), pp.7903-7909.

Hiruta, O., Futamura, T., Takebe, H., Satoh, A., Kamisaka, Y., Yokochi, T., Nakahara, T. and Suzuki, O., 1996. Optimization and scale-up of γ-linolenic acid production by Mortierella ramanniana MM 15-1, a high γ-linolenic acid producing mutant. *Journal of fermentation and bioengineering*, *82*(4), pp.366-370.

Izmirlioglu, G. and Demirci, A., 2012. Ethanol production from waste potato mash by using *Saccharomyces cerevisiae*. *Applied Sciences*, *2*(4), pp.738-753.

Junker, B.H., 2004. Scale-up methodologies for Escherichia coli and yeast fermentation processes. *Journal of bioscience and bioengineering*, *97*(6), pp.347-364.

Khawla, B.J., Sameh, M., Imen, G., Donyes, F., Dhouha, G., Raoudha, E.G., Oumèma. N-E., 2014. Potato peel as feedstock for bioethanol production: A comparison of acidic and enzymatic hydrolysis. *Industrial Crops and Products,* 52, 144–149.

Klöckner, W., Gacem, R., Anderlei, T., Raven, N., Schillberg, S., Lattermann, C. and Büchs, J., 2013. Correlation between mass transfer coefficient k_{La} and relevant operating parameters in cylindrical disposable shaken bioreactors on a bench-to-pilot scale. *Journal of biological engineering*, *7*, pp.1-14.

Marques, M.P., Cabral, J.M. and Fernandes, P., 2010. Bioprocess scale-up: quest for the parameters to be used as criterion to move from microreactors to lab-scale. *Journal of Chemical Technology & Biotechnology*, *85*(9), pp.1184-1198..

Moodley, P. and Kana, E.G., 2019. Bioethanol production from sugarcane leaf waste: Effect of various optimized pretreatments and fermentation conditions on process kinetics. *Biotechnology Reports*, *22*, p.e00329.

Ogbonna, J.C., Mashima, H., Tanaka, H., 2001. Scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor. *Bioresource Technology,* 76, 1-8

Oniscu, C., Galaction, A.-I., Cascaval, D., Ungureanu, F., 2002. Modeling of mixing in stirred bioreactors 2. Mixing time for non-aerated broths. *Biochemical Engineering Journal* 12, 61–69

Palmqvist, E. and Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresource technology*, *74*(1), pp.17-24.

Pérez, R.P., Suárez, J.G., Diaz, E.N., Rodríguez, R.S., Menéndez, E.C., Balaguer, H.D., Lasa, A.M., 2018. Scaling-up fermentation of *Escherichia coli* for production of recombinant P64k protein from *Neisseria meningitidis. Electronic Journal of Biotechnology* 33, 29–35.

Pietruszka, M., Pielech-przybylska, K., Szopa, J.S., 2010. Synthesis of higher alcohols during alcoholic fermentation of rye mashes. *Food Chemistry and Biotechnology*, 1081 (74), 51-64.

Qazizada, M.E., 2016. Design of a batch stirred fermenter for ethanol production. *Procedia Engineering,* 149, 389 – 403

Romero-Güiza, M.S., Vila, J., Mata-Alvarez, J., Chimenos, J-M., Astal, S., 2016. The role of additives on anaerobic digestion: *A review Renewable and Sustainable Energy Reviews* 58, 1486–1499.

Rorke, D.C., Suinyuy, T.N. and Kana, E.G., 2017. Microwave-assisted chemical pre-treatment of waste sorghum leaves: Process optimization and development of an intelligent model for determination of volatile compound fractions. *Bioresource technology*, *224*, pp.590-600.

Sanusi, A.I., Faloye, F.D., Kana, E.G.B., 2019. Impact of Various Metallic Oxide Nanoparticles on Ethanol Production by *Saccharomyces cerevisiae* BY4743: Screening, Kinetic Study and Validation on Potato Waste. *Catalysis Letters*, 149 (7), 2015-2031.

Sanusi, I.A., Suinyuy, T.N., Lateef, A. and Kana, G.E., 2020. Effect of nickel oxide nanoparticles on bioethanol production: process optimization, kinetic and metabolic studies. *Process Biochemistry*, *92*, pp.386-400.

Suinyuy, T.N., Donaldson, J.S. and Johnson, S.D., 2013. Patterns of odour emission, thermogenesis and pollinator activity in cones of an African cycad: what mechanisms apply?. *Annals of Botany*, *112*(5), pp.891-902.

Tyo, K.E., Kocharin, K. and Nielsen, J., 2010. Toward design-based engineering of industrial microbes. *Current opinion in microbiology*, *13*(3), pp.255-262.

Vincent, A. and Meneguzzi, M., 1994. The dynamics of vorticity tubes in homogeneous turbulence. *Journal of Fluid Mechanics*, *258*, pp.245-254.

Whiting, G.C., 1976. Organic acid metabolism of yeasts during fermentation of alcoholic beverages—A review. *Journal of the Institute of Brewing*, *82*(2), pp.84-92.

Wimonsong, P., Nitisoravut, R., 2015. Comparison of Different Catalysts for Fermentative Hydrogen Production. *Journal of Clean Energy Technologies*. 3 (2), 128–131.

Xia, J., Wang, G., Lin, J., Wang, Y., Chu, J., Zhuang, Y. and Zhang, S., 2016. Advances and practices of bioprocess scale-up. *Bioreactor Engineering Research and Industrial Applications II*, pp.137-151.

Zhu, R., Gao, X., Xiao, Y., Yang, N. and Jin, Y., 2019. Improved performance in γ-polyglutamic acid production by Bacillus subtilis LX on industrial scale by impeller retrofitting and its unstructured microbial growth kinetics model. *Preparative Biochemistry and Biotechnology*, *49*(3), pp.307-314.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

5.1 Conclusions

This study was undertaken with the aim to optimize biosurfactant production with antifungal activity by *B. subtilis* BS20. The research also explored the impact of nanoparticles on *B. subtilis* BS20 for biosurfactant production. In addition, the feasibility of a preliminary scale up of the optimized process was evaluated. The key findings of this study and their significance are summarized below:

- The response surface methodology (RSM) was used as a mathematical and statistical tool to model glucose concentration (10 – 30 g/L), temperature (25 – 45°C) and incubation times (24 – 96 h) for optimum biosurfactant production. The model suggested optimal process set points of 11.5 g/L, 24 h and 41ºC for substrate concentration, incubation time and incubation temperature, respectively, that promoted *B. subtilis* BS20 metabolic activities and biosurfactant production. Evaluating the pairwise interactive effects of the input parameters and the obtained optimal set points clearly resulted in increased biosurfactant production with high antifungal activity. Model validation gave biosurfactant with antifungal activity of 68 mm resulting in a 13% increase in the zone of inhibition. The results highlight the importance of key operational parameters on enhancing biosurfactant production These findings could pave the way for large scale production of biosurfactant with high antifungal activity. The scaling up of biosurfactant with high antifungal activity will accelerate its commercialization and contribute to the implementation of sustainable and environmentally friendly pathogen control. Therefore, it is important to conduct findings on the scale up viability of this process to fully understand the process

complexities of production of biosurfactant with high antifungal activity from these optimized process conditions.

- Additionally, seven nanoparticles were evaluated for their catalytic potential to promote biosurfactant production by *B. subtilis* BS20. Upon analysis the nanoparticles exhibited significant positive effects on *B. subtilis* BS20 growth resulting in high biomass concentration. The supplementary inclusion of nanoparticles favored increased biomass yield but biosurfactant with high antifungal activity was not obtained. This is an indication that metabolic pathway that favors high cell proliferation do not promote the formation of biosurfactant with high antifungal activity.
- Furthermore, this research work undertook semi-pilot scale assessment of biosurfactant production by *B. subtilis* BS20. Process scaling up intricacies might considerably impact the process kinetics, product formation, and consequently the process productivity. Scaling-up criteria of constant impeller tip speed (V_{tip}), Reynold number (*Re*) and constant power consumption (*P/V*) were employed in this study. Implementing constant V_{tip}, *P/V* and *Re* at 41°C, gave biosurfactant antifungal activity of 65 mm, 55 mm, and 47 mm in the 10 L scale bioreactor, respectively. This is comparable to the antifungal activity (68 mm) obtained in the 1 L bioreactor. Moreover, maintaining constant V_{tip} decreased shear stress by 0.77-fold in the 10 L bioreactor which resulted in low cell damage and high cell viability. Constant impeller tip speed provided the most desirable process conditions especially the mixing regime that favored the production of biosurfactant with high antifungal activity. Insufficient mixing has been recognized as a focal challenge in bioprocess scaling up. Desirable pumping capacity ($V_P = 3.4 \times 10^{-4}$ $m^3/\mathbf{s}$) and circulation time (t_c=5.2 s) were attained in this study to achieve considerable process performance. Pumping capacity and circulation time are important mixing properties that are required for efficient mixing behavior in bioprocess.

- Additionally, the logistic function was fitted using experimental data from the biomass dry weight over time for both scales, with correlation coefficients (R^2) for Reynolds number, power consumption, impeller tip speed, and the 1 L bioreactor of 0.64, 0.781, 0.894, and 0.952, respectively. This demonstrates the logistic model's effectiveness on describing growth of BS20 using these scale-up techniques. Thus, the scale up results in this study provided significant insights on the production of biosurfactant with high antifungal activity towards achieving its commercialization.

5.2 Recommendations for future studies

Modelling several parameters for improved production of biosurfactant with high antifungal activity is an intricate process that requires the evaluation of many parameters.

- In this study it was demonstrated that optimal substrate concentration, operational temperature, and incubation time enhanced process performance to produce high antifungal activity biosurfactant. To improve on the efficiency of biosurfactant production in this research, additional process parameters could be evaluated.
- Additional research into the interaction of the metallic oxide nanoparticles with *B. subtilis* BS20 to improve the formation of biosurfactant with high biomass yields in this study would provide knowledge on the impact of nanoparticles on *B. subtilis* BS20 metabolic activities. This study observed increased biomass yields when supplemented with nanoparticles more significantly nickel oxide. Further exploration may include iron (II) oxide as well as nickel oxide with optimization strategies to find the optimal concentration required for end product requirement.
- Moreover, to enhance capabilities of *B. subtilis* BS20 for high antifungal activity biosurfactant production, metabolically engineered strain of *B. subtilis* BS20 might be required, as well as explore the use of genetic engineering for selective enhancements of fencing, a lipopeptide biosurfactant.

- In addition, to increase the industrial feasibility of *B. subtilis* BS20 cultivation, evaluation of co-production can be investigated. This set up could be carried out with the production of another highly valuable bioproduct alongside biosurfactant. Research has shown the possibility of biosurfactant production alongside enzyme production such as lipase and protease.