Enhancing Biomolecule Production from *Bacillus velezensis* PhCL Using Low-Cost Agricultural Residues as Feedstock and Improving Biomass Hydrolysis through Enzyme Mutagenesis

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Abstract of the Dissertation

Bioproduction have been considered an alternative option for obtaining useful and eco-friendly chemicals for industrial applications. *Bacillus velezensis*, a trending bacterium first isolated in 2005, is well-known for its ability in producing various economic and environmentally-friendly biomolecules, such as industrial enzymes, biosurfactants, antioxidants, antibiotics, etc. However, most of the studies nowadays use edible sugar for biomolecule production which increased the production cost meanwhile raises concerns regarding hunger and food-energy competition. Meanwhile, several genomic sequencing studies suggested that various strains of *B. velezensis* have the ability in producing various lignocellulase, such as xylanase and CMCase. However, very few studies use agricultural wastes as feedstock for producing biomolecules. Therefore, for advancing the utilization of the lignocellulosic biomass, a newly isolated *B. velezensis* PhCL was characterized, and its potential for bioremediation was evaluated. Furthermore, various agricultural wastes were used as a fermentation feedstock for producing various biomolecules, and the amylase production was optimized via response surface methodology. The remaining biomass residue was also converted to biochar for further utilization. Moreover, for advancing the utilization of agricultural waste, a GH 11 xylanase was *in silico* analyzed and several mutants were constructed based on the analysis. The mutant showed better hydrolysis efficiency and released more reducing sugar from wheat straw. This study explored another approach for utilizing *B. velezensis* for value-added biomolecule production, which could be used for bioremediation and various industrial applications, from low-cost agricultural waste. Moreover, this study also contributed to the understanding of utilizing advanced computer

programs for improving enzymatic performance meanwhile exploring the undermined structure-function relationship of xylanase.

A newly isolated bacterium, *Bacillus velezensis* PhCL, was identified and characterized. The fermentation conditions for producing amylase were optimized via response surface methodology. The optimal amylase activity of 35.626 ± 0.87 U/mL could be achieved at medium pH 8, temperature 40 °C, and fermentation time 72 hours. Furthermore, various bioactive compounds were purified using sequential precipitation by ammonium sulfate. The partially purified enzymes exhibited their outstanding characteristics for bioremediation and industrial applications. Moreover, the crude biosurfactant exhibited a better performance in engine oil bioremediation compared to commercial surfactants. This study explored the potential of the *B .velezensis* PhCL and demonstrated that sequential precipitation could be a potential approach for purifying multiple biomolecules at low cost.

Moreover, fermentation of *Bacillus velezensis* PhCL was examined using various agricultural wastes as feedstock. After the initial screening and the optimization of fermentation conditions, the optimal amylase activity 43.94 ± 0.51 U/mL could be achieved with a fermentation temperature of 33.5 °C, pH 8, and fermentation time of 68 hours with a medium composed of 35% rice husk, 0.375% $(NH_4)_2SO_4$, and 0.5% of peptone. Moreover, besides the production of other biomolecules, the rice husk used as feedstock was hydrolyzed and further used for producing biochar. The results suggested that the hydrolyzed rice husk would have better adsorption compared to the control and the potential of using this biochar for enzyme immobilization was evaluated. This study explored the potential of using agricultural wastes as feedstock for producing various useful biomolecules from *B. velezensis*

and the further utilization of fermented residues was evaluated.

Furthermore, this study also included the overexpression and characterization of a novel GH 11 endo-β-1, 4 xylanase from *Bacillus sp.* P3. The sequence of the protein had been used for constructing the homologous protein structure and the structural changes were simulated using molecular dynamics. After analyzing the results from molecular dynamics, several mutants were constructed for lowering the protein flexibility, and the wild-type and mutants were characterized. The results suggested that the mutant N159I would have a better catalytic efficiency and substrate binding affinity compared to the wild-type. Moreover, the structural difference also allowed mutant N159I to release more reducing sugar from wheat straw. This study explored the potential of using advanced computer programs for protein engineering and advancing our understanding of the structure-function relationship of xylanase.

Overall, this study has explored the production, characteristics, and applications of various biomolecules produced by a newly isolated *B. velezensis* PhCL and a glycoside hydrolase family 11 xylanase was subjected to mutagenesis based on the molecular dynamics simulation. The mutant has shown better catalytic efficiency and released more reducing sugar from wheat straw compared to wild-type xylanase. This study provided a further exploration into utilizing agricultural wastes for energy and chemical production.

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Introduction of the Thesis

Microorganisms are an essential part of nature and are existing everywhere on Earth, even in outer space (Horneck et al., 2010). Microorganisms have several important and irreplaceable contributions to nature and human beings. They are well-known as decomposers and play an important role in natural nutrient cycles including the carbon cycle, nitrogen cycle, phosphorus cycle, etc (Barsdate et al., 1974; Schulze, 2000). Besides their significance in the natural ecosystem, they are also having critical roles in or outside of the human body. Studies have suggested that the human microbiome is affecting our food consumption, mental health, and is even related to severe disease (Hill et al., 2014; Lucas, 2018; Shreiner et al., 2015). Moreover, microorganisms have been widely applied in human activities and technology for a long time.

Even though the word "Biotechnology" was first introduced by Karl Ereky in 1919 (Verma et al., 2011), humans have been already using microorganisms for food processing, alcohol and enzyme production, and soil fertilization since ancient times. The first fermentation can be traced back to 7000 BC when Sumerians and Babylonia were producing beer by converting sugars to alcohol (Demain, 2010). Meanwhile, wine was also produced in China (McGovern et al., 2004). Besides the beverage, microorganisms also have been used for producing other fermentation products such as bread, cheese, and vinegar. At this ancient biotechnology stage, most of the techniques were applied without understanding the scientific mechanism behind them.

After the discovery of microorganisms and vaccination, scientists started to develop biological techniques based on scientific knowledge and observation, and thus, classic

biotechnology was further established in the early 19 century. Most of the studies at this stage was focusing on the potential of using organisms or microorganisms for producing value-added products. However, due to the limitation of the understanding of microbiology, these research findings did not apply to industrial production yet.

As time passed, scientists have been exploring the potential of fermentation using microorganisms and have overcome several issues in techniques and sterility. In the early 20 century, several microbial fermentations have been developed such as the production of citric acid, glycerol, gluconic acid, etc., and these fermentations can be up-scaled and controlled for industrial purposes (Lee et al., 2016). Furthermore, the law of heredity suggested by Mendel brought new insight into the inheritance in organisms and further developed into the concept of "genetics" which has made a significant impact on biotechnology development.

Nowadays, biotechnologies using genetic-modified microorganisms have been widely applied in our society and various industrial activities including fermentation, bioremediation, and the production of bio-based materials. Furthermore, the enzymes and secondary metabolites produced by microorganisms are also commercially available and attracted various industrial interests, such as amylase, laccase, lignocellulase, and antibiotics. However, the production of these enzymes and bioactive compounds would require edible resources such as mono-sugar, starch, lipid, etc., and the utilization of food could raise the concern about hunger and food-energy competition (Harvey and Pilgrim, 2011). Therefore, the utilization of non-edible biomass, lignocellulose, has become one of the promising alternative approaches for producing sustainable and green energy and chemicals. Therefore, agricultural wastes, as a low-cost and widely available lignocellulose, have been attracting numerous

research attention and various studies had been using them as fermentation feedstock.

In this study, a novel strain of *Bacillus velezensis* PhCL was isolated from the phenolic crystal waste based on its ability in using lignin as the sole carbon source for growth. The characterization of the strain suggested that it had the ability in producing various bioactive compounds such as amylase, lignocellulase, biosurfactant, and antioxidants. However, using agricultural wastes as feedstock to produce these bioactive compounds from *Bacillus velezensis* was seldom reported. Therefore, this study intended to explore and characterize the potential of using agricultural waste as feedstock for producing biomolecules from *Bacillus velezensis* PhCL.

Therefore, to advance the utilization of agricultural wastes biomass, several hypotheses had been proposed:

(1) The newly isolated *Bacillus velezensis* could produce various highly active biomolecules such as biosurfactants and enzymes in the liquid medium;

(2) Enhancing the catalytic efficiency of xylanase could contribute to hydrolyzing agricultural waste;

(3) Agricultural waste could be utilized as feedstock for *Bacillus velezensis* fermentation and various biomolecules such as biosurfactants and enzymes could be produced.

Therefore, to verify these hypotheses and achieve the goal, several objectives had been proposed:

(1) The optimization, purification, and characterization of the biomolecules produced by *Bacillus velezensis* PhCL;

(2) The utilization of advancing computer science technology for improving catalytic

efficiency of the lignocellulase and evaluation of the potential in hydrolyzing biomasses;

(3) The utilization of agricultural wastes for biomolecule production such as biosurfactants and enzymes and the utilization of the remaining residues as adsorbent.

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Chapter 1. Literature review

This chapter is partially adapted from my recent publication:

Chio, Chonlong, Mohini Sain, and Wensheng Qin. "Lignin utilization: A review of lignin depolymerization from various aspects." Renewable and Sustainable Energy Reviews 107 (2019): 232-249.

1. Overview of the trending bacterium, *Bacillus velezensis*

1. 1 Introduction

Bacillus is one of the most studied genera of bacteria. It is widely distributed in nature and played several important roles in various fields such as agriculture, industrial production, medication, biomaterial, etc (Radhakrishnan et al., 2017; Sibponkrung et al., 2020). There are more than 200 species under *Bacillus* genus and some of them are well-studied due to their extraordinary characteristics. *Bacillus subtilis* is one of the well-characterized species and it shows several interesting properties including producing value-added products such as vitamins (Acevedo-Rocha et al., 2019) and poly β-Hydroxybutyrate (PHB) (Singh et al., 2013a), protecting plants and crops from pathogens (Wang et al., 2018), treating wastewater (Hlordzi et al., 2020), etc. *Bacillus cereus* is also a well-studied species due to its relationship with food poisoning and diseases (Baldwin, 2020; Ehling-Schulz et al., 2019). *Bacillus thuringiensis* can produce the delta-endotoxin which is an effective pesticide and also known as Cyt proteins (Fernández-Chapa et al., 2019). *Bacillus amyloliquefaciens* is also widely studied regarding its outstanding ability in hydrolyzing starch, produce amylase, and against several plant pathogens (Farooq et al., 2021b; Shafi et al., 2017; Xu et al., 2013). Among the bacteria listed above, *Bacillus velezensis* recently is also attracting worldwide researcher's

attention due to its extraordinary ability in biocontrol and plant growth-promoting. The number of publications and genomic sequencing of *B. velezensis* is rapidly increasing annually since 2015 (Figure 1).

Publication regarding
***Bacillus velezensis* in each year**
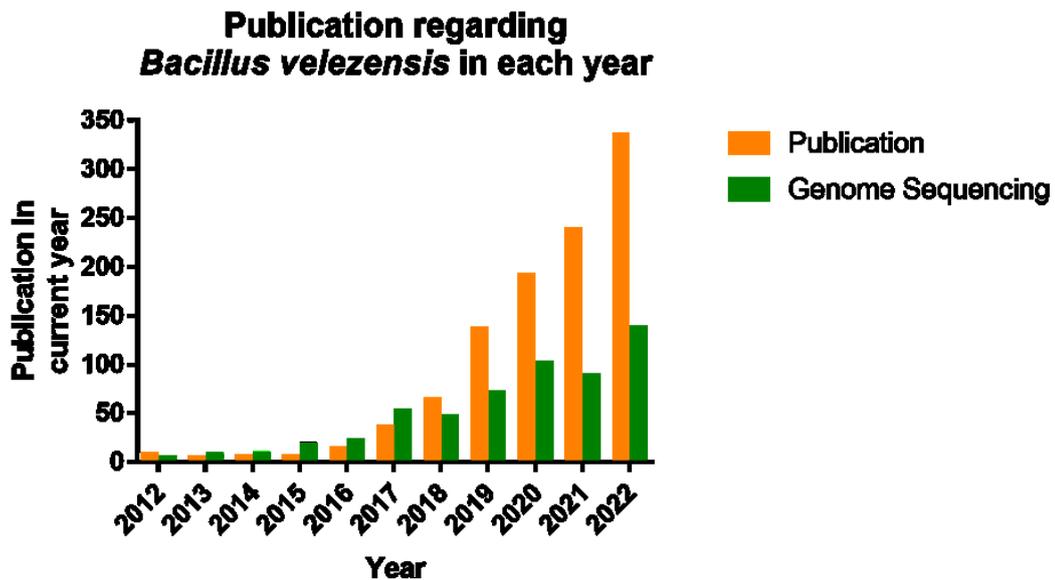
Figure 1 The papers and genome sequencing published from 2012 to 2022. The data of publication was retrieved from the Web of Science using "Bacillus velezensis" as the keyword for searching and the genome data was searched in the NCBI database.

Bacillus velezensis was named due to that the species was first isolated from the river Vélez which is located in southern Spain and it was first described by Ruiz-Garcia et al in 2005 (Ruiz-Garcia et al., 2005). *B. velezensis* is an aerobic, gram-positive bacterium with a rod shape and had the ability in forming an ellipsoidal endospore. The size of the *B. velezensis* usually is around 1.5 to 3.5 μm (Ruiz-Garcia et al., 2005). The morphology of the bacterial colonies usually is cream white but it could be different under different environments. Some strains of the *B. velezensis* could convert the colonies from non-mucoid

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to mucoid status or vice versa. Choi et al. (2021a) showed that the mucoid *B. velezensis* GH1-13 was more sensitive to antibiotics and stress. Furthermore, they also demonstrated the difference in metabolites and gene expression patterns between the non-mucoid and mucoid *B. velezensis*.

B. velezensis can be growing within the range of temperature from 25 to 50 °C, pH from 5 to 10, and sodium chloride from 0% to 15% (Meena et al., 2018; Myo et al., 2019; Torres et al., 2020; Yao et al., 2019). The ability to adapt to various environmental conditions could be beneficial to various industrial applications (Becker and Wittmann, 2018).

1. 2 Applications

Bacillus velezensis is well-known for its ability in producing various bioproducts with numerous applications. Figure 2 summarized 661 publications (from 2012 to 2021) regarding *Bacillus velezensis* and the keywords or important terms in the publication. The keywords and abstract were analyzed and grouped based on the relevant and mention frequency. Based on the network analysis, 3 major functions had been widely discussed: Biocontrol, plant promotion, and production of biopolymers. Besides these widely discussed functions, the enzymes, especially the lignocellulase, produced by *B. velezensis* would also be discussed.

1. 2. 1 Plant growth-promoting and biocontrol agent

Bacillus velezensis is well-known as a plant growth-promoting rhizobacteria (PGPR) and it could colonize or assist the colonization of other plant probiotics, thus promoting plant growth (Becker and Wittmann, 2018). The study conducted by Sibponkrung et al. (2020)

indicated that the co-culture of *Bacillus velezensis* S141 and *Bradyrhizobium diazoefficiens* USDA110 could enlarge the nodules which resulted in higher nodulation and nitrogen-fixing efficiency. Moreover, Meng et al. (2016) indicated that the nitrogen fixation of *Bacillus velezensis* BAC03 could significantly enhance the growth of the leaf and root of radishes, carrots, and beets. Besides being directly involved in the plant-promoting activity, interestingly, the study from Sun et al. (2022) indicated that the interaction between *Bacillus velezensis* SQR9 and plant-promoting bacterium *Pseudomonas stutzeri* could promote the growth of cucumber by inducing *Pseudomonas stutzeri* and the biofilm from *Bacillus velezensis* SQR9 would be essential for activating the *Pseudomonas stutzeri*.
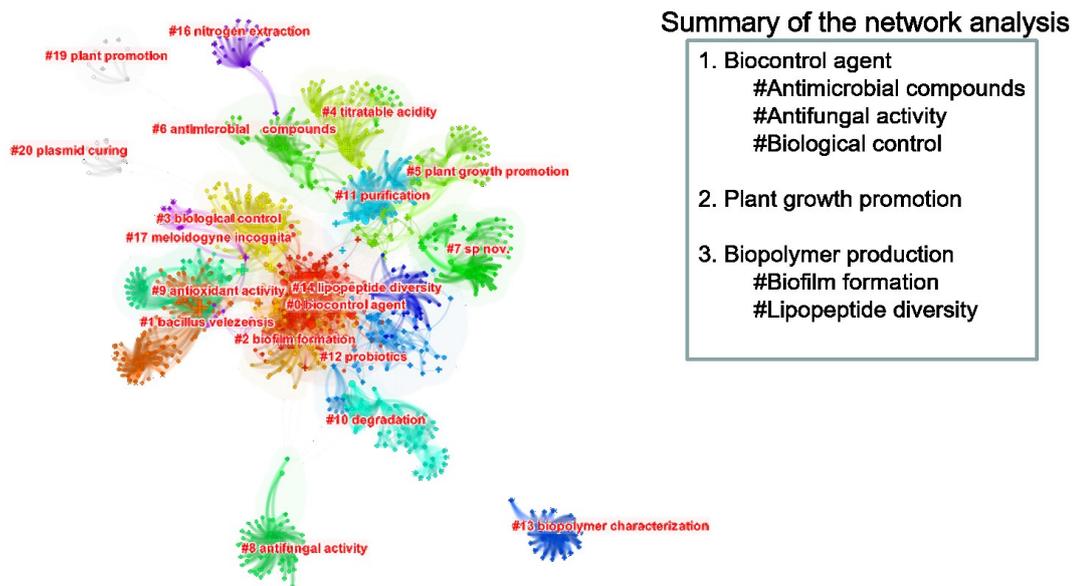
Figure 2 The keywords grouping of 661 publications regarding Bacillus velezensis using Citespace (Chen, 2014). The title, keywords, and abstract of the publication were retrieved from the Web of Science.

The production of various cytokinins and metabolites could also be one of the routes for promoting plant growth. Idris et al. (2007) reported that *Bacillus amyloliquefaciens* FZB42

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(later renamed as *B. velezensis* FZB42) could produce a significant amount of indole-3-acetic acid (IAA) which is well-known as plant hormone for plant growth and the presence of tryptophan could enhance 5 times of the IAA production. Moreover, Zhang et al. (2007) indicated that a closely related species *B. amyloliquefaciens* IN937a could produce volatile organic compounds, and the auxin homeostasis of the *Arabidopsis* would be regulated via the compounds emission. Furthermore, *B. velezensis* also could modulate and control the hormone homeostasis and growth gene expression in plants (Hasan et al., 2022; Sharma et al., 2022).

Besides producing inducers or inducing plant growth, *B. velezensis* also could promote plant growth by inhibiting the growth or metabolism of plant pathogens, which is also well-known for its biocontrol ability. Moon et al. (2021) showed that *B. velezensis* CE100 could inhibit root rot diseases by controlling the growth of various *Phytophthora spp*. The growth of pathogens and their myelin would be inhibited by the glucanase and protease which would hydrolyze their oomycetes cell wall and result in abnormal growth. Moreover, some strains of *Bacillus velezensis* would have the ability in producing an antimicrobial lipopeptide, bacillomycin D, and it had shown its extraordinary antimicrobial activity against several common plant pathogens, such as *Fusarium graminearum* which was causing Fusarium ear blight (Gu et al., 2017), *Rhizopus stolonifer* (Lin et al., 2019), *Colletotrichum gloeosporioides* (Penz.) which was causing anthracnose (Jin et al., 2020), etc.

Moreover, besides directly acting on the plant pathogens, glucanases and bacillomycin D can stimulate the induced systemic resistance (ISR) which stimulated the plant to defend against the pathogens and produce other antimicrobial compounds. Moreover, Chowdhury et

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al. (2015) further confirmed that the presence of lipopeptides could be the inducer of the IRS. Xue et al. (2023) revealed that bacillomycin D could stimulate the ISR in cherry tomatoes by activating the synthesis of phenolic acids and lignin, expressing other defense-related genes, and accumulating hydrogen peroxide. Similarly, Lin et al. (2019) also reported that the presence of bacillomycin D could stimulate the ISR in cherry tomatoes and increase the activities of plant defense-related enzymes such as chitinase, glucanase, and peroxidase, etc.

1. 2. 2 Production of biopolymers

Bacillus velezensis is capable of producing various biopolymers such as lipopeptides, exopolysaccharides, bacterial nanocellulose, etc. but no report on the production of polyhydroxyalkanoates at all. Binmad et al. (2022) reported that *B. velezensis* P1 was able to produce a novel exopolymeric substance with strong antifungal activity. Interestingly, this novel exopolymeric substance was composed of mainly protein, carbohydrate, and ash (up to 30 %) with a crystallinity index of 87% and it could also remain stable until 500 ℃. Cao et al. (2020b) reported that *B. velezensis* SN1 was able to produce an exopolysaccharide composed of glucose, mannose, and fructose with a high molecular weight of 2.21×10^5 Da, and this exopolysaccharide exhibited very strong antioxidant activity and thermal stability. Flores et al. (2020) reported the production of poly(γ-glutamic acid) by *B. velezensis* 83, which could be used for food and medical industry and wastewater treatment.

Besides these polymers, the major polymer produced by *B. velezensis* was the bioactive lipopeptide. Various lipopeptides have been used as antimicrobial agents for controlling pathogens, such as bacillomycin D mentioned above, and there were 3 major groups of the

lipopeptide produced by *B. velezensis*: surfactin family, iturin family, and fengycin family. Commonly they were considered and used as an antimicrobial agent (Fazle Rabbee and Baek, 2020; Liu et al., 2020), but recently these lipopeptides were widely used as emulsifiers and biosurfactants for bioremediation which involved removing hydrocarbon pollutants, poly aromatic hydrocarbon, and other low-solubility substance (Zahed et al., 2022). Quijano et al. *in silico* simulated the hexadecane biodegradation process and found that the limiting step of the biodegradation was the hexadecane entering into the bacterium and biosurfactant could significantly improve the contact between the hexadecane and microbes (Quijano et al., 2010). Meena et al. (2021) showed that *B. velezensis* KLP2016 could produce surfactin meanwhile, utilizing the surfactin to enhance the emulsion of the engine oil and improve the biodegradation efficiency. Similarly, Jakinala et al. (2019) demonstrated that the presence of surfactin could enhance the solubility of toxic organic pollutant atrazine and result in enhancing the biodegradability of the atrazine by *B. velezensis* MHNK1. Moreover, Prakash et al. (2021) demonstrated that applying biosurfactants would significantly enhance the bio-electrokinetic remediation of oil-contaminated soil.

1. 2. 3 Production of enzymes

The enzyme was another economic biomolecule that would be attractive to industries. There are several reports regarding the enzyme production from *B. velezensis*, such as protease, cellulase, amylase, etc. (Ye et al., 2018). Lu et al. (2021) characterized and purified a fibrinolytic enzyme from *B. velezensis* SN14 using yeast extract, glucose, and beef extract as nutrients and the purified enzyme could be activated using Fe^{2+} with a 7.3-fold activity

enhancement. Ghosh et al. (2021) also suggested adding purified xylan would stimulate the expression of the xylanase from *B. velezensis* AG20 and result in a higher activity of xylanase. Zhang et al. (2018) reported that *B. velezensis* ZY-1-1 could express high-activity xylanase and cellulase using xylan and cellulose as medium substrates. Moreover, the complete genome sequence was analyzed and revealed that strain ZY-1-1 contained multiple lignocellulase including xylanase, cellulase, laccase, and other glycosidic hydrolases. This indicated that strain ZY-1-1 could have the ability to completely degrade lignocellulose. However, Chen et al. (2018) sequenced the genome of 24 different strains of *B. velezensis* and the result suggested that several cellulase, xylanase, and pectin lyase were found across the strains, but limited lignin-degrading enzymes were found which implied that these 24 strains of *B. velezensis* would lack the ability in degrading lignin.

As mentioned above, most of these enzymes and biomolecules were produced using edible sugar or nutrition as feedstock. This would increase the production cost meanwhile raise the concern about the food-energy competition and world hunger problem and one of the promising solutions was changing the feedstock from edible resource to non-edible biomass, lignocellulose. Even though genomic sequencing reports were stating that *B. velezensis* contained various lignocellulase, however, seldom reports were using raw lignocellulose for enzyme or biomolecule production, especially submerged fermentation. Bhatt et al. (2020) used moong husk and soybean cake with the addition of 1.5% fructose as feedstock for producing amylase from *B. velezensis* KB2216. Li et al. (2020b) and Tang et al. (2021) indicated that *B. velezensis* LC1 could utilize bamboo shoots as the carbon source for producing cellulase and xylanase, and the bioavailability of the hydrolyzed bamboo shoot

was higher and thus, more bioethanol could be converted by yeast. However, more studies such as screening with various lignocellulosic biomasses, the pretreatment of the lignocellulose, and the syngeneic effect with other microbes on the biodegradation of the lignocellulose.

1. 3 Summary and perspectives

Bacillus velezensis has the ability in producing various useful biomolecules. Besides the products discussed above, it was also able to produce vitamin K2 (Zhao et al., 2021) and other functional small molecules (Saravanan et al., 2022). However, although most of the lignocellulose study was only conducted at the genome sequencing level, the study focusing on the production and optimization of functional biomolecules utilizing lignocellulose biomass as feedstock was limited. Moreover, the potential of *B. velezensis* in bioremediation could be further expanded since it is considered an eco- and plant-friendly species. The expanded understanding of utilizing lignocellulose with *B. velezensis* could advance our bioremediation application meanwhile lowering the cost of producing various useful biomolecules.

2. Lignocellulose

2.1 Introduction

Since the industrial revolution, the need for energy resources has been dramatically increasing. Furthermore, the increasing energy demand also resulted in progressively increasing the emission of greenhouse gases which are considered one of the reasons for

worldwide climate change (Stone et al., 2019). Therefore, the development of an alternative energy resource that is sustainable, eco-friendly, and cost-effective is one of the popular topics in various research fields. Since there are numerous feedstocks of lignocellulose in nature and it can be regenerated through the carbon cycles, lignocellulose is being widely studied as a renewable and sustainable raw material for producing biofuel, bioproducts, and other value-added fermentation products (Sharma et al., 2019). Lignocellulose has attracted numerous interests as a sustainable natural resource which had a high potential in replacing fossil resources for producing energy and other bio-carbon-based material meanwhile avoiding the food-energy competition (Zoghlami and Paës, 2019).
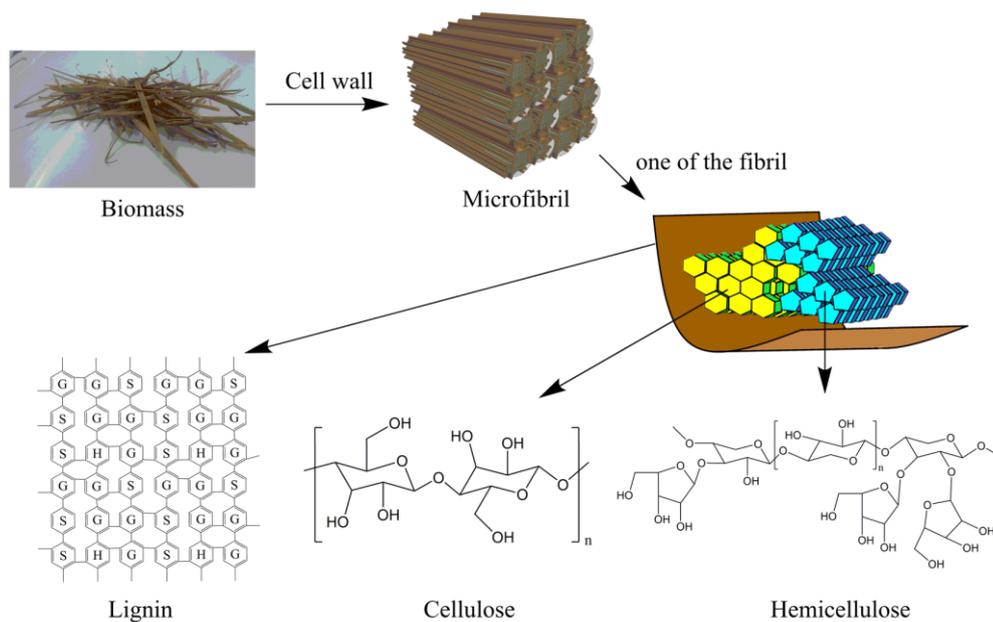
Figure 3 The composition of lignocellulose.

2.2 Composition and structure

Lignocellulose is one of the major compositions in dry plant biomass. It is usually

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considered the most abundant heterogeneous polymer in nature and it is composed of

cellulose, hemicellulose, and lignin (Figure 3). Different sources of lignocellulose contain

different ratios of these constructive polymers. In hardwood stem, the xylem usually contains

40-55% of cellulose, 24-40% of hemicellulose, and 18-25% of lignin, while the softwood

stem contains 45-50% of cellulose, 25-35% of hemicellulose, and 25-35% of lignin (Howard

et al., 2003a).

2.3 Cellulose

Cellulose is the most abundant polymer on Earth and it is composed of glucose which is

linearly linked via β-1, 4 glycosidic bonds. Since it is mainly composed of glucose, it is

considered a promising resource for producing energy and bio-based chemicals (Chang and

Zhang, 2011). Hydrolyzing cellulose had been studied for more than decades and it was still

one of the attractive research topics (Tomme et al., 1995; Zhou et al., 2023b). Besides glucose

and ethanol, various useful biochemicals were also investigated. Lo et al. (2020) combined

phosphoric acid, enzymatic hydrolysis, and microbial fermentation for converting cellulose to

succinic acid which could be used for further chemical synthesis. Huang et al. (2022)

summarized the application of pyrolysis in converting cellulose to other value-added

chemicals. They indicated that cellulose after pyrolysis would be converted to levoglucosan

and other similar structure chemicals, glucose, and different furans which could be served as

platform chemicals for further synthesis.

Besides hydrolyzing it to glucose, cellulose and its derivatives had been used for various

applications, such as hydrogel and aerogel (De France et al., 2017), additives in plastic (Siró

and Plackett, 2010), drug delivery (Xue et al., 2017), packaging (Azeredo et al., 2017), etc.

Nguyen et al. reported that nanocellulose fiber could be used as the printing material for 3D

printing. Moreover, they utilized this technology incorporating a stem cell culture into a

physiological environment (Nguyen et al., 2017). Zhang et al. (2021) demonstrated that

utilizing pyrolysis could effectively turn cellulose into different biochar with various

functions by controlling the pyrolysis temperature. Imato et al. (2017) reported that the

modified nanocellulose could be used as a self-healing material due to the presence of

reversible covalent bonds.

2.3.1 Cellulase

The enzyme which can cleave cellulose would be referred to as cellulase, which can be

further divided into endoglucanases (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91). Cellulase

can be utilized in various industrial applications, such as the paper and pulp industry, juice

extraction, biofuel production, etc. (Singhania et al., 2010; Siqueira et al., 2020). Even though

animals and plants are also able to produce cellulase, research, and industries preferred using

microorganisms for cellulase production due to the low production cost and short cultivation

period (Zhang and Zhang, 2013). Moreover, there were several attempts to improve the

production of cellulase meanwhile lowering the production cost. One of the common

methods is solid-state fermentation using low-cost lignocellulosic biomass as feedstock

(Couto and Sanromán, 2006). Solid-state fermentation is not only easy to operate but also can

produce more enzymes with lower energy consumption (Bowyer et al., 2020; Cerda et al.,

2017). Besides optimizing the production method and process, mutagenesis (Singhania et al.,

2021), metabolic engineering (Bhati et al., 2021), computer simulation (Pramanik et al., 2021), and co-culturing with other microbes (Lin et al., 2011) are also considered potential direction for lowering the production cost and enhancing the cellulase activity.

Besides traditional cellulase, recently there is another group of enzymes with polysaccharide degradation ability, lytic polysaccharide monooxygenase (LPMO). LPMO is different compared to other common glycosidic hydrolases, LPMO catalyzes the oxidative cleavage of the glycosidic bond and modifies the highly resistant crystalline cellulose region, thus the cellulase can degrade cellulose more efficiently (Eibinger et al., 2014). Moreover, interestingly, Rodríguez-Zúñiga et al. (2015) indicated that the higher content of lignin in the lignocellulose would induce the activity of the LPMO.

2.4 Hemicellulose

Hemicellulose is considered the second most abundant polysaccharide in nature and it is a heterogeneous polymer mainly composed of a xylan backbone with other sugar such as arabinose and mannose at the side chain. Unlike cellulose, hemicellulose is composed of different sugar with a non-specific structure and thus hemicellulose from different plants source could have different properties with a lower mechanical strength (Saha, 2003). Therefore, the hydrogel composed of hemicellulose would have lower mechanical properties, loading capacity, and lower electrical conductivity (Myung et al., 2008). However, hemicellulose is still useful for producing various chemicals, such as bioethanol, 2,3-butanediol, furans, and xylitol (Luo et al., 2019). However, even though the mechanical strength is weak, hemicellulose is considered an appropriate material for packaging due to its

hydrophilic properties and highly biodegradable (Farhat et al., 2017; Huang et al., 2021).

2.4.1 Hemicellulase

Xylanase is the most common and widely studied hemicellulase and the same as cellulase, it also can be divided into endoxylanase and exoxylanase and mainly recovered from microbial fermentation (Walia et al., 2013). There are several potential applications of xylanase, such as in textile, biorefinery, food, and feed industries, and bio-bleaching and ink-removing in pulp and paper industries (Bhardwaj et al., 2019a). Xylanase can be obtained from multiple approaches, submerged fermentation and solid-state fermentation would be the most common route for obtaining low-cost xylanase using agricultural waste as feedstock (Ding et al., 2018; Liu et al., 2018; Yadav et al., 2018). Recombinant xylanase is another potential approach since a cellulase-free enzyme mixture is desired in paper and pulp industries, due to the major component in paper is cellulose (Chaudhary et al., 2021). Another important function of xylanase is used for biofuel production, whether directly involved in the production of xylose which later converted to biofuel, or be used as pretreatment for lowering the recalcitrance of lignocellulose (Sun and Cheng, 2002).

However, unlike cellulose, there are very few reports on using lytic polysaccharide monooxygenase to degrade hemicellulose (Agger et al., 2014). Koskela et al. (2022) indicated that the content of hemicellulose would significantly affect the properties of the LPMO which could be one of the potential reasons that LPMO was not widely applied to the hemicellulose degradation. Moreover, Tõlgo et al. (2023) demonstrated that the side chain of the xylan would also affect the xylan-degrading activity of LPMO.

2.5 Lignin

Lignin has a complicated cross-linking structure and contains several functional groups within the molecule, including aliphatic hydroxyl, phenolic hydroxyl, and methoxyl groups. These functional groups affect the reactivity and chemical properties of lignin, especially the hydroxyl groups and aromatic structure are the most critical functional groups to determine the characteristics of the polymers (Cateto et al., 2009; Pandey and Kim, 2011). The aliphatic hydroxyl group usually is the most abundant hydroxyl group in lignin polymer. However, the ratios of these hydroxyl groups in different sources of lignin could be various (Li et al., 2015).
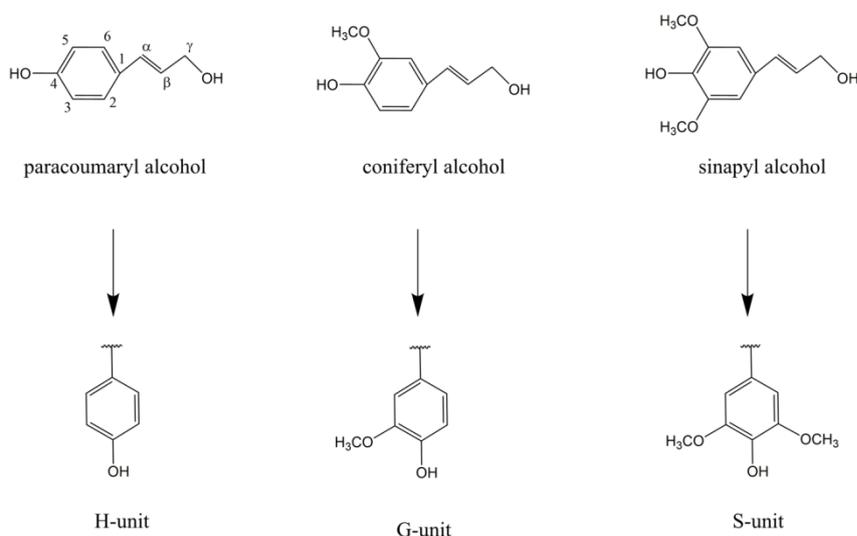
Figure 4 The precursors and basic unit in lignin molecule.

The three major precursors of the lignin polymer are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 4). Lignin from different plants is constructed by various percentages of these precursors. For instance, the lignin from softwood mainly contains coniferyl alcohol, around 90-95%, while the lignin from hardwood usually contains coniferyl and sinapyl alcohols, around 25-50% and 50-75%, respectively, and the lignin from grass

typically contains all three monomer alcohols (Dorrestijn et al., 2000; Zhongzheng, 2012).

There are two major linkages between these monomers: carbon-carbon linkages, also known

as condensed linkages, and ether linkages (Figure 5). The dominant linkage in the lignin

polymer is ether linkages representing 56% or more in total linkages (Pu et al., 2008). Due to

the different ratios of these monomers in different sources of lignin, the exact ratios of

linkages are also different in various species, like the most common ether linkages, β-aryl

ether (β-O-4), represents around 50% and 60% of total linkages in softwood and hardwood,

respectively (Chen and Wan, 2017). Aryl ether linkage is easier to cleave when compared to

condensed linkages during the lignin depolymerization and conversion (Yuan et al., 2010),

thus the cleavage of the β-O-4 is also considered a critical step of lignin depolymerization for

utilizing lignin as raw materials to produce other chemicals (Reiter et al., 2013).
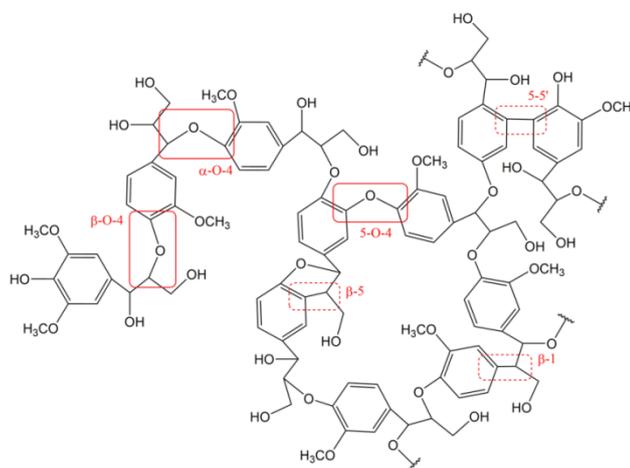
Figure 5 Several typical linkages within the lignin molecule. The linkages marked by

solid squares are ether linkages. The linkages marked by dot squares are condensed linkages.

2.5.1 Bioconversion of lignin

Lignin is the most abundant phenolic polymer in nature and during a long time of

evolution, numerous organisms have developed an effective metabolic system and methods to degrade and converted lignin to aromatic compounds and further converted these compounds to energy by multiple pathways (Fuchs et al., 2011).

2.5.2 Bacteria

Bacteria are considered as less effective than fungi in lignin degradation and one of the reasons is that fewer species of bacteria could degrade lignin. However, several bacteria strains have been identified to be suitable for lignin depolymerization and conversion. *Rhodococcus jostii* RHA1 is one of the well-studied lignin degraders and it has a great performance in consuming lignin to other compounds with the assistance of various enzymes (Ahmad et al., 2011). Sainsbury et al. (2015) suggested that *R. jostii* RHA1 could cleave the β-O-4 with the help of dye-decolorizing peroxidase (DyP) and result in producing vanillin as the product. Furthermore, Kitagawa et al. (2001) and Seto et al. (1995) also indicated that *R. jostii* RHA1 could degrade polychlorinated biphenyl and further genomic studies also showed that *R. jostii* RHA1 contained gene *benABCD* which could translate to various enzymes like 2-hydro-1,2-dihydroxybenzoate dehydrogenase for cleaving the linkage between biphenyl. Therefore, *R. jostii* RHA1 also showed great potential for cleaving one of the common condensed linkage 5-5' within the lignin molecule. Moreover, Sainsbury et al. (2013) also knocked out the vanillin dehydrogenase from *R. jostii* RHA1, and the mutated *R. jostii* RHA1 could accumulate a certain level of vanillin in the cell. Salvachúa et al. (2015) also indicated that *R. jostii* RHA1 was a robust bacteria for genetic engineering because the mutated bacteria could grow under a high cell-density environment with limited nutrients and could

tolerate toxic metabolites.

Except for *R. jostii* RHA1, *Pseudomonas putida* KT2440 is also known as an excellent lignin-degrading bacterium (Salvachúa et al., 2015). Previous studies indicated that *P. putida* could be a good candidate for degrading lignin to low molecular-weight molecules, then producing and accumulating polyhydroxyalkanoates (PHAs), one of the potential raw materials for bioplastic production, by converting lignin-derived aromatic compounds from lignin-rich medium (Linger et al., 2014; Salvachúa et al., 2015; Xu et al., 2018). Further genetic studies were conducted including domestication and pathway engineering. Martínez-García et al. (2014) eliminated 300 genes in the *P. putida* KT2440 genome and the engineered strain had significant improvement in almost all of the physiological statuses, including reducing the lag phase, increasing the biomass yields, growth rate, and tolerance to oxidative stress (Lieder et al. 2015). Further pathway studies indicated that mutated *P. putida* could accumulate PHAs as metabolic intermediates by using several aromatic compounds as raw materials. However, using lignin as the carbon source for *P. putida* culture had not been tested yet (Olivera et al., 2001). Except for PHAs, pyruvate, and *cis, cis* muconic acid (MA), which had great potential for various bioplastic production, was able to be converted from lignin-derived aromatic compounds by introducing additional enzymes. These products could be further accumulated in the cell by blocking the specific metabolic pathway (Johnson and Beckham, 2015; Vardon et al., 2015).
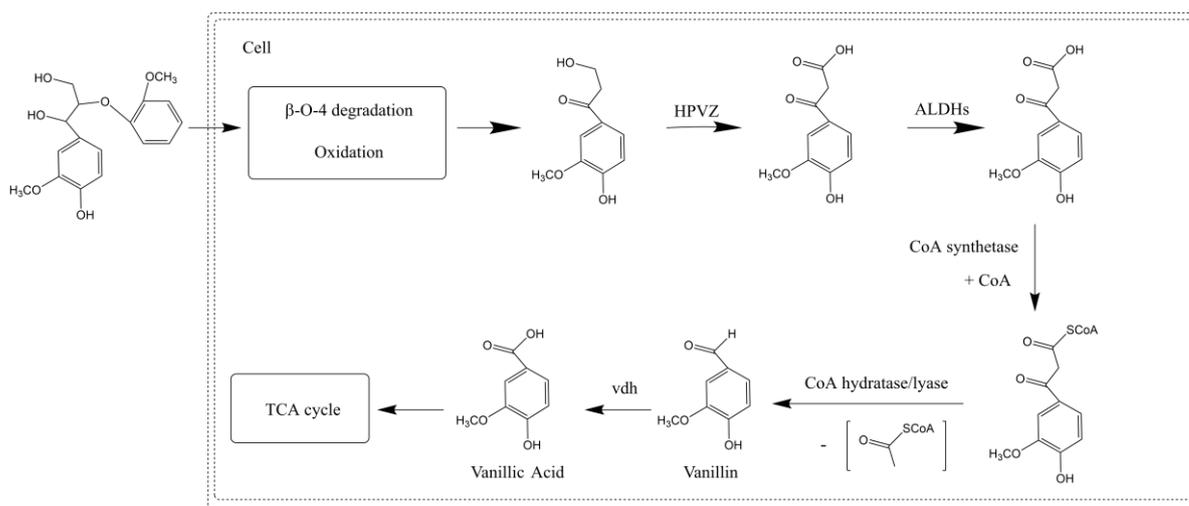
Figure 6 The purposed pathway of converting lignin-derived compounds to energy

Amycolatopsis sp. was widely studied due to its effective depolymerization ability and high conversion rate of high molecular weight lignin when compared to other bacteria (Salvachúa et al., 2015). Due to the well-understanding of several aromatic metabolism pathways in *Amycolatopsis* sp. (Achterholt et al., 2000; Davis et al., 2012), several metabolic engineering studies had been completed recently. Similar to *R. jostii* RHA1, Fleige et al. (2013) identified and cloned the vanillin dehydrogenase (*vdh*) from *Amycolatopsis sp.* 39116 genome. After the verification, they successfully increased the yield of vanillin 2.3 times by knocking out the *vdh*. Due to the gene knockout, the bacterium was not able to use vanillin as the sole carbon resource for energy production, thus vanillin produced from ferulic acid was accumulated in the cells (Figure 6). However, the study also indicated that vanillin could enter an alternative pathway that could slowly oxidize vanillin to energy (Fleige et al., 2013). Except for vanillin, MA also is one of the valuable chemicals that could be recovered from lignin depolymerization. Barton et al. (2018) used genetically engineered *Amycolatopsis sp.* 39116 to produce and accumulate MA from depolymerized lignin lysate. The gene *catB*,

which was an essential enzyme for utilizing MA for further metabolism, was knocked out, thus the MA could not be further oxidized and accumulated in the cell. Even though the deletion affected the cell growth rate, the mutant could accumulate 3 times more MA than the wild-type.

Although various bacteria show their potential in lignin degradation and conversion, the conversion efficiency of bacteria was much lower than fungi, especially white- and brown-rot fungi. Ahmad et al. (2010) reported that the activities of the extracellular enzyme for lignin degradation in bacteria were significantly lower than the enzyme from fungi. Furthermore, Salvachúa et al. (2015) showed that after 7 days of incubation, *R. jostii* was only able to convert 20% of dark-shaded bars lignin and 27% of it with the presence of glucose. Moreover, genetic modification could not be applied to all bacteria species due to the lack of related genetic information (Beckham et al., 2016).

2.5.3 Fungi

The fungus is one of the most studied microbes for lignin depolymerization and degradation. White rot basidiomycetes have been extensively studied in various aspects. They showed a higher conversion rate and depolymerization efficiency when compared to bacteria (Salvachúa et al., 2015). Due to their strong ability in degrading lignin, white rot fungi have been applied to various industrial applications, including removing phenolic compounds from pollutants, delignification of biomass and increasing the cellulose ratio, and improving biomethane production (Asgher et al., 2008; Nizami et al., 2009; Reddy, 1995; Shi et al., 2009; Zhi and Wang, 2014).

White rot fungi are the most effective microbe for degrading the native lignin in the wood (Rytioja et al., 2014). Its great ability for degrading lignin might strongly relate to that white rot fungi could produce various extracellular oxidases including lignin manganese, laccases, and phenol oxidases (Pérez et al., 2002). The excessive amounts of these oxidase secretions also result in the white rot fungi having an excellent performance in lignin degradation. *Phanerochaete chrysosporium* showed very high efficiency in removing lignin from biomass or waste, up to 99% (Chen and Wan, 2017; Sharari et al., 2011). Koncsag et al. (2012) indicated that the strain *Pleurotus ostreatus* could effectively depolymerize lignin and produced several useful compounds like ferulic acid, and syringyl alcohol. Some fungi could depolymerize lignin and use the low molecular weight lignin or monomers as the carbon source for lipid synthesis. *Aspergillus fumigatus* was used for the fermentation of the wheat straw lignin-rich fraction and after the fermentation, various valuable chemicals were detected including syringic acid and vanillic acid, several short-chain fatty acids, including acetic acid and butyric acid, also detected (Baltierra-Trejo et al., 2015). However, Xie et al. (2015) could not demonstrate that the fatty acid was mainly converted from lignin or another carbon source. A similar result had been presented by using a unique strain *Cunninghamella echinulata* FR3 which could effectively accumulate lipids by degrading lignocellulose. Moreover, Fenseca et al. also indicated that *C. echinulata* FR3 could degrade lignin with higher efficiency than cellulose or hemicellulose.

There are several genetic, proteomic, and pathway studies focusing on improving fungi performance and our understanding of how fungi depolymerize lignin. A laccase-encoding gene *lac I* had been isolated and identified from *Phlebia brevispora*. This gene was further

cloned and transformed into *Pichia pastoris* for protein expression. The expression study indicated that the lac I enzyme showed a high tolerance to various salt and solvents which showed its potential for use in various industrial applications (Fonseca et al., 2018). Moreover, a novel laccase gene *lcc1* was isolated from *Ganoderma tsugae*. The knockout experiments show that *lcc1* played an important role in lignin degradation. Furthermore, the depletion of *lcc1* also had significant effects on the development of the mycelium and fruitbodies (Jin et al., 2018). Proteomic studies indicated that manganese peroxidases and laccases were the most common lignin-degrading enzymes in basidiomycetes. Interestingly, several laccases showed that they have the optimal reaction pH at 7 but usually, these laccases also performed efficiently and were active in many different industrial environments (Kinnunen et al., 2017). Recently a novel β-etherase, which was involved in the specific cleavage of β−O−4 linkage, was found in the *Dichomitus squalens*. Interestingly, a similar gene could be widely detected in various fungi or bacteria genomes but only a few of these species could exhibit the enzyme activity (Marinović et al., 2018; Mathieu et al., 2013). However, even though numerous genetic and pathway studies had been completed, unlike bacteria, a limited amount of studies were using metabolic engineering for improving valuable chemical production.

Extracellular laccase and various peroxidase are the major approaches for fungi to degrade lignin. However, even though fungi are more effective in lignin degradation when compared to bacteria due to the powerful extracellular enzyme, similar to other biological lignin depolymerization, the efficiency of the enzyme was much lower than in chemical catalysis. Furthermore, the oxidoreductases produced by basidiomycetes also caused the

lignin fragments' repolymerization (Munk et al., 2015).

2.5.4 Enzymes

Enzymes that could effectively degrade lignin have been isolated from the fungi or bacteria and these enzymes have been applied in several *in vitro* experiments for lignin depolymerization or conversion study. Using *in vitro* enzymatic reaction could avoid several drawbacks including reducing the culturing time and the direct encounter between microbe and substrate (Zhang, 2015). Most of the *in vitro* experiments were conducted by using a single enzyme for cleaving lignin-model molecules as substrate. Other more complicated enzymatic digestion systems needed a further understanding of molecular mechanisms (Picart et al., 2015). According to their reaction mechanism and environment, the enzyme for lignin degradation could be further classified as *in vitro* enzyme, mainly peroxidase, and laccases, and *in vivo* enzyme.

2.5.5 Extracellular enzymes

Most of the enzyme we found which could degrade lignin is non-specific cleavage. These enzymes mostly come from two enzyme families: peroxidase and laccase. Both of these enzymes catalyze the lignin by oxidation. Instead of catalyzing specific substrates or linkages, they attacked the lignin molecule randomly. Two groups of peroxidases have been well-studied, lignin peroxidases (LiP) and manganese-dependent peroxidases (MnP). Recently, versatile peroxidase (VP) and dye-decolorizing peroxidase (DyP) also attracted various studies' interest due to their versatile properties.

2.5.6 Laccases
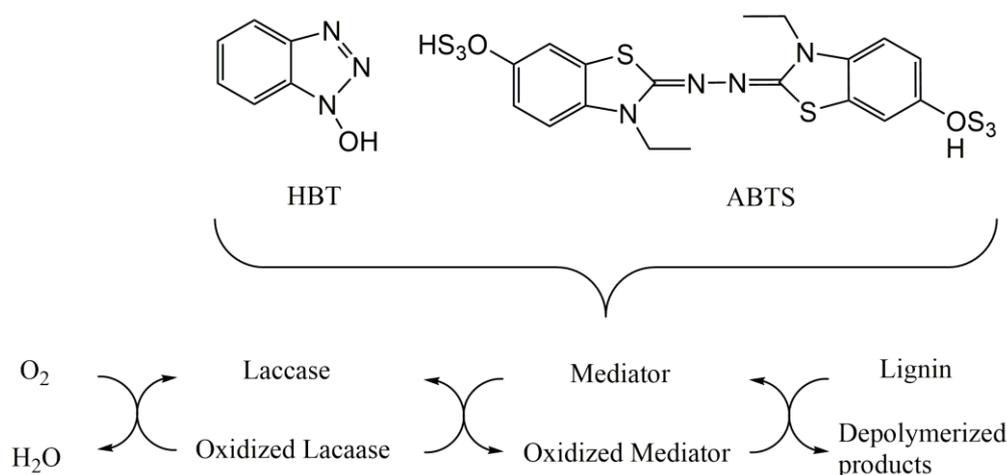
Figure 7 The mechanism of laccase-mediated lignin degradation

Laccase is one of the common oxidases that could be isolated from various fungi and

bacteria (Leonowicz et al., 2001). The most effective lignin-degrader white wood fungal also

is one of the major laccase producers (Kinnunen et al., 2017). Laccase is a blue-copper

phenoloxidase that can use oxygen as an electron acceptor and oxidize phenolic compounds

(Figure 7). The oxidized phenolic compounds could be converted into phenoxyl free radicals

which was an unstable intermediate and it could further lead to polymer cleavage (Gianfreda

et al., 1999).

Even though the activity of laccase was limited to phenolic compounds, laccase also

could cooperate with the mediator and degrade non-phenolic compounds (Bourbonnais and

Paice, 1990). The mediators were some small molecules that could transfer the electron, such

as 2, 2'-azinobis (3 - ethylbenzothiazoline - 6-sulphonic acid) (ABTS) and 1-hydroxy

benzotriazole (HBT). These accompanying molecules could assist the laccase to form a stable

intermediate with the substrate (Hilgers et al., 2018). With the help of the mediator, laccase could degrade almost 80-90% of the lignin structure (Camarero et al., 2004). Numerous studies tried to increase our understanding of the laccase at the genetic level and improve its performance and stability. Laccase also could be applied in several treatments except for depolymerization, including lignin-like chemical conversion, and delignification (Chen et al., 2012; Li et al., 2018b; Lim et al., 2018). Interestingly, even though laccase played an important role in lignin depolymerization, laccase was not an essential enzyme for microbes to depolymerize lignin. Previous studies indicated that even with a limited amount or without the presence of laccase, the microbes were still able to degrade lignin (Martinez et al., 2004; Thurston, 1994).

2.5.7 Lignin peroxidases
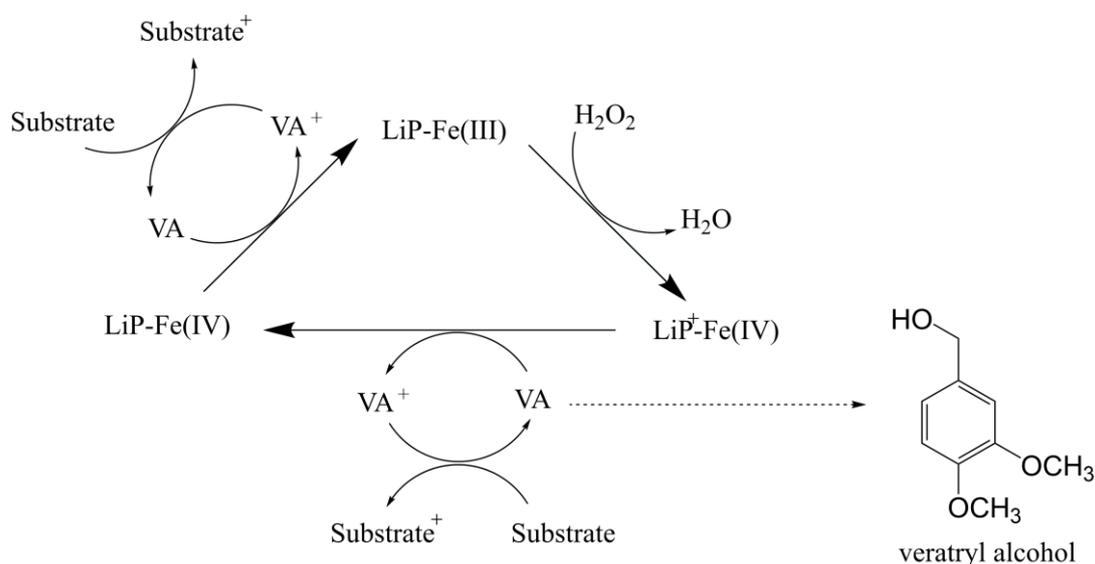
Figure 8 The mechanism of LiP-mediated lignin degradation

Lignin peroxidase (LiP) also has been isolated from various fungi. The LiP is a

25

glycoprotein and it contains a heme group in its active center and it has a range of molecular weight from 38-43 kDa (Pérez et al., 2002). It requires hydrogen peroxide for initiating and catalyzing the non-phenolic compounds and phenolic compounds (Datta et al., 2017) (Figure 8). It also requires veratryl alcohol as an electron donor and cofactor to complete the catalytic cycle (Wong, 2009). LiP is also known as the most effective peroxidase when compared to other peroxidases because it has a high redox potential which made LiP able to oxidize various substrates which other peroxidases could not oxidize (Sigoillot et al., 2012). Except using for lignin depolymerization, LiP was also used for delignification due to its great efficiency in removing lignin (Vandana et al., 2018; Zhang et al., 2015c).

2.5.8 Manganese dependant peroxidases
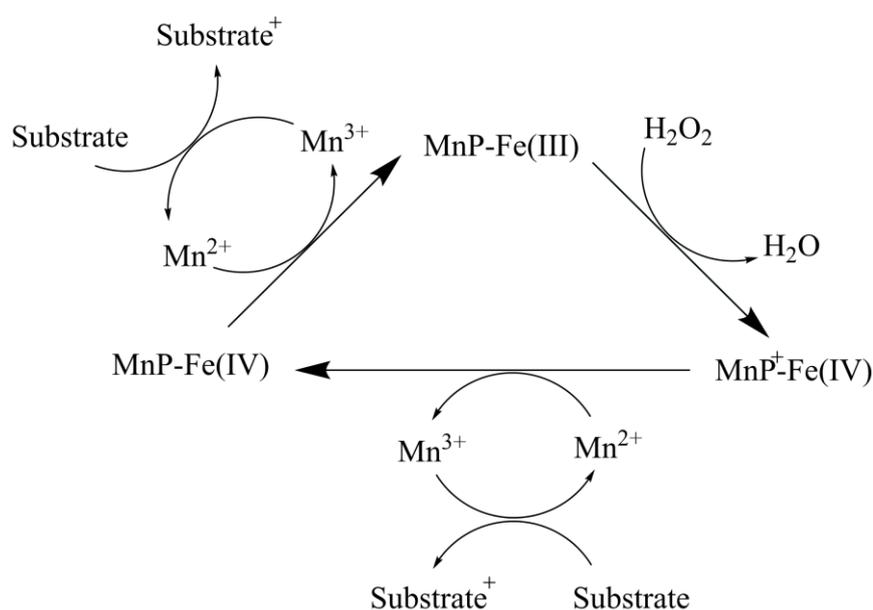
Figure 9 The mechanism of MnP-mediated lignin degradation

Manganese-dependant peroxidase (MnP) is an enzyme very similar to LiP. It is a glycosylated protein and needs hydrogen peroxide as an oxidant to initiate the catalytic cycle

(Figure 9). Afterward, MnP uses Mn^{2+} as a reducing substrate and converted it into Mn^{3+}. The Mn^{3+} is a strong oxidant and it diffuses from the enzyme and starts to oxidize the lignin phenolic compounds. Therefore, the MnP can convert lignin phenolic compounds to phenoxy-radicals by Mn^{3+} and the phenoxy-radicals can cause lignin depolymerization (Hofrichter, 2002). Similar to laccase, MnP played an important role in the initial lignin depolymerization (Guillén et al., 2005). Furthermore, *in vitro* experiments indicated that the addition of MnP present in the system could enhance the effectiveness of the depolymerization process (Hettiaratchi et al., 2014; Jayasinghe et al., 2011). Usually, MnP only could oxidize phenolic compounds, but MnP is also able to oxidize non-phenolic lignin model compounds with the presence of additional Mn^{2+}, previous studies also indicated that a high level of Mn^{2+} could enhance the activity of MnP to degrade lignin in solid (Kirk and Cullen, 1998; Rothschild et al., 1999). However, repolymerization is also observed during the use of LiP and MnP for depolymerizing the synthetic lignin polymer (Hammel et al., 1993; Wariishi et al., 1991).

2.5.9 Other peroxidases

Several peroxidases can be used for lignin depolymerization. Versatile peroxidase (VP) can be widely found in fungi *Bjerkandera* and *Pleurotus* and it has some similar catalytic properties to MnP and LiP (Guillén et al., 2005; Moreira et al., 2007). VP is a bifunctionality enzyme. It can oxidize Mn^{2+} like MnP, and it is also able to oxidize various subtracts which had high or low redox potentials like LiP (Camarero et al., 1999; Ruiz-Dueñas et al., 2008). However, unlike MnP, VP could oxidize Mn^{2+} independently (Perez-Boada et al., 2005).

The protein crystal structural studies also explain the bifunctionality of VP (Perez-Boada et al., 2005; Sáez-Jiménez et al., 2015; Zeng et al., 2017) and due to its bifunctionality, VP has attracted several research interests. Besides using VP for lignin depolymerization, VP also can be used for the delignification of biomass and decolorization of industrial waste (Baratto et al., 2015; Kong et al., 2017). The genetic studies related to VP also have been studied for a decade. The gene *mnp2* has been identified that it is responsible for encoding the VP and the inactivation of *mnp2* reduced the effectiveness of fungi in lignin degradation (Salame et al., 2014). VP has been cloned and expressed in heterogeneous hosts, including *E. coli* and yeast, for the large-scale production and mutagenesis study (Garcia-Ruiz et al., 2012; Mohorčič et al., 2009; Pollegioni et al., 2015).

Dye-decolorizing peroxidase (DyP) is another type of peroxidase which have been widely studied for lignin depolymerization. The first DyP was isolated from *Bjerkandera adusta* in 1999 (Kim and Shoda, 1999). The following studies also indicated that DyP, unlike other peroxidases which are mainly found in fungi, can be found widely in various bacteria (de Gonzalo et al., 2016; van Bloois et al., 2010). Even though the sequence and structure of DyP are different from other peroxidases, they share similar catalytic properties and mechanisms by using hydrogen peroxide and mediator for substrate oxidation (Brown et al., 2012; Liers et al., 2014; Sugano et al., 2007). According to the sequence characteristics, DyPs could be classified into four classes (Fawal et al., 2012). Type A, B, and C can be widely found in bacteria and type D is mostly produced by fungi (Abdel-Hamid et al., 2013). Usually, type A and B DyP are produced by bacteria and they have a smaller size and lower activity. However, type C DyP is similar to type D DyP, both type C and D DyP have a higher activity

for substrate oxidation (Brown and Chang, 2014). These four classes of DyP all have peroxidase activity and characteristics. However, even though Mn^{2+} is a necessary mediator for type B DyP oxidation, some type A DyPs doesn't have the Mn^{2+} oxidation activity and it might oxidize substrates by other routes (Rahmanpour et al., 2016; Singh et al., 2013b). Interestingly, there is a novel DyP had been recently identified and it could oxidize the substrate with the oxygen in the air and without the presence of hydrogen peroxidase (Avram et al., 2018). The mutagenesis study also had been conducted in DyP from *Pseudomonas putida* MET94 to improve the DyP performance in the industrial application by direct evolution (Brissos et al., 2017).

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Chapter 2. Optimization and Purification of Bioproducts from *Bacillus velezensis* PhCL Fermentation and Their Potential in Industrial Application and Bioremediation

Abstract:

Bioproduct is considered a promising solution of obtaining useful and green chemicals. However, the downstream process of biomolecules has been one of the major difficulties in upscaling the application of bioproducts due to the high purification cost. Acid precipitation is the most common method for purifying biosurfactants from the fermentation broth with high purity. However, the use of strong acids and organic solvents in solvent extraction has limited its application. In this study, a new *Bacillus velezensis*, strain PhCL, was isolated from phenolic waste, and its amylase production was optimized via response surface methodology. After that, various biomolecules were purified by sequential ammonium sulfate precipitation and the result suggested that even though the purified crude biosurfactant had a lower purity compared to the acid precipitation, the yield was higher and other bioactive compounds also could be recovered for lowering the purification cost. Moreover, the purified amylase and crude biosurfactant were characterized and the results suggested that the purified crude biosurfactant would have a higher emulsion activity and petroleum hydrocarbon removal rate compared to SDS and Triton X100. This study provided another approach for purifying various bioactive compounds from the same fermentation broth and further explored the potential of *Bacillus velezensis* in the bioremediation of polycyclic aromatic hydrocarbons and petroleum hydrocarbons.

1. Introduction

Recovering bioproducts from microbe's fermentation broth has been considered an alternative and eco-friendly way of obtaining useful green chemicals and replacing petroleum-based chemical production. Various *Bacillus* species have been considered generally regarded as safe (GRAS) (Schallmey et al., 2004) and used for producing several green bioproducts such as enzyme cocktail (Farias et al., 2021), 2, 3-butanediol (Wang et al., 2021), menaquinone-7 (Liao et al., 2021), and even the cell could be directly used as a tool for bioremediation and biocontrol (Kim et al., 2021; Sun et al., 2022). Among all the *Bacillus* species, *Bacillus velezensis* has recently been attracting more attention due to its interesting and outstanding properties.

Bacillus velezensis was first isolated from the river in Spain and reported by Ruiz-Garcia et al in 2005 (Ruiz-Garcia et al., 2005). Even though there were debates regarding the species classification (Rabbee et al., 2019; Wang et al., 2008), *B. velezensis* has been currently recognized as an individual species (Dunlap et al., 2016) and grouped with its highly similar species *Bacillus amyloliquefaciens* and *Bacillus siamensis* to form the "Operational Group *B. amyloliquefaciens*" (Fan et al., 2017). One of the most outstanding properties of *B. velezensis* is its ability in promoting plant growth and crop yield. Studies have shown that *B. velezensis* can assist plant growth by producing indole-3-acetic acid (IAA) and ammonia via nitrogen fixation (Meng et al., 2016; Myo et al., 2019; Sibponkrung et al., 2020). Furthermore, *B. velezensis* has also shown its antimicrobial activity which can assist plants to defend against the invasion of plant pathogens by producing various cyclic lipopeptides and inducing plant

systemic resistance (Fan et al., 2018; Rabbee et al., 2019). Thus, *B. velezensis* is also well-known as plant growth-promoting rhizobacteria (PGPR) (Rabbee et al., 2019).

Besides promoting plant growth, *B. velezensis* is also good at producing various bioproducts and enzymes. Zhao et al. (2021) reported that *B. velezensis* ND could produce a significant amount of menaquinone-7 using raw soybean flour as fermentation feedstock. da Rosa et al. (2022)have shown that *B. velezensis* P45 could produce and secrete various lipopeptides and biosurfactants including surfactin, fengycin, and iturin via feather meal fermentation, and these lipopeptides have shown their antimicrobial activities against *Listeria monocytogenes*. Besides antimicrobial activity, these lipopeptides are also involved in several important applications such as soil bioremediation, emulsifying agent, etc (Bhatt et al., 2021). Moreover, various enzymes have been identified by genomic sequencing and heterogeneously expressed or purified from the crude culture medium, including amylase, protease, laccase, cellulase, xylanase, and chitinase (Ghosh et al., 2021; Li et al., 2020a; Lu et al., 2021; Nair et al., 2018; Tran et al., 2022; Zeng et al., 2021; Zhang et al., 2021). These enzymes could be commercially beneficial and applied in various industrial applications such as manufacturing beverages and detergents, hydrolysis of lignocellulose, increasing soluble protein in biomass, etc. (Farias et al., 2021; Farooq et al., 2021a; Han et al., 2021; Zeng et al., 2021).

There are several strategies for purifying the biomolecules from the culture medium, including precipitation (Shah et al., 2016), solvent extraction (Nataraj et al., 2021), fractionation (Hubert et al., 2012), crystallization (Li et al., 2019), ultrafiltration (Aghajani et al., 2018), and chromatography (Meena et al., 2021). These purification processes have their

own advantages and disadvantages and among them, precipitation of biomolecules could be considered the easiest approach for the purification of biosurfactants (Shah et al., 2016; Venkataraman et al., 2021). The precipitation of the biomolecules usually is due to the changes in pH or hydrophobicity in the solution and eventually the molecules would be aggregated and form a high-weight solid which precipitated in the solution bottom (Baker and Chen, 2010). Thus, the most common reagents being used for precipitation are acids and ammonium sulfate. However, even though the operation is much easier than other purification strategies, precipitation is usually considered as cost-ineffective and the use of strong acids is raising concerns regarding environmental and safety problems (Baker and Chen, 2010).

Hence, even though there are a lot of papers reporting the purification of the enzymes or crude bioproducts from the culture medium, there are seldom reports that purifying multiple products or compounds from the same culture medium for lowering the production cost. In this study, a new strain of *Bacillus velezensis* PhCL was isolated from phenolic waste crystal and identified by 16s rRNA gene sequencing. The production of amylase was optimized via response surface methodology and the produced amylase was characterized which was seldom reported in other *Bacillus velezensis* studies. Moreover, another bioproduct, biosurfactants, was recovered from the same fermentation broth via sequential ammonium sulfate precipitation. Moreover, its potential for bioremediation of polycyclic aromatic hydrocarbons (PAHs) and petroleum hydrocarbon-contaminated soil was evaluated. This study provided a step-forward understanding of better utilization and production of useful biochemicals from an eco-friendly and economic aspect and further explored the potential of

B. velezensis in various industrial and bioremediation applications.

2. Materials and methods

2.1 Isolation of the bacterium and reagents used in the study

The *B. velezensis* PhCL was isolated from the phenolic waste crystal stored in our lab. In short, 0.1 g of the phenolic waste crystal was suspended in 10 mL of 0.9% (w/v) autoclaved NaCl solution, and the well-mixed solution was serially diluted. Around 50 μl of each diluted solution was spread on the Luria–Bertani (LB) agar plates and these plates were kept at 35 °C for 48 hours. All the chemicals and reagents used in the study were purchased from Sigma and Fisher Scientific with at least ACS grade except specifically indicated.

2.2 Identification of *B. velezensis* PhCL via 16s rRNA gene sequencing and the construction of its phylogenetic tree

The partial 16s rRNA gene sequence of the *B. velezensis* PhCL was used for molecular identification. In short, the region of 16s rRNA gene between 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') was amplified using *Pfu* polymerase (Biobasic Inc, Canada). The genomic DNA was extracted based on the freeze-thaw cycle method (Chen et al., 2020) and used as the PCR template. The amplified 16s rRNA gene fragment was sent to Eurofins Genomics for Sanger sequencing and the sequence of *B. velezensis* PhCL has been uploaded to the NCBI database with the accession number OP185368. The 16s rRNA gene sequence of *B. velezensis* PhCL was aligned with the existing data in National Center for Biotechnology Information (NCBI)

database via the basic local alignment search tool (BLAST). Meanwhile, the sequence was also aligned with other type strains in similar species such as *B. amyloliquefaciens* and *B. pumilus* by MEGA X, and analyzed with the phylogenetic analysis. The phylogenetic tree was constructed using the Maximum-Likelihood method with the strength of 1000 bootstrap using MEGA X (Tamura et al., 2021) and the 16s rRNA gene sequence of *Pseudomonas putida* is used as the outer group for constructing the phylogenetic tree.

2.3 Culture medium and production of enzymes and bioproducts by *B. velezensis* PhCL

LB and minimal salt medium (MSM) M9 were used in the study as culturing media for seed culture and fermentation. The M9 medium was composed by: 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NaCl, and 20 g/L of soluble starch (Biobasic, Canada) was used as carbon source in the medium. For the production of enzymes and bioproducts, in short, a colony of *B. velezensis* PhCL was picked up from the LB agar plate, then inoculated in 5 mL of LB liquid medium and cultured at 35 ℃ for overnight (18 hours) with a rotation speed 220 rpm. After the incubation, the overnight seed culture was loaded into the M9 medium at a ratio of 1% v/v. The bacterium was incubated for 5 days with different temperatures and pH and 0.2 mL of the culture medium was taken out from the fermentation broth for the enzymatic and metabolite analysis every 24 hours. The same rotate speed was used in all the experiments. All the experiments have been conducted at least in triplicate.

2.4 Analysis of the enzymatic activities

The measurement of the enzymatic activities of amylase was referred to as the

dinitrosalicylic acid (DNS) method (Miller, 1959b) and the 96-well microplate method in our lab (Wang et al., 2019b). Soluble starch (Biobasic, Canada) and beechwood xylan (Cedarlane, Canada) were used as substrates for the reaction of amylase and xylanase respectively, and each of the substrates was dissolved in 0.1 M Tris-HCl pH 7 buffer with concentration at 1% (w/w). In short, 10 μL of diluted enzyme solution was mixed with 20 μL of 1% substrates and reacted in a 50 °C water bath for 10 mins. After the reaction, the mixture was immediately cooled down and 60 μL of DNS reagent was added to the mixture then the mixture was boiled in boiling water for 5 mins. After the boiling, the mixture was instantly cooled down again and 200 μL of water was loaded into the mixture and eventually, 200 μL of the mixture was taken out and the absorbance at 540 nm of samples was measured using Epoch Microplate Spectrophotometer (BioTek, USA). The release of the reducing sugars is determined by the standard curve using corresponding mono-sugars as standard. The enzymatic activity was expressed as the International Unit (IU): One unit of activity referred to the amount of enzyme required for releasing 1 μmol of maltose and xylose in 1 min under specific conditions.

2.5 Box-Behnken design (BBD) for optimizing the amylase production

The production of the amylase in the culture medium was optimized via the response surface methodology (RSM). Three factors and three levels were selected for the BBD based on the results from the single-factor experiments. The RSM model was constructed by the SYSTAT 12 (Systat Software, Inc., USA) using the BBD table. For evaluating the goodness of fits of the second-order polynomial model equation, the coefficient R^2 and the lack of fit

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were used for indicating by an F-test at a 5% level of significance. Furthermore, the highest enzymatic production and the corresponding culture condition were calculated based on the RSM model.

2.6 Purification of the enzyme and bioproducts via different methods

The methods used for purifying enzymes and bioproducts are based on ammonium sulfate precipitation (ASP) (Meena et al., 2021) and acid precipitation (ACP) (Zhao et al., 2021). The ammonium sulfate precipitation is based on disrupting the hydrogen bond between the biomolecule and water. In short, 20 mL of culture medium was collected and centrifuged at 2600 g for 15 mins to remove the cells, and then 20%, 40%, 60%, and 80% (saturation percentage) of ammonium sulfate was added into 20 mL of cell-free culture medium for precipitating the biomolecules, and the precipitates from the different concentration of ammonium sulfate were collected by centrifugation at 2600 g for 15 mins. Then, the pellets are dissolved in 5 mL 0.1 M Tris-HCl pH 7 buffer.

The acid precipitation was based on modifying the charges of protein and the denaturation and aggregation of the biomolecules. In short, 20 mL of culture medium was collected and centrifuged at 2600 g for 15 mins to remove the cells. Then, the pH of the cell-free culture medium was adjusted to 2 by adding hydrochloride acid and incubated in a 4 °C fridge overnight. The precipitate was collected by centrifugation at 2600 g for 15 mins and the pellet was extracted by hexane-isopropanol solution (2:1, v:v) by vortex for 1 mins. The mixture was centrifuged again at 2600 g for 15 mins and the supernatant would be separated into 2 fractions: isopropanol fraction and hexane fraction. The content in each fraction was

weighted by evaporating the organic solvent and the substances were re-dissolved in methanol.

The amount of the biosurfactant was determined by weighting the dry weight. In short, a pellet or sample solution was loaded on a clear petri dish and the dishes were dried in a 70 °C oven until constant weight. The concentration of the enzyme was determined based on the Bradford reagent (Biobasic, Canada) (Kruger, 2009) and bovine serum albumin was used as the standard curve. The yield of enzymes after the purification and the purification fold was calculated based on the following equations:

$$\text{Protein Yield (\%)} = \frac{Total\ enzymatic\ activity\ after\ purification}{Total\ enzymatic\ activity\ before\ purification} \times 100\%$$

$$\text{Purification fold} = \frac{Specific\ activity\ after\ purification}{Specific\ activity\ before\ purification}$$

2.7 Characterization of the purified amylase

The enzyme mixture purified from the 60% ASP was used for the enzymatic characterization. The determination of optimal reaction temperature, reaction pH, and effect of metal ions was measured as mentioned above, with different reaction temperatures, pH buffers, and the addition of metal ions. The thermostability was determined based on the half-life of the enzyme (the incubation time remaining 50% maximum activity) incubating at various temperatures (50 °C and 70 °C). The effect of the metal ions was tested as chloride salt dissolved in the 0.1 M Tris-HCl pH 7 buffer at different concentrations (2.5mM, 5mM, 10 mM).

2.8 Measurement of the antioxidant activity

The activity of the antioxidant was evaluated based on the Trolox equivalent antioxidant capacity assay (Arts et al., 2004). In short, for preparing the oxidized solution, an equal volume of 7 mM of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution and 2.4 mM of potassium persulfate solution was mixed well, and incubated at room temperature for 14 hours. After that, 20 µl of the antioxidant solution was mixed with 200 µL of the oxidized ABTS solution and the mixture stood at room temperature for 7 mins and the absorbance at 734 nm was measured. The decolorization rate was calculated based on the following equation:

$$\text{Decolorization rate} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100\%$$

$Abs_{control}$ is the absorbance of the oxidized ABTS after incubation without the antioxidant and Abs_{sample} is the absorbance of the oxidized ABTS after incubation with the antioxidant. The decolorization rate was further converted into the amount of Trolox equivalent antioxidant and standardized using Trolox as a standard curve.

2.9 The emulsion activity of the purified biosurfactant and its potential in dissolving PAHs

The biosurfactant purified from the ammonium sulfate was used for measuring the emulsion activity and other common surfactants such as Tween 20, Triton 100, and sodium dodecyl sulfate (SDS) were used as a comparison. The emulsion activity was measured based on the method reported by Pathak and Keharia (2014) with minor modifications. In short, the same amount of surfactant was added to a mixture containing 0.6 mL of water and 20 µL of

olive oil. After suspending all the mixture by vortexing for 2 mins, the mixture was incubated at 35 °C for 1 hour and the turbidity was determined by measuring the optical density (OD) at 600 nm. One unit of the emulsion activity was defined as the amount of surfactant that obtaning 0.1 reading at OD 600 nm.

The PAHs assay was conducted based on the solubility enhancement of naphthalene and phenanthrene in various concentrations of surfactants (0.3 mg/mL, 0.5 mg/mL, and 1 mg/mL). In short, 0.5 mg of naphthalene or phenanthrene was loaded into the water with various concentrations of common surfactant (Triton X-100, SDS) and biosurfactant. After shaking at room temperature for 24 hours, the absorbance of 280 nm of the solution was measured to determine the amount of dissolved naphthalene and phenanthrene. Water without any surfactants and water without PAHs were used as background control.

2.10 Evaluating the potential of crude biosurfactant in the bioremediation of used-engine oil-contaminated soil

The particle size distribution and soil organic matter (SOM) of the soil were shown in supplementary material Figure S1. The determination of the soil organic matter was based on the method from Salehl et al. (2019) with slight modification. In short, the soil was dried at 105 °C overnight, and the de-moisturized soil was combusted at 360 °C (preventing the loss of carbonate and meanwhile burning most of the organic carbon) for 2 hours and the weight loss was measured as SOM.

The potential for bioremediation of soil was evaluated based on the method reported by Phulpoto et al. (2020) with minor modifications. In short, 2 g of used engine oil was mixed

with 20 g of de-moisturized soil and stay for overnight. Then, the contaminated soil was suspended in 60 mL of water and the same amount of the biosurfactant, Triton X-100, and SDS are added into the soil solution reaching the final concentration at 40 mg/L, respectively. Then, the soil solution was shaken at room temperature with a rotation speed of 120 rpm for 24 hours. After the incubation, the samples were centrifuged at 2600 g for 15 mins and the supernatant was extracted with n-hexane then after evaporating the organic phase, the oil from n-hexane extraction was considered as the oil removed by the surfactants.

2.11 Data processing and statistical analysis

All the experiments had been conducted at least in triplicates and the data presented in the study would be mean ± SD. The significant test was carried out using the one-way ANOVA in GraphPad Prism 7.0 and the Tukey's multiple comparisons test was used for the comparative analysis. The group with p-value less than 0.05 was considered as statistically significant.

3. Results and discussion

3.1 Morphology and identification of the *B. velezensis* PhCL via 16s rRNA gene sequencing and its phylogenetic tree

The isolated bacteria strain PhCL had an extraordinary ability in producing biofilm on the agar plate (Figure 1A), even on the water-air interface of the liquid medium. Interestingly, the bacteria colony would be mucoid or non-mucoid due to different culturing conditions and bacterial status, even though the exact condition that caused the mucoid was remaining

unknown. The mucous bacteria colony contained a certain amount of sticky liquid (Figure 1B) in the bacteria colony and the colony with or without mucoid significantly affected the production of enzymes and biopolymers (data not shown). The result suggested that using a mucous bacteria colony for sub-culture and fermentation would have a significantly higher amount of enzymes and bioproducts. Thus, the mucous bacterial colony was used for further experiments and fermentation.
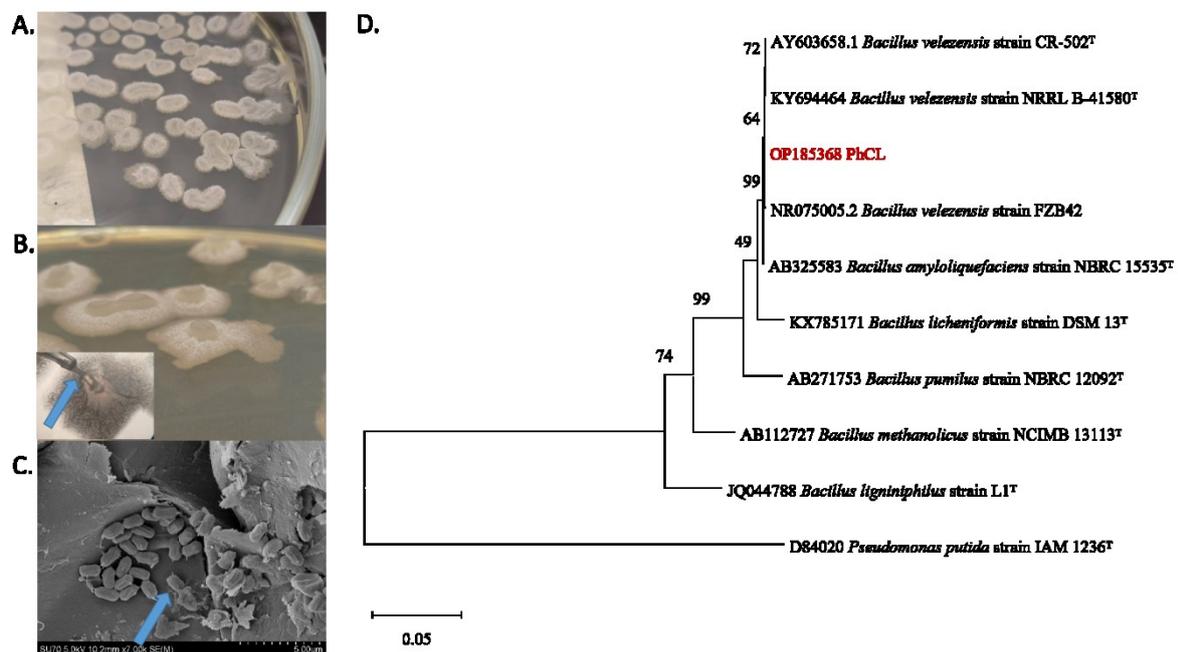
Figure 1. The characterization and identification of the B. velezensis. (A) The morphology of B. velezensis PhCL colony and the produced biofilm on the agar plate. (B) The mucoid status of the B. velezensis PhCL colony. (C) The morphology of B. velezensis PhCL under the scanning electron microscopy and the bacterium was pointed with the arrow. (D) The phylogenetic analysis using the 16s rRNA gene of B. velezensis PhCL alignment with other related species type strains (indicated by the supercritical letter T). The phylogenetic tree was constructed using the Maximum-Likelihood method with the strength of 1000 bootstrap and *Pseudomonas putida* is used as the outer group.

Biofilm was one of the well-known properties of *B. velezensis* and its related functional

group. Biofilm is a self-synthesized extracellular polymeric matrix such as polysaccharides, peptides, lipids, etc. produced by the bacteria (Costerton et al., 1987) and it is considered highly related to their biocontrol function (Rabbee et al., 2019). Studies indicated that the biofilm formation of *B. velezensis* SQR9 could stimulate the production of various antimicrobial metabolites (Huang et al., 2023; Weng et al., 2013; Zhang et al., 2015a), meanwhile, a recent study from Sun et al. (2022) indicated that the biofilm produced by *B. velezensis* SQR9 was essential for promoting the plant health and salt stress tolerance via a synergic interaction with other plant-promoting bacteria at the transcriptome and metabolism level. Moreover, the applications of biofilm in the bioremediation of environmental pollutants were also widely studied. One of the common ways was providing fixation, protection, and nutrition to the biodegrading bacteria in the waste-treatment tank which eventually improved the biodegradation (Mitra and Mukhopadhyay, 2016). A recent study from Liu et al. (2022b) also indicated that *B. velezensis* C5 could rapidly degrade untreated commercial polyethylene with biofilm-assisted micro-colonization. Furthermore, Maheswaran et al. (2023) also indicated that the biofilm formation would be beneficial to the colonization of polyethylene terephthalate and result in promoting biodegradation. Therefore, the ability in producing an excessive amount of biofilm would imply that strain PhCL could have the potential in various bioremediation and biocontrol application.

However, even though there were numerous studies related to the biofilm from various *B. velezensis* strains, the mucous status of *B. velezensis* and its characteristic was seldom reported. Choi et al. (2021b) reported that the mucous status of *B. velezensis* would be related to several bacterial phenotypes such as antibiotic sensitivities. Moreover, multi-omics

analysis suggests that the native conjugative plasmid pBV71 would interact with the mucous status and play important roles in biofilm formation, catabolite repression, and biosynthesis of various extracellular bio-compounds, which would be similar to our observation since the expression of amylase could be under the regulation of the catabolism regulator *CcpA* (Lorca et al., 2005; Stülke and Hillen, 1999). However, more investigations would be necessary in revealing the mechanism and detail of how mucous status affects catabolism and could be utilized for biotechnological application.

The morphology of the strain PhCL was also observed under scanning electronic microscopy (Figure 1C). The image clearly showed the typical morphology of *Bacillus*, rod-shaped with a size of around 1.5 to 3.5 μm (Ruiz-Garcia et al., 2005). To identify the isolated strain PhCL, the 16s rRNA gene of strain PhCL was sequenced and aligned with the existing data in the NCBI database via BLAST. The result suggested that the strain PhCL would be likely associated with the species *Bacillus velezensis* compared to other *Bacillus* species. For further confirming the species, the sequence was further aligned with other *Bacillus*-type strains listed in the List of Prokaryotic names with Standing in Nomenclature and performed the phylogenetic analysis. The result indicated that strain PhCL had a closer relationship with the type strain of *B. velezensis* compared to *B. amyloliquefaciens* and the operational group (Figure 1D). Moreover, it showed a closer relationship with the most famous strain FZB42 and thus, strain PhCL could be considered *Bacillus velezensis* based on the phylogenetic analysis.

Strain FZB42 is well-known as a biocontrol agent and plant fertilizer. There were more than 160 publications on this strain regarding its extraordinary performance using as a

biocontrol agent and bio-fertilizer (Fan et al., 2019). Strain FZB42 was identified as *Bacillus amyloliquefaciens* and further sequencing suggested that it should be reclassified as a sub-species of the *B. amyloliquefaciens* and renamed as *B. amyloliquefaciens* subsp. plantarum FZB42 (Borriss et al., 2011). After years of debates and advances in phylogenetic analysis, the strain FZB42 has found its taxonomy position and has been classified as *Bacillus velezensis* FZB42 after 2016 (Schoch et al., 2020). Therefore, based on the current phylogenetic analysis, even though the newly isolated strain PhCL might not be tightly related to the type strain of *B. velezensis* which isolated in 2005 (Ruiz-Garcia et al., 2005), the result showed that strain PhCL would be close to the famous strain FZB42 and could be considered as *B. velezensis* as well.

3.2 Effects of fermentation conditions on amylase production

To optimize the amylase production, various fermentation factors were evaluated and optimized through single-factor optimization, including carbon source (Figure 2A), carbon concentration (Figure 2B), initial medium pH (pH, Figure 2C), fermentation temperature (Figure 2D), and fermentation time. The result suggested that using starch as the sole carbon source (21.93 ± 1.27 U/mL) would significantly increase (P<0.0001) around 1.67 folds compared to other mono-sugar carbon sources (13.12 ± 1.13 U/mL on average) at the same mass concentration (2% w/v). Moreover, using 2% starch would have the highest amylase production which was significantly higher than 0.5%, 1%, and 1.5%. However, there was no significant difference between 2.5% compared to other concentrations (P>0.05). It could be due to that the standard deviation in 2.5% starch is much higher than other experiment groups

which were related that the starch was not evenly dissolved in the medium and the un-dissolved starch affected the sampling and measurement. Therefore, 2% starch was selected for further experiments.
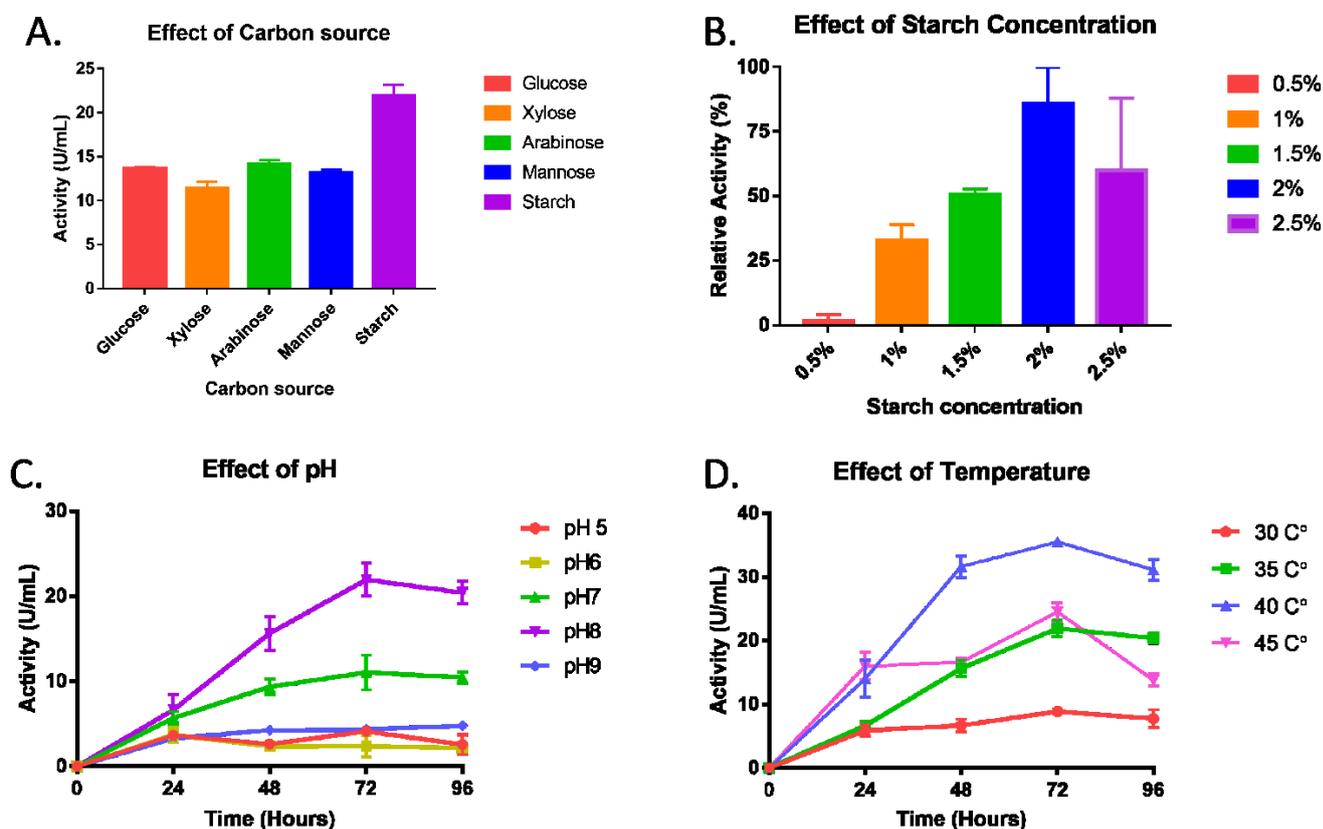
Figure 2. The single-factor optimization on amylase production (A) Carbon source, (B) Starch concentration, (C) Initial medium pH, and (D) Fermentation pH.

When culturing at neutral or slightly alkaline conditions (pH 7 and 8), strain PhCL showed a relatively good amylase production. Both conditions showed the highest amylase production after 72 hours of incubation. However, amylase production was much lower under the acidic condition (pH 5 and 6) and more alkaline conditions (pH 9). The initial pH 8 medium could produce the highest amylase production meanwhile it also had the most dramatic production changes during the fermentation compared to other pH.

The highest amylase production could be achieved at 40 °C with the initial medium pH 8 and a relatively high amylase production (>60% of highest activity) could be achieved under 35 to 45 °C. Moreover, both pH and temperature optimizations showed the highest production after 72 hours of incubation and the production was decreasing after that.

Therefore, the result from the single-factor experiments suggested that the primary optimal fermentation conditions under the tested range would be 2% of starch in M9 medium culturing at 40 °C with initial pH of 8 for 72 hours. Moreover, among all the conditions, the fermentation temperature, medium initial pH, and fermentation time were selected as independent variables for further optimizing the amylase production (response variable) via response surface methodology.

Various sugars had been selected as the sole carbon source for fermentation and amylase production in this study, including glucose, xylose, arabinose, mannose, and starch. It was not surprising that under the same mass concentration, using starch would be producing the highest amylase production. It could be that starch, unlike other mono-sugar, cannot be utilized directly, and thus, the lack of feasible sugar would repress the expression of *abrB*, a repressor of starvation-induced process, and stimulate the transcription and expression of the amylase gene (Robertson et al., 1989; Yu et al., 2018; Zhang et al., 2022b).

Interestingly, the strain PhCL also could effectively utilize pentoses such as xylose and arabinose for growing and producing amylase. Since pentose cannot be directly involved in the glycolysis and tricarboxylic acid (TCA) cycle, pentose usually generates lower energy for microbes and this process is also energetically unfavorable (Hochster and Watson, 1954; Lawford and Rousseau, 1995; Tadioto et al., 2022). Thus, pentose usually was considered not

suitable for fermentation. However, Chun et al. (2019) have compared multiple metabolism pathways of various type strains in the *B. amyloliquefaciens* operational group at the genomic level and they indicated that only *B. velezensis* showed the genes that were related to pentose and glucuronate interconversions and they suggested that this would be a unique metabolic feature could be used for identifying *B. velezensis* among other group members. The pentose and glucuronate interconversions could convert pentose into glucuronate and this metabolic pathway was not only involved in the pentose phosphate pathway but also is well-known that related to the production of energy and metabolites (Mazumdar et al., 2013; Xie et al., 2018; Zhou et al., 2023a). Thus, strain PhCL might be benefitted from this metabolic pathway and become able to utilize pentose effectively.

The concentration of the carbon source also played an important role in microbial growth and fermentation since it would determine the accessible energy for microbes and the osmotic effect in the medium (Mizzi et al., 2020). Starch was a common substrate for amylase production, however, there were not many studies have optimized the starch concentration in the fermentation medium. Even though reports were indicating that the higher concentration of starch would improve the production of amylase (Anbu et al., 2020; Gangadharan et al., 2008), most of the studies using 10 g/L starch and even some studies indicated that increasing starch concentration over than 10 g/L would not significantly enhance or even lower the amylase production in *Bacillus* fermentation (Bajpai and Bajpai, 1989; Konsula and Liakopoulou-Kyriakides, 2004; Wind et al., 1994). Thus, strain PhCL might have the ability to effectively digest and metabolize a higher concentration of starch and sugar.

Even though there were reports regarding the amylase production from *B. velezensis*,

most of the studies focus on the role of amylase in plant defense or biocontrol ability (Myo et al., 2019; Yin et al., 2022) and the fermentation condition for amylase production was seldom optimized but it had been optimized in other closely related species. Anbu et al. (2020) optimized the amylase production of *B. amyloliquefaciens* AMY02 with the fermentation condition of a temperature of 30 ℃, initial pH of 7, and fermentation time of 48 hours. Saad et al. (2021) reported that *B. licheniformis* WF67 would achieve the maximum amylase production with a fermentation condition temperature of 45 ℃, initial pH of 6.33, and fermentation time of 50 hours. Du et al. (2018) optimized the amylase production from *B. amyloliquefaciens* BH1 via RSM and the result indicated that the optimal fermentation condition would be a temperature of 40 ℃, initial pH 7, and fermentation time of 42 hours. Compared these studies to strain PhCL, even though strain PhCL might take longer times for maximizing amylase production, strain PhCL could produce amylase under alkaline fermentation conditions and be a good candidate for genetic engineering to produce amylase and hydrolyze starch under alkaline condition.

3.3 Optimization of amylase production via RSM

The production of amylase was further optimized based on the BBD design and the design matrix, corresponding experimental and forecasted results are listed in Table 1. The fermentation temperature (X_1: 35℃, 40℃, 45℃), initial medium pH (X_2: 7, 8, 9), and the fermentation time (X_3: 48 h, 72 h, 96 h) were selected as the independent factors and the amylase production was set as the response value. After the statistical analysis by response surface methodology, the relationship between the amylase production and individual factors

can be expressed as the following formula:

$$y = -10.767X_1^2 - 18.398X_2^2 - 8.122X_3^2 + 1.389X_1X_2 + 1.422X_2X_3 - 1.893X_1X_3$$

$$- 1.401X_1 - 1.796X_2 + 0.711X_3 + 35.513$$

Table 1 . Box-Behnken design for optimizing amylase production

Run	Temp (X$_1$) / °C	pH (X$_2$)	Time (X$_3$) / h	Activity (U/mL)	Forecasted value
1	35 (-1)	8 (0)	96 (1)	20.422	20.629
2	35	8	48 (-1)	15.641	15.421
3	35	9 (1)	72 (0)	4.440	4.564
4	35	7 (-1)	72	11.045	10.934
5	40 (0)	9	96	9.663	9.33
6	40	7	96	10.173	10.078
7	40	8	72	36.008	35.513
8	40	8	72	35.381	35.513
9	40	8	72	35.151	35.513
10	40	9	48	4.968	5.064
11	40	7	48	11.168	11.5
12	45 (1)	8	96	13.823	14.041
13	45	8	48	16.612	16.405
14	45	9	72	4.428	4.54
15	45	7	72	5.478	5.354

The analysis of the variance (ANOVA) of the model is listed in supplementary material Table S1. The squared multiple R is 0.999 which indicated that most of the response value (dependent variable) could be explained by the independent variable and equation. The p-value of the regression and lack-of-fit test was >0.001 and 0.594 which indicated that the established quadratic equation was relatively reliable and without lack of fitness in explaining the amylase production with these independent factors. The 2-D contour plots of

the equation shown in Figure 3 indicated that the optimal amylase production could be achieved within the tested condition and it is also affected by the interaction between the independent factors since all the contour plots appeared as ellipses. Based on the equation and contour plots, the initial medium pH would be having the most significant effect on the amylase production and then fermentation temperature and time. However, the interaction between temperature and time would contribute more effect on amylase production compared to the interaction between pH and other factors.
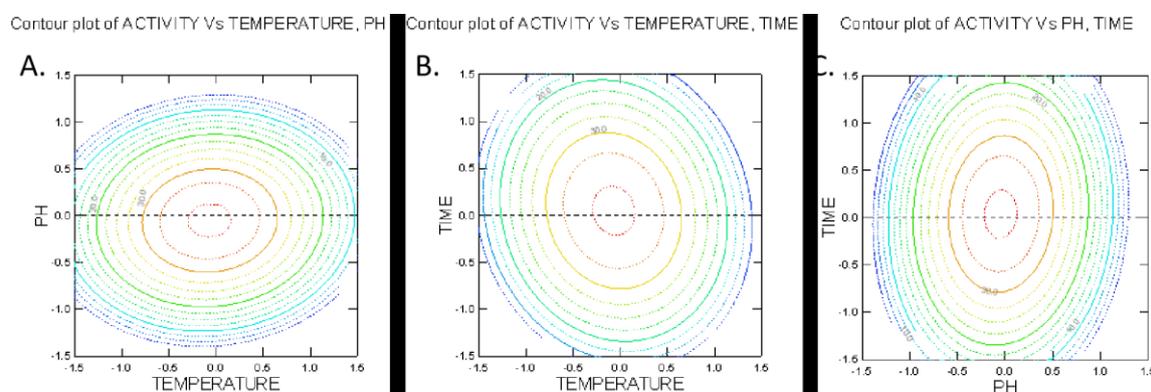
Figure 3. The 2-D counterplots of the established RSM model indicate the interaction between different individual factors: (A) pH vs Temperature, (B) Fermentation time vs Temperature, and (C) Fermentation time vs pH.

Based on the equation within the range of the BBD design, the forecasted highest amylase production 35.626 ± 0.87 U/mL (with a 95% confidence interval from 35.008 to 36.243) can be achieved under the fermentation temperature 39 ℃, initial medium pH 7.9, and fermentation time 73 hours which was no significant difference compared to the central point of BBD (run 7, 8, and 9 have average production of 35.513 ± 0.44 U/mL). Therefore, the equation was reliable for predicting the amylase production within the tested range and it would be expected that fermentation conditions for optimal amylase production could be

slightly flexible, especially in fermentation temperature and time, without significantly affecting the production which would be favorable to the industrial application.

Response surface methodology is one of the most common statistical approaches for optimizing enzyme production since it could optimize the production meanwhile explaining how the factors were interacting with other factors. The study from Bandal et al. (2021) analyzed the effect of various factors on amylase production from *Bacillus sp.* H7 including the concentration of carbon source and nitrogen source, salt, initial medium pH, fermentation temperature and time, and inoculum ratio. They concluded that the production of amylase would be significantly affected by the initial medium pH, concentration of starch, and the fermentation time based on the analysis from Placket–Burman Design. Similar to our result, they also indicated that the medium pH and fermentation time would be playing the most important role in affecting the amylase yields based on the RSM analysis. A similar result was also reported by Saad et al. (2021). Their study had shown that among the concentration of carbon and nitrogen source, initial medium pH, fermentation time, medium pH and fermentation time would have the most significant effect on the amylase production based on ANOVA. It was no surprise that the medium pH would be playing an important role in amylase production. Not only would the medium pH affect the enzymatic hydrolysis and bacteria catabolism (Tobisch et al., 1999; Wilks et al., 2009), but also the pH shifting due to the production of catabolites would also inhibit the expression of functional extracellular amylase (Kachan and Evtushenkov, 2021). Therefore, timely pH monitoring and adjustment were highly recommended for industrial amylase production.

3.4 Purification of crude bioproducts and enzymes from fermentation broth via sequential precipitation

After optimizing the amylase production via RSM, various biomolecules in the medium were also investigated under the optimal fermentation conditions, and their activities were shown in Figure 4A. Besides amylase, the activities of xylanase and cellulase were also detected. However, the cellulase production was very low (data not shown) compared to other numbers in the figure, and thus it was not further discussed in this study. The result showed that both amylase and antioxidant activity reached the maximum activity after 72 hours of fermentation. The activity of the biosurfactant was continuously increasing during the fermentation period and unlike other biomolecules, xylanase production reached its maximum after 24 hours of fermentation and continuously decreased at the later stage. Moreover, the crude biomolecules were partially purified with 2 different precipitation strategies: acid precipitation (ACP) followed by solvent extraction and sequential ammonium sulfate precipitation (ASP), and the yields and purification fold from each fraction were listed in Table 2.
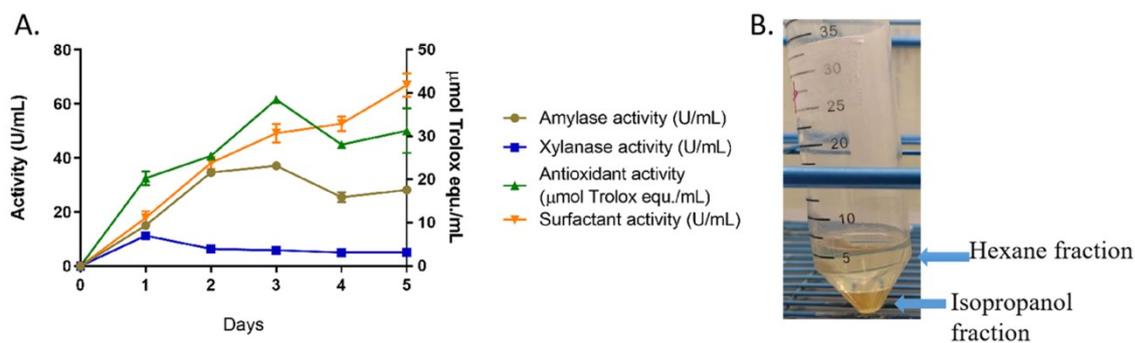
Figure 4. (A) Activities of various biomolecules under the RSM optimized fermentation condition. (B) The phase separation after Hexane-isopropanol extraction of ACP precipitates.

Table 2. The yield and activity of purified bioproducts via different purification methods

| | | Ammonium sulfate precipitation | | | | | | | Acid precipitation | | | | | |
| | Medium | 40 % precipitation | | | 60 % precipitation | | | Isopropanol fraction | | | Hexane fraction | | |
	Specific Activity	Specific Activity	Yield (%)	Purification fold	Specific Activity	Yield (%)	Purification fold	Specific Activity	Yield (%)	Purification fold	Specific Activity	Yield (%)	Purification fold
Enzymes													
Amylase (U/mg)	68.90 ± 5.96	37.19 ± 3.08	9.93	0.54	132.07 ± 21.22	66.83	1.92	/	/	/	/	/	/
Xylanase (U/mg)	58.53 ± 2.74	5.15 ± 3.64	1.62	0.088	124.76 ± 7.74	74.31	2.13	/	/	/	/	/	/
Bioactive compounds													
	Specific Activity	Specific Activity	Yield (g/L)	Purification fold	Specific Activity	Yield (g/L)	Purification fold	Specific Activity	Yield (g/L)	Purification fold	Specific Activity	Yield (g/L)	Purification fold
Antioxidant (mmol Trolox equ./ mg)	0.83 ± 0.12	0.95 ± 0.19	3.87	1.15	0.34 ± 0.02	3.23	0.41	3.06 ± 1.05	0.54	3.69	3.31 ± 0.77	0.36	3.99
Biosurfactant (U/mg)	1.28 ± 0.33	2.34 ± 0.36		1.82	0.46 ± 0.07		0.36	3.89 ± 1.31		3.03	1.05 ± 0.07		0.82

Even though series concentrations (20%, 40%, 60%, and 80%) of ammonium sulfate were used for precipitating the biomolecules, most of the biomolecules precipitated at the concentration of 40% and 60%, and thus, data from other concentrations was not listed in Table 2. Compared to the ACP, all the precipitates recovered from the ASP were highly soluble and easily dissolved in a buffer solution. Compared to the precipitates from 2 different concentrations, the crude enzymes were mainly recovered in the 60% fraction (yields of amylase and xylanase were 66.83% and 74.31%, respectively) and the specific activity of the enzymes were also improved (purification fold of amylase and xylanase were 1.92-fold and 2.13-fold, respectively). The specific activity of the crude biosurfactant and antioxidant was higher in the 40% fraction compared to the 60% fraction which implied that the purity of the crude biosurfactant would be higher in the 40% fraction (purification fold of crude biosurfactant and antioxidant were 1.82-fold and 1.15-fold, respectively). Moreover, the yield of the crude biosurfactant (3.87 g/L) in the 40% fraction is higher than the 60% fraction (3.23 g/L). Furthermore, the yield of the enzymes and crude biosurfactant were both higher in APS compared to ACP.

Since the precipitates from ACP were insoluble in water, the pellet from the ACP was further extracted with an organic solvent (hexane: isopropanol=2:1) for solubilizing the bioactive compounds (Zhang et al., 2022a). Moreover, since the solvent used for extraction was purely organic, no enzymatic activities were detected in the organic solvent. The organic solvent was separated into 2 different layers after centrifuge, namely isopropanol fraction and hexane fraction (Figure 4B). The remaining pellet was refolded in the 8M urea solution for recovering enzymes. However, the efficiency of dissolving in urea and enzyme recovery was very low and thus the data was not listed in Table 2. The specific activity of the antioxidant was higher in the hexane fraction (3.99-fold) compared to the isopropanol fraction (3.69-fold). However, the highest purification fold of the crude biosurfactant (3.03-fold) was obtained in

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the isopropanol fraction among all the precipitation which implied that there would be the highest purity of biosurfactant in the isopropanol fraction among other precipitation fractions.

Bacillus velezensis was well-known for with ability to produce various biomolecules such as useful enzymes, antioxidants, antibiotics, and biosurfactants. As mentioned above, optimizing the production of the enzymes from *B. velezensis* was seldom reported. Besides amylase, xylanase activity was also detected in the purified enzyme solution. There were studies indicated that *B. velezensis* had the ability in producing different glycoside hydrolases which were able to hydrolyze cellulose and hemicellulose, however, most of the studies conducted at the genome analysis and lack of further exploration during the fermentation and lignocellulose hydrolysis (Chen et al., 2023; Li et al., 2020b; Srivastava et al., 2023). Interestingly, the maximum xylanase activity was achieved within 24 hours of incubation, which was similar in other *Bacillus* species. Bakry et al. (2022) indicated that *Bacillus haynesii* K6 also had the maximum xylanase activity after 24 hours of incubation using xylan and peptone as a nutrient source. Limkar et al. (2019) also indicated that *Bacillus licheniformis* NRRL 14209 would have the highest xylanase activity within 24 hours using wheat bran as a carbon source. The fast production of xylanase could potentially be beneficial to the industrial lignocellulose hydrolysis process since long-period incubation was also considered one of the critical factors in the high production cost (Gençkal and Tari, 2006).

Antioxidant activity was also detected in the culture medium and the antioxidant activity was converted into the activity of an equivalent amount of Trolox (vitamin E). There were studies from various aspects indicated that *B. velezensis* would have the ability in producing various metabolites with antioxidant activity and their function in plant promotion and defense. Tung et al. (2021) reported that the genome mining of *B. velezensis* VTX20 had revealed its ability in producing biotin, riboflavin, and other vitamin B family substances. The study from Zhao et al. (2021) indicated that *B. velezensis* ND could produce a more

valuable vitamin K_2 (menaquinone-7) using yeast extract and glycerol as nutrients. Besides the vitamins, the lipopeptides, biosurfactants, and exopolysaccharides produced by *B. velezensis* also exhibited antioxidant activity (Cao et al., 2020a; Kumar et al., 2021).

Biosurfactants was one of the typical bioproducts from *B. velezensis* and studies have been focusing on the identification, extraction, and application of these biosurfactants for decades. Surfactin, iturin, and fengycin were the major and common lipopeptide families produced by *B. velezensis*. These cyclic lipopeptides play an important role in antimicrobial activity and bioremediation. Studies indicated that these cyclic lipopeptides produced by *B. velezensis* could inhibit several lethal plant pathogens including *Podosphaera fusca*, *Colletotrichum gloeosporioides*, and *Burkholderia contaminans* via different mechanisms of action such as attacking the microbial cell membrane and alternating the microbial metabolism (Chen et al., 2022; Han et al., 2022; Jumpathong et al., 2022; Romero et al., 2007). Besides the antimicrobial activity, the production of biosurfactants also contributed to bioremediation such as the removal of organic compounds and heavy metals in soil (Meena et al., 2021; Mishra et al., 2021). Since the biosurfactant could lower the interfacial tension and result in enhancing the solubility of various substances in water, the presence of biosurfactants could enhance the bioavailability of the organic compounds and thusly improve microbial degradation and water removal (Kreling et al., 2020; Sajadi Bami et al., 2022). Moreover, some of the anionic biosurfactants such as surfactin constituted negative charges and thusly had a strong binding capacity with some heavy metal ions such as Cd^{2+}, Pb^{2+}, Hg^+, and Zn^{2+} (Mishra et al., 2021; Sajadi Bami et al., 2022).

As mentioned above, there are several strategies for purifying the crude biomolecules from the culture medium, and precipitation with acids and ammonium sulfate was considered one of the promising methods due to the easy operation. ACP was the most common method used for precipitating the crude biosurfactant and in this study, the acid precipitate was

further extracted by the solvent mixture hexane-isopropanol. Since the major biosurfactant compounds such as surfactin and iturin were polar molecules due to the presence of the negatively charged amino acid group (Théatre et al., 2021), the cyclic lipopeptide biosurfactant would have a higher solubility in the weak polar organic solvent compared to water or non-polar solvent, and thus the precipitate was further extracted by the weak polar solvent isopropanol and result in the phase separation (isopropanol fraction and hexane fraction) after centrifuge (Figure 4B). Shen et al. (2019) reported a new solvent extraction strategy by using the solvent mixture hexane-methanol and their result suggested that the new solvent extraction system could recover up to 90% of the biosurfactant meanwhile achieving almost 100% purity in the methanol fraction. A similar result has been shown in this study (Table 2) that after extracting with hexane-isopropanol, the isopropanol fraction would have the highest purification fold among all the treatments which implied that it would be having the highest biosurfactant purity and other organic impurities such as antioxidant have been extracted and maintained in the hexane fraction.

ASP is another approach for extracting and purifying crude biomolecules from the culture medium. However, unlike ACP, the ammonium sulfate purification would be precipitating other high molecular biomolecules and thusly resulting in a relatively low purity (Banat et al., 2010; Sarubbo et al., 2022). However, compared to the ACP, the sequential ammonium sulfate precipitation could effectively separate the biosurfactant (mainly in 40% ASP fraction) and industrial enzyme (mainly in 60% ASP fraction). Even though the purity of the biosurfactant from ASP was lower compared to ACP, the yield is much higher in ASP compared to ACP and the purity could be further optimized by further purification meanwhile lowering the amount of organic solvent being used in the extraction. Moreover, highly active enzymes could be further purified with sequential ASP for lowering the production cost but meanwhile, ACP would denature the protein and result in losing parts of the valuable

biomolecules.

3.5 Characterization of the purified amylase

The amylase purified from 60% ASP was further characterized and the potential for industrial application was evaluated with the optimal reaction pH (Figure 5A), temperature (Figure 5B), thermostability (Figure 5C), and effect of metal ions (Figure 5D). The purified amylase had the optimal reaction pH at 5, 7, and 8 without significant differences ($p > 0.05$). Moreover, the amylase also exhibited the ability in adapting a broad range of environment pH, from 4 to 11, and remaining at least 50% of the maximum activity. The optimal reaction temperature would be 50 °C to 70 °C without significant difference ($p > 0.05$) and around 50% of the maximum activity can be remained at 40 °C, 80 °C, and 90°C.
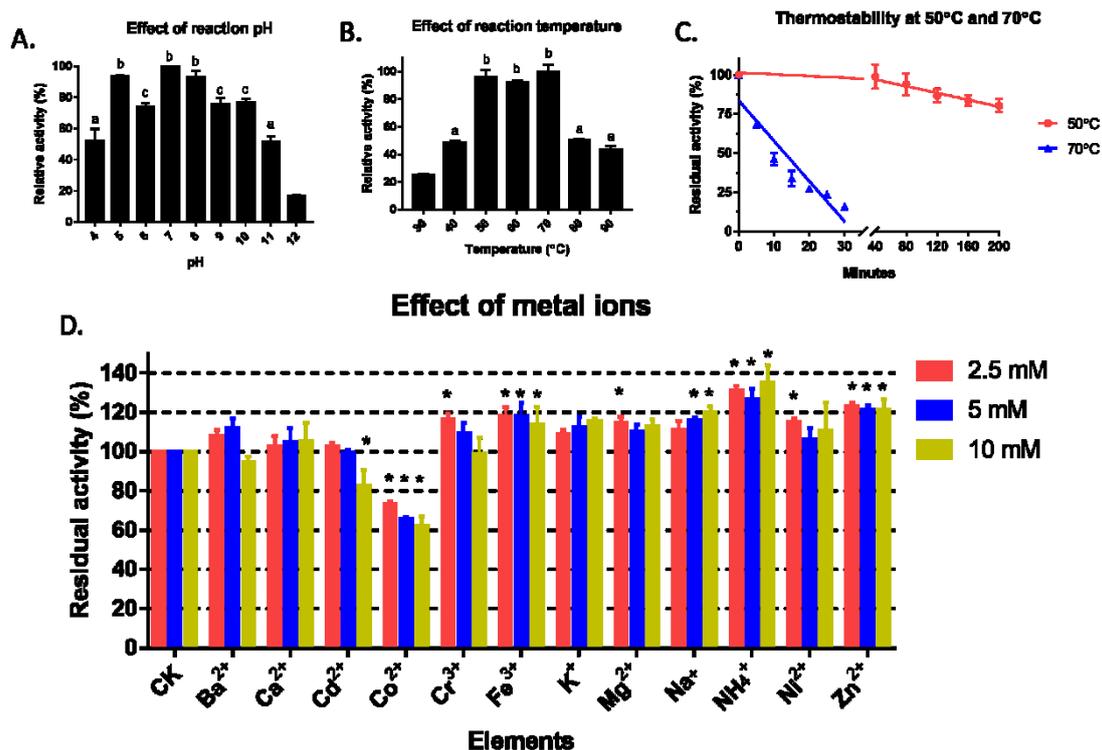
Figure 5.The effect of various factors on the activity of purified amylase. (A) reaction pH, (B) reaction temperature, (C) thermostability at 50 °C and 70 °C, and (D) the metal ions as chloridic salt. The same letter indicated that there were no significant differences between these data ($p > 0.05$) and "*" indicated that there was significant difference between this data and the control group (CK) ($p < 0.05$).

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The optimal reaction pH and temperature would be the critical factors that affect the enzyme application. The purified amylase from this study has a similar reaction profile as other reported amylase from *B. velezensis* or other related species, having optimal reaction temperature around 50 °C to 60 °C with a board pH from acid to alkaline (Bhatt et al., 2020; Hu et al., 2022; Ratnayake and Jackson, 2008). One of the major applications of amylase was hydrolyzing the gelatinized starch solution. The condition for the gelatinization of raw starch would be various depending on the source and molecular structure of the starch but usually around the range of 60 °C to 70 °C. The purified amylase would be suitable for combining with the gelatinizing process of wheat starch, oat starch, lentil starch, and chickpea starch which had a relatively lower gelatinization temperature (Ratnayake and Jackson, 2008). Combining the gelatinization and hydrolysis process would lower the energy and equipment consumption and simplify the procedure which would be beneficial to lowering the operation cost.

For further evaluating the potential in industrial applications, the thermostability at the edge of the optimal reaction temperature, 50 °C and 70 °C, was measured. The result suggested that the half-life at 50 °C and 70 °C was 473 mins and 13 mins, respectively. Besides the thermostability, the effect of metal ions was also evaluated at different ions and concentrations. Among all the tested ions, only Cd^{2+} and Co^{2+} have shown a significant and dose-dependent inhibition effect on the amylase activity ($p<0.05$). In the contract, several ions had significantly promoted the enzymatic activity ($p<0.05$) including Fe^{3+}, Na^+, NH_4^+, and Zn^{2+}. Interestingly, Ca^{2+} didn't play a significant role in affecting the amylase activity.

A thermostable enzyme would be much more attractive to industries. The result suggested that the purified amylase would be very stable at 50 °C, with a half-life of around 7 to 8 hours, compared to other reported amylase (Ratnayake and Jackson, 2008), although the

stability at 70 °C would need to be further improved. The high stability at 50 °C with a broad range of reaction pH implied that the purified amylase could be useful in the textile treatment and baking (Balajia et al., 2018; Trabelsi et al., 2019). The effect of metal ions is another critical factor regarding the amylase characterization since some of the amylases would require the presence of Ca^{2+} or other metal ions for the essential activity (Sivaramakrishnan et al., 2006). In this study, the purified amylase didn't require the presence of Ca^{2+} and even the addition of Ca^{2+} is not significantly promoting the amylase activity since Ca^{2+} is generally considered one of the promoters for the amylase activity due to the presence of the Ca^{2+} binding pocket in the amylase protein structure (Gupta et al., 2003). Interestingly, the activity of purified amylase was promoted by the presence of Fe^{3+} and Zn^{2+} which were generally considered the inhibitors for amylase activity (Gupta et al., 2003). Liao et al. (2019) reported that the presence of the Zn^{2+} would disrupt the outer rim of the catalytic pocket and further bury the active site and limit the residue rotation under the molecule dynamic simulation. The result from this study implied that further molecular analysis of the purified amylase could provide valuable information regarding the ions tolerance from a molecular aspect.

3.6 Characterization of the biosurfactant and its potential in bioremediation

The emulsion activity of the purified crude biosurfactant in water was measured and other common surfactants were used for comparison (Figure 6A). The result indicated that the biosurfactant exhibited a significantly higher emulsion activity compared to other common surfactants (p<0.05). Among them, Triton X100 had a second high emulsion activity and was thusly chosen for further analysis. Moreover, instead of emulsifying oil into water, SDS would prefer forming foam on top of the water and the oil could be suspended in the foam instead of the water. It was not surprising that an anionic surfactant such as SDS might not be that effective as an emulsifier compared to a non-ionic or biosurfactant (Dickinson and

Ritzoulis, 2000; Fei et al., 2020; Tadros, 2014). However, the addition of substance containing charges into the water, such as the mixture of the anionic surfactant and non-ionic surfactants, would significantly enhance its emulsion activity toward oil in water (Kralova and Sjöblom, 2009). Among them, the purified crude biosurfactant could contain both types of surfactant, surfactin served as an anionic surfactant and iturin served as a non-ionic surfactant, and the combination of surfactin and iturin could be further enhanced the emulsion activity (McClements and Jafari, 2018). Moreover, since the crude biosurfactant was purified from the ASP, the high molecular weight of the biosurfactant would also contribute to the emulsion of oil since these polymeric surfactants could easily be coating the oil droplet compared to the low molecular weight biosurfactant and form a stable emulsion in the water solution (Muthusamy et al., 2008).

In order to further explore the potential of the purified crude biosurfactant in bioremediation, the petroleum hydrocarbon (used engine oils) and model PAHs (naphthalene and phenanthrene) were used as pollutants and the bioremediation potential was evaluated based on the enhancement in removal ability or solubility.

The potential of using biosurfactants for removing petroleum hydrocarbon from soil was evaluated under a simulation situation (Figure 6B). The result indicated that all the surfactants could significantly improve the removal of used engine oil from the soil and the purified crude biosurfactant (61.48%) was significantly higher ($p<0.05$) than the other two surfactants (55.89% and 54.04% for Triton X100 and SDS, respectively) which were similar to the result in emulsion activity measurement and study from Phulpoto et al. (2020). The presence of the surfactant could enhance the bioavailability of the petroleum hydrocarbon for microbial degradation by emulsifying the oil into the water and its emulsion activity would be highly related to its biodegradation (Bezza and Chirwa, 2015; Von Lau et al., 2014). Moreover, a study from Yin et al. (2023) indicated that *B. velezensis* BSA1 could produce

biosurfactant for enhancing the solubility of the low permeability oil meanwhile regulating the microbial community for enhancing the biodegradation of the oil. However, study from Akbari et al. (2021) also indicated that the alternation of microbial community could result in lowering the biodegradation efficiency. Therefore, B. velezensis PhCL would have a strong potential for petroleum hydrocarbon bioremediation but further on-site trait was necessary for further expanding its application on soil bioremediation and agricultural improvement.
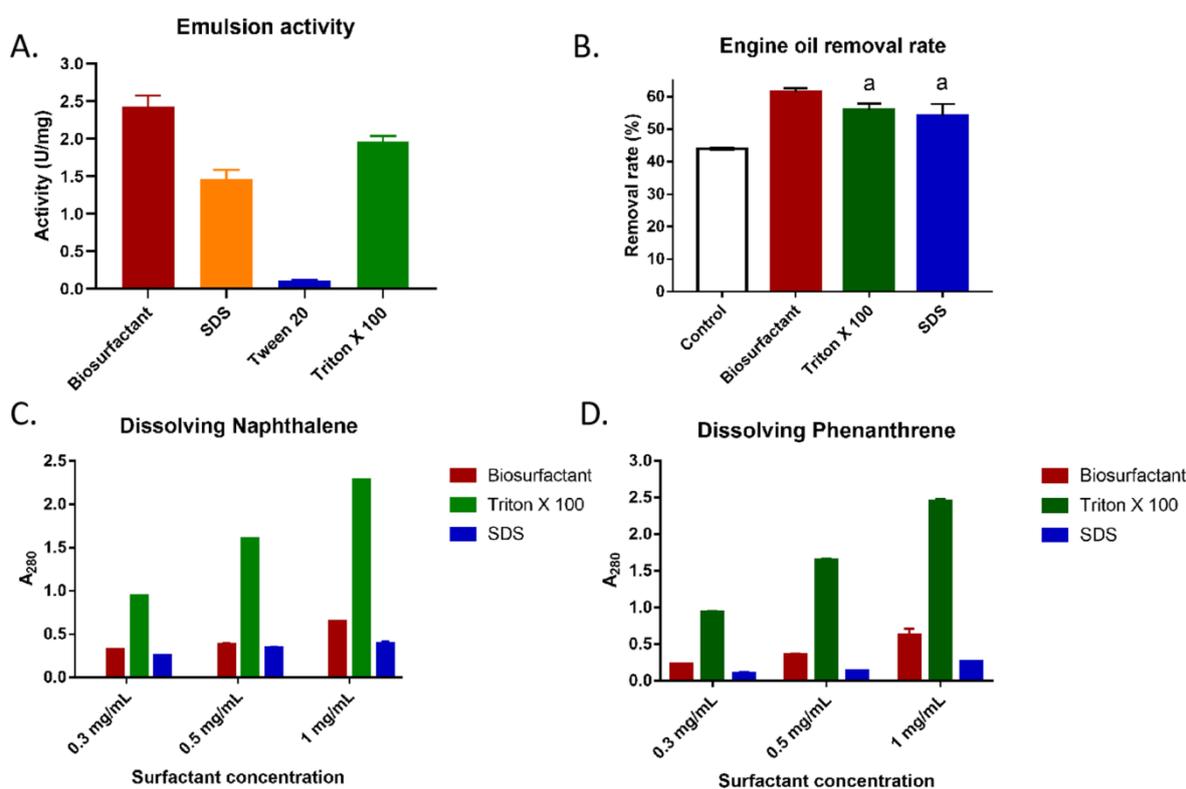
Figure 6. The characterization of the purified crude biosurfactant. (A) The emulsion activity of various surfactants, (B) The used engine oil removal rate of the biosurfactant, SDS, and Triton X100. The same letter indicated that there were no significant differences between these data ($p>0.05$), (C) The effect of various surfactants on improving the solubility of naphthalene, and (D) The effect of various surfactants on improving the solubility of phenanthrene.

However, even though all the surfactants could improve the solubility of PAHs in water along with increasing the surfactant concentration, the result indicated that the biosurfactant and SDS didn't enhance much solubility of PAHs in water compared to Triton X100 (Figure

6C, 6D). It could be due to that not only because the non-ionic surfactant had a better solubilization effect on the PAHs (Shih et al., 2020), but also due to the low dosage of SDS in the solution. Even though the hydrophobic/hydrophilic balance of SDS was higher compared to Triton X100, the critical micelle concentration (CMC) of anionic surfactant (e.g. CMC_{SDS}=8.1 mM) would be higher than the non-ionic surfactant (e.g. $CMC_{Triton-X100}$=0.31 mM) and therefore the performance of the SDS could be further improved with a higher SDS concentration (Hong et al., 2018; Mohamed and Mahfoodh, 2006). Similarly, as mentioned above, the purified crude biosurfactants would have a higher molecular weight compared to Triton X100, and thus the molar concentration of the biosurfactant could be much lower than Triton X100 and thus result in that biosurfactants would have a much lower improvement in solubility. Interestingly, even though using the surfactants could enhance the solubility of the PAHs and thusly enhance the bioavailability to microbes, it wasn't absolutely improving the biodegradation of the PAHs, especially in soil. A study from Yuan et al. (2000) indicated that the use of Triton X100 indeed enhanced the solubility of PAHs, but meanwhile, the Triton X100 and increasing solubilized PAHs could be also the toxic factors that inhibit the microbial growth and degradation which result in delaying the PAHs' degradation. Vaidyanathan et al. (2022) also indicated that the use of Triton X100 could lower soil organic matter and porosity of the soil compared to the surface-active bio-compounds and result in a decrease in the microbial population. Similar to the hydrocarbon biodegradation, applying biosurfactants for PHAs bioremediation also would alternate the microbial community and Cazals et al. (2020) indicated that the alternation in their study had improved the biodegradation of the PAHs. Therefore, further research and investigation was necessary for evaluating the bioremediation potential of the purified biosurfactant.

4. Conclusion

A novel strain of *B. velezensis* PhCL was isolated from the phenolic waste based on its ability in degrading lignin. The newly isolated strain exhibited several enzyme activities including amylase and xylanase meanwhile it could produce several useful bioproducts such as biofilm, antioxidants, and biosurfactants. The amylase production was optimized based on the response surface methodology and statistical analysis revealed that the initial medium pH would play an important role in the amylase production. Moreover, the enzymes and biosurfactants were purified with sequential ammonium sulfate precipitation which is seldom reported before and the result suggested that even though the purity of the crude biosurfactant was not as high as the common acid precipitation, it still can be used for partially purifying high molecular weight biosurfactant and highly active enzymes which was not able to be recovered by acid precipitation. The characteristic of the purified enzymes and crude biosurfactant was determined and the result suggested that the purified amylase was suitable for a broad range of reaction pH and temperature meanwhile having a good thermostability at the optimal reaction temperature and highly adapted to several metal ions. The evaluation of bioremediation suggested that the purified crude biosurfactant would have a much higher emulsion activity and remove more engine oil from the soil compared to the common surfactants SDS and Triton X100. Even though the biosurfactant could not enhance the PAHs solubility as high as Triton X100, the potential of enhancing the biodegradation of PAHs would need further evaluation. The findings in this study could further step forward in the utilization of the useful bioproducts from *Bacillus velezensis* for environmental bioremediation and industrial application and development of an environmentally and economically sustainable bioproduction.

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Particle size distribution after filtering
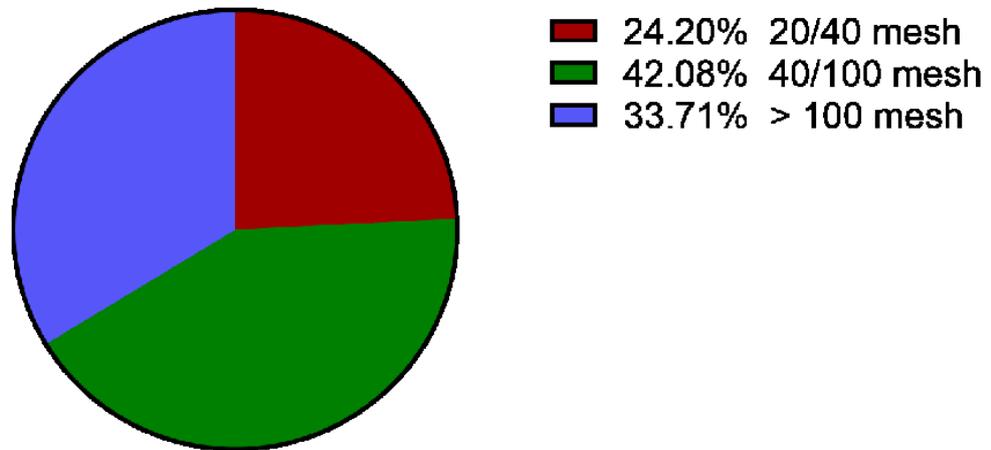
Particle size distribution after filtering
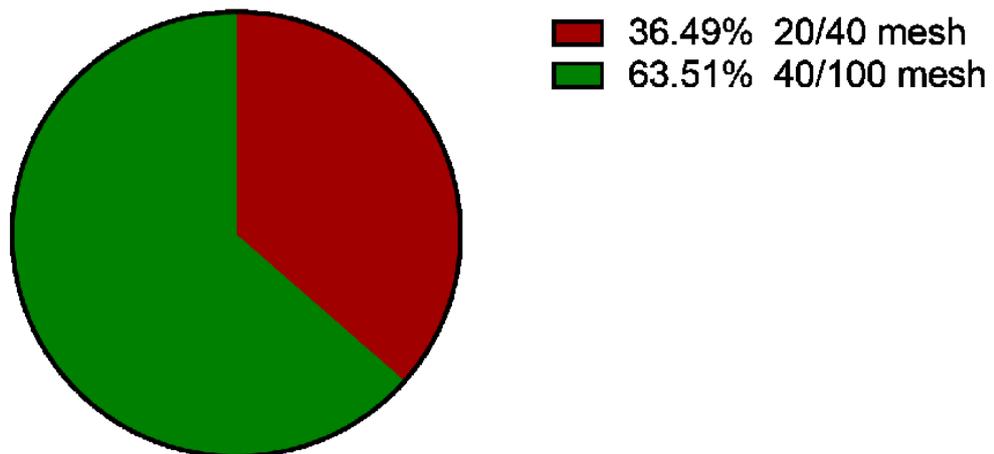
Figure S1. The particle size distribution of the soil particles before and after filtering. The SOM of the soil was 10.352 ± 0.94 %.

Table S1. The coefficients and statistical analysis of the RSM model

Estimates of the Regression Coefficients				
Effect	Coefficient	Standard Error	t	p-value
CONSTANT	35.513	0.241	147.419	<0.001
X_1	-1.401	0.148	-9.497	<0.001
X_2	-1.796	0.148	-12.173	<0.001
X_3	0.711	0.148	4.821	0.005
$X_1 * X_1$	-10.767	0.217	-49.583	<0.001
$X_2 * X_2$	-18.398	0.217	-84.728	<0.001
$X_3 * X_3$	-8.122	0.217	-37.401	<0.001
$X_1 * X_2$	1.389	0.209	6.656	0.001
$X_2 * X_3$	1.422	0.209	6.818	0.001
$X_1 * X_3$	-1.893	0.209	-9.071	<0.001

Analysis of Variance					
Source	df	Type I SS	Mean Squares	F-ratio	p-value
Regression	9	1,786.443	198.494	1,140.112	<0.001
Linear	3	45.545	15.182	87.202	<0.001
Quadratic	3	1,710.764	570.255	3,275.440	<0.001
Interaction	3	30.133	10.044	57.694	<0.001
Residual Error	5	0.871	0.174		
Total Error	14	1,787.314			
Lack of Fit Test					

Analysis of Variance					
Source	**df**	**Type I SS**	**Mean Squares**	**F-ratio**	**p-value**
Source	**df**	**SS**	**Mean Squares**	**F-ratio**	**p-value**
Lack of Fit	3	0.477	0.159	0.808	0.594
Pure Error	2	0.394	0.197		
Residual Error	5	0.871	0.174		

Chapter 3. Production of Biomolecules and Optimization of Amylase Production from *Bacillus velezensis* PhCL using Low-value Agricultural Waste as Feedstock and the Potential of the Remaining Residue using as Biochar

Abstract:

Biomolecule is considered an alternative way to obtain useful and green chemicals for society development. However, the production of the biomolecules would require edible-resource and result in a higher production cost. Therefore, utilizing non-edible lignocellulose for biomolecule production would be a promising approach to sustainable development. In this study, various agricultural wastes were used for the production of amylase and other bioactive compounds from *Bacillus velezensis* PhCL, and the fermentation process was optimized by response surface methodology. The result suggested that the rice husk would be the most suitable biomass for amylase production with optimized fermentation conditions, amylase production 43.94 ± 0.51 U/mL could be achieved and several biomolecules such as biosurfactant and antioxidants could be recovered from the fermentation broth. Moreover, for further utilizing the remaining residue, the hydrolyzed rice husk was converted to biochar and the result suggested that the hydrolyzed biochar would have faster adsorption compared to the control and it could be potentially used for immobilizing enzymes. This study demonstrated another approach for producing bioactive compounds from *Bacillus velezensis* using low-cost agricultural wastes and the potential of using hydrolyzed biomass for biochar production.

1. Introduction

Bioproduct is considered an alternative solution for obtaining useful chemicals and

reagents through a sustainable and green process (Ncube et al., 2023; Rogers and Kerton, 2022). Various bioproducts have been developed recently such as biosurfactants (Jimoh and Lin, 2019), bioplastics (Onen Cinar et al., 2020), biofuel (Sharma et al., 2020), bioabsorbent (Ghafghazi and Taghavi, 2022), etc. Among these bioproducts, the enzyme is one of the well-studied and widely used bioproducts with large economic and environmental benefits (Cipolatti et al., 2019).

Amylase is one of the most common commercialized enzymes worldwide and it is the largest ratio (around two-thirds) in the global enzymes market since starch is also one of the most abundant natural polysaccharides on earth (Escaramboni et al., 2022). It can catalyze the hydrolysis between the α-1, 4-glycosidic bond in the starch molecules and yield dextrin and maltose which could be further utilized in various industrial applications (Visvanathan et al., 2020). Amylase is classified as a member of glycosidic hydrolase family 13 (GH 13) and it can be further divided as α, β, and γ-amylase based on the substrate recognization and acting position (Akinfemiwa et al., 2022). Besides breaking down starch into sugars for baking and beverage, amylase is also widely used in the textile industry for managing the fabric and modifying the starch which could be used for biomedical and absorption later (Garcia et al., 2020; Li et al., 2012; Salem et al., 2021).

However, the production of biomolecules is usually related to microbial fermentation which would need the nutrition for the growth of microbe and the bioconversion between the feedstock to bioproducts. Some of the common feedstock including starch, mono-sugar, and edible plants increased the concerns regarding the food shortage and food-energy competition. Therefore, using non-edible and sustainable resources for biomolecules products has been widely studied (Ghani et al., 2019; Talebi et al., 2022) and one of the promising feedstock is the lignocellulose from the non-edible plants and agricultural wastes (Ko et al., 2020; Prasad et al., 2022).

In this study, various agricultural wastes were used as feedstock for producing amylase by fermenting with *Bacillus velezensis* PhCL, and the fermentation conditions were optimized via response surface methodology (RSM). Furthermore, various bioproducts were detected and measured during the optimal fermentation, and the remaining lignocellulosic residue was further converted into biochar. The result suggested that the biochar generated from microbial hydrolyze rice husk would have a faster absorption. This study provided a further investigation on producing valuable bioproducts from *Bacillus velezensis* using various biomasses as feedstock and contributed another aspect for lowering the cost related to bioproduction.

2. Materials and methods

2.1 Bacteria strain and reagents

The *Bacillus velezensis* PhCL was isolated previously and stored in our lab (Chapter 2). The agricultural wastes (wheat straw, oat straw, goldenrod stem, spent grains, and rice husk) were collected or purchased from local farms and markets. The obtained agricultural wastes were dried in an open space until constant weight, then the biomasses were ground and passed through a 20 mesh sieve. The filtered biomass powder was stored in a bag without any other treatments. All the chemicals and reagents (at least ACS grade) used in the study were purchased from Sigma and Fisher Scientific except those specifically mentioned.

2.2 The screening of biomasses and fermentation conditions for bioproducts production by *B. velezensis* PhCL

Luria–Bertani (LB) and minimal salt medium M9 were used as the culture medium in this study. The screening of suitable biomasses for producing bioproducts was conducted by modifying the carbon source in the M9 medium. The M9 was composed of 6 g/L Na_2HPO_4, 3

g/L KH$_2$PO$_4$, 0.5 g/L NaCl, 20 g/L of biomass, and 5 g/L (NH$_4$)$_2$SO$_4$. In short, *B. velezensis* PhCL was cultured in LB medium at 35 °C for overnight with a rotation speed of 250 rpm and this would be used as seed cultured. Biomasses and M9 would be autoclaved at 121 °C for 30 mins separately and mixed together after cooling down to room temperature. After that, the seed culture would be inoculated into the M9 at the ratio of 1:100. The M9 medium with inoculum and biomass would be incubated and shaken at 35 °C for 5 days and 0.5 mL of the culture medium was collected from the fermentation broth every 24 hours for measuring enzymatic and metabolites activity. The same rotate speed was used in all the experiments except specifically indicated.

2.3 The determination of enzymatic activity

The determination of the amylase and xylanase activity was according to the dinitrosalicylic acid (DNS) method (Miller, 1959b) with minor modification (Wang et al., 2019a). Soluble starch, carboxymethyl cellulose, and beechwood xylan (Cedarlane, Canada) dissolved in 50 mM pH 7 potassium phosphate buffer were used as the substrate for amylase, CMCase, and xylanase reaction, respectively. Briefly, 10 µl of diluted enzyme solution was mixed with 20 µl of the substrate and reacted at a 50 °C water bath for 10 mins, and 60 µL of DNS reagent was added to the mixture to stop the reaction. After that, 90 µL of the mixture was boiled in boiling water for 5 mins and immediately cool down after boiling. Eventually, 200 µL of water was added to the mixture and the same amount of mixture was transferred to the 96 wells microplate, and the absorbance at 540 nm was measured using Epoch Microplate Spectrophotometer (BioTek, USA). The absorbance was converted into the concentration of maltose, glucose, and xylose based on the corresponding standard curve. The enzyme activity was expressed as the International Unit which was defined as 1 unit of activity referring to the amount of enzyme required for releasing 1 µmol of maltose (for amylase), glucose

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(CMCase), and xylose (for xylanase) in 1 min under specific conditions.

2.4 The optimization of medium composition and fermentation conditions for amylase production and further optimized via Box-Behnken design (BBD)

The effect of medium composition such as biomass concentration, nitrogen concentration, and additives, and fermentation conditions such as fermentation temperature, initial medium pH, and fermentation time were investigated and the amylase production was optimized via single-factor experiments. After the single-factor experiment, three factors and three levels would be selected for the BBD and the RSM model would be constructed using SYSTAT 12 (Systat Software, Inc., USA) based on the BBD table. The coefficient R^2 and the lack of fit were used for indicating the goodness of fits and reliability of the model equation by an F-test at a 5% level of significance. After that, the optimal amylase production and corresponding fermentation condition would be calculated and validated based on the RSM model.

2.5 The measurement of the emulsion and antioxidant activity

The measurement of biosurfactant emulsion activity was referred to the publication by Pathak and Keharia (2014). In short, 0.1 mL of the surfactant was loaded into a mixture containing 0.6 mL of water and 20 μL of olive oil. After suspending the mixture by vigorous shaking for 2 mins, the suspension was incubated in a water bath at 35 °C for 1 hour and the turbidity was measured based on the reading of optical density (OD) at 600 nm. One unit of the emulsion activity was defined as the amount of surfactant causing 0.1 reading at OD 600 nm in the mixture.

The measurement of antioxidant activity was according to the method from Arts et al. (2004) with minor modifications. The activity was determined based on the ability in scavenging the radical of oxidized 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

(ABTS). Briefly, the 7mM of ABTS solution was mixed with an equal volume of 2.4 mM of potassium persulfate solution and incubated at 4 °C for 18 hours. After that, 20 µl of the medium broth was added to 200 µL of oxidized ABTS solution, and the mixture was incubated at room temperature for 7 mins, then the absorbance at 734 nm was measured. The oxidized ABTS decolorization rate was converted to the amount of Trolox equivalent antioxidant unit based on the standard curve using Trolox as standard antioxidant performing the same experiment.

2.6 The production and characterization of biochar using hydrolyzed biomass and its potential in the application of adsorption

The biomass residue obtained from the fermentation was washed with water 3 times and dried in the oven at 105 °C until constant weight. The biochar was produced by pyrolyzing dried biomass in a muffle furnace at 500 °C for 3 hours and cooled down to room temperature. X-ray diffraction (XRD) was used for characterizing the biomass and biochar. The XRD pattern was recorded using a PANalytical X'pert Pro diffractometer (PANalytical, Holland) equipped with a conventional X-ray tube (CuKα 40 kV, 20 mA, line focus) in transmission mode. The intensity 2θ between 10° to 100° was measured with the step of 0.0263° and 37.74 s/step. The crystallinity index (CrI) was calculated according to Segal et al. (1959) method as follows:

$$CrI = \frac{I_{200} - I_{am}}{I_{200}} \times 100$$

I_{200} referred to the intensity of the crystalline peak corresponding to the crystalline plane 200 (2θ=22.4°). I_{am} referred to the intensity of the amorphous peak between the plane 200 and 110 (2θ=18.8°). The intensity was calculated by the function "Area under the curve" in GraphPad Prism.

The potential of microbial hydrolyzed biochar (HBC) in applications was evaluated based on the ability in removing Congo red and immobilize enzymes. In short, 50 mg/mL of biochar was loaded in 100 ppm Congo red solution and the mixture was shaken at room temperature with a rotation speed of 200 rpm. The sample was taken every 5 mins and the HBC was removed by centrifugation at 12, 000 g for 3 mins. The absorbance at 498 nm of the supernatant was measured and converted to Congo red concentration based on the standard curve. The biochar (BC) without microbial hydrolysis (incubated without microbe) was used as a control. The removal rate of HBC and BC under acidic or alkaline conditions was evaluated. In short, 50 mg/mL of BC and HBC loaded into 100 ppm Congo red solution with different pH (5, 7, 9) and shaken at room temperature for 30 mins with a rotation speed of 200 rpm. After that, the supernatant was collected by centrifugation at 12, 000 g for 3 mins and the absorbance at 498 nm was measured.

The potential of enzyme immobilization was evaluated based on the residual xylanase activity after several times of re-use. In short, 50 mg/mL of HBC was suspended in a crude xylanase solution obtained from Chapter 4. After shaking at room temperature for 30 mins, 0.1 mL of biochar solution was centrifuged at 12, 000 for 3 mins and the remaining biochar was suspended in a 50 mM pH 7 potassium phosphate buffer for washing unbound protein. After that, the biochar was suspended in 0.2 mL of beechwood xylan solution (dissolved in 50 mM pH 7 potassium phosphate buffer) and incubated at 50 ℃ for 10 mins, then the supernatant was collected and used for measuring the released reducing sugar using DNS method as mentioned above. The remaining biochar was suspended in potassium phosphate buffer again for washing the products from the biochar and then the biochar was mixed with beechwood xylan solution again for the next round of reaction.

2.7 Data processing and statistical analysis

All the experiments and measurements were conducted at least triplicated and the data presented in the study was mean ± SD. The analysis of variance (ANOVA) and Tukey's multiple comparisons were used for evaluating the significance and conducting the comparative analysis. The data with a p-value less than 0.05 was considered a significant difference.

3. Results and discussion

3.1 Screening of suitable biomass for amylase production

Various agricultural wastes (wheat straw, oat straw, goldenrod stem, spent grains, and rice husk) were used as feedstock for the amylase production and the result was shown in Figure 1. The result suggested that most of the biomasses had the maximum amylase production after 72 hours of incubation except the goldenrod stem which was after 48 hours. The highest amylase production could be obtained from using rice husk as feedstock (1.834 ± 0.036 U/mL), followed by spent grains (1.689 ± 0.045 U/mL), goldenrod stem (1.121 ± 0.068 U/mL), oat straw (0.946 ± 0.017 U/mL), and wheat straw (0.809 ± 0.022 U/mL). Therefore, rice husk was selected as the feedstock for optimizing the amylase production from *B. velezensis* PhCL, although there was no significant difference in amylase activity between rice husk and spent grains (p<0.05).
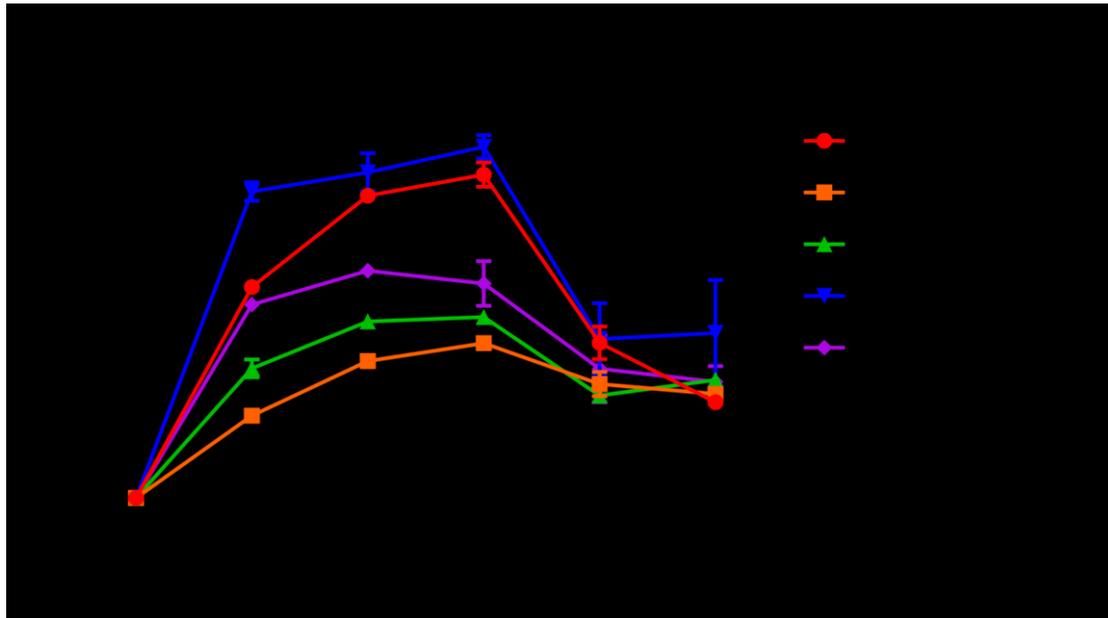
Figure 1. The screening of various agricultural wastes as potential feedstock for amylase

production (35 °C, pH7, 2% biomass, 0.5% (NH4)2SO4)

Rice husk was one of the major agricultural wastes from paddy fields and for each ton of rice being harvested, around 140 kg of rice husk would be generated (Moraes et al., 2014). The rice husk is composed of around 35% of cellulose, 25% of hemicellulose, 20% of lignin, and 20% of ash which was mainly composed of silicon (Ugheoke and Mamat, 2012). Compared to other common agricultural wastes, rice husk contained a significantly higher amount of silica and thus, some of the studies applied the rice husk ash as fertilizer and production of nano-silica particles (Pode, 2016; Thuadaij and Nuntiya, 2008). Rice husk was also commonly used as feedstock for producing enzymes such as amylase, laccase, lignocellulase, and protease (Aqeel and Umar, 2010; Iqbalsyah et al., 2019; Negi et al., 2020; Perdani et al., 2020), and the other value-added biomolecules such as poly(3-hydroxybutyrate), biosurfactant, and antioxidant (Lee et al., 2023; Rodríguez et al., 2021; Tlais et al., 2020). Moreover, studies indicated that the addition of rice husk in the medium could stimulate amylase production (Ashraf et al., 2003; Deljou et al., 2018).

Therefore, rice husk could be used as a fermentation feedstock for producing enzymes and multiple biomolecules.

3.2 The effect of medium composition on amylase production

The effect of medium composition including the concentration of rice husk (2% to 35%, Figure 2A), $(NH_4)_2SO_4$ (0.25% to 1%, Figure 2B), and additives (Figure 2C) on amylase production was investigated.
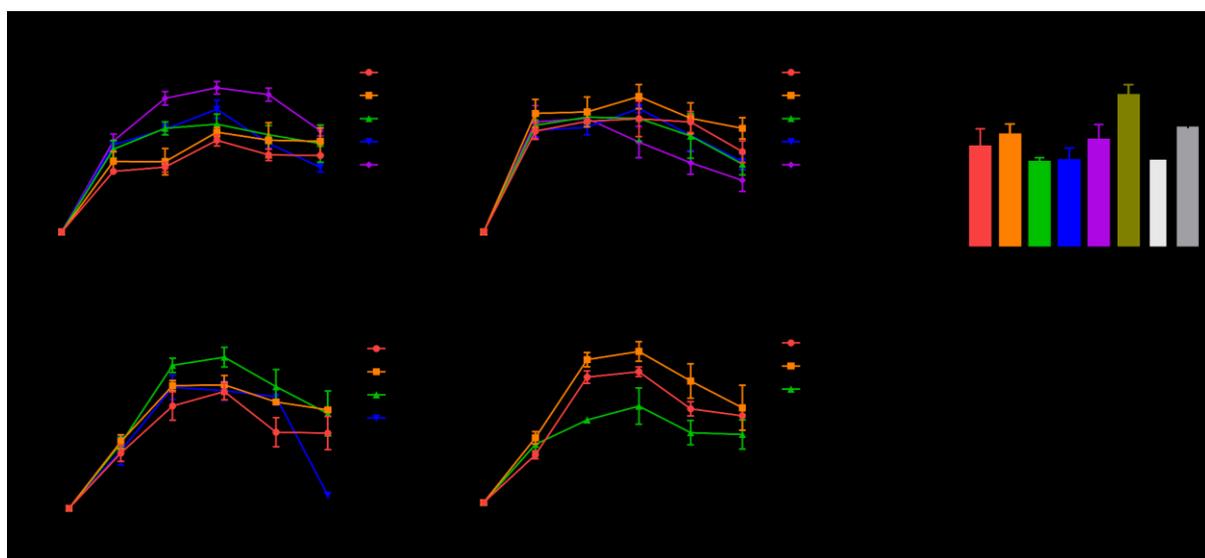
Figure 2. The effect of the medium composition and fermentation conditions on the amylase production. (A) Rice husk concentration (35 °C, pH 7, 0.5% $(NH_4)_2SO_4$); (B) $(NH_4)_2SO_4$ concentration (35 °C, pH 7, 35% biomass); (C) additives (35 °C, pH 7, 35% biomass, 0.375% $(NH_4)_2SO_4$); (D) medium pH (35 °C, 35% biomass, 0.375% $(NH_4)_2SO_4$, 0.5% peptone); and (E) fermentation temperature (35% biomass, pH 8, 0.375% $(NH_4)_2SO_4$, 0.5% peptone)

The results suggested that the amylase production would be increasing along increasing in rice husk concentration, and the highest tested rice husk concentration (35% w/v) achieved the highest amylase production (21.07 ± 0.538 U/mL) which was significantly higher than other rice husk concentration (p<0.05). The further increase in the rice husk concentration

would result in a sticky culture medium which would lose the liquid medium for enzyme assay (data not shown) and thus, 35% of the rice husk was used for further optimization. The concentration of $(NH_4)_2SO_4$ was also optimized with the range of 0.25% to 1%. The result suggested that similar to the result above, the highest amylase production could be achieved after 72 hours of fermentation except in 1% $(NH_4)_2SO_4$ and the concentration of 0.375% (w/v) would have the amylase production (23.75 ± 2.127 U/mL) slightly higher than other concentration, although the difference between different concentration was not statistically significant ($p>0.05$). After optimizing the concentration of rice husk and $(NH_4)_2SO_4$, various additives (glucose, xylose, peptone, and yeast extract) with different concentrations (0.05% and 0.5% w/v) were added to the fermentation broth for enhancing the amylase production (Figure 2C). The result indicated that only 0.5% of peptone could significantly enhance around 1.6-fold of the amylase production after 72 hours of incubation compared to the control ($p<0.05$). Even though glucose and yeast extract also slightly enhanced the amylase production, the enhancement was not significant ($p>0.05$). Therefore, the optimized medium was composed of 35% of rice husk, 0.0375% of $(NH_4)_2SO_4$, and 0.5% of peptone and this medium would be used for optimizing the fermentation conditions and RSM optimization.

Biomass concentration was one of the critical factors affecting biomolecule production and fermentation. Not only would the excess biomass concentration or osmotic stress lead to the inhibition of enzyme production and microbial growth (Klinke et al., 2004), but also would change the growth model to solid-state fermentation which would significantly alternate the genetic expression and physiological activity (Barrios-González, 2012). In this study, the rice husk concentration was much higher (35%) compared to other studies that used other biomasses such as wheat straw (around 6%) (Tsegaye et al., 2019), agave (around 3%) (Wang et al., 2019b). It is not surprising that the concentration of rice husk would be higher than other biomasses since most of the studies using rice husk for fermentation or

enzyme production were conducted as solid-state fermentation with a minimal biomass concentration of 50% w/v (Razali et al., 2021; Rodríguez et al., 2021; Sala et al., 2020; Tosuner et al., 2019) or using rice husk hydrolysate instead of rice husk particle (Zhang et al., 2022c). However, a study using rice husk in a liquid fermentation was seldom reported which could provide a different metabolism pattern compared to the solid-state fermentation (Sankar et al., 2023). Therefore, it would be possible that amylase production could be further increased with a higher rice husk concentration, even in solid-state fermentation. Moreover, by comparing the liquid culture from this study and the solid-state fermentation, the difference in the metabolites and bioproducts could provide more insights into advancing the utilization of agricultural waste and *B. velezensis*.

Interestingly, only 0.5% of peptone could significantly enhance the production of amylase since yeast extract could offer more growth factors such as vitamin and mineral elements which could be beneficial to microbial growth and enzyme production (Cheng et al., 2015; Galvagno et al., 2011; Pereira et al., 2019). It could be due to that since the rice husk could not be utilized directly, the addition of peptone could provide an immediate and feasible carbon source for microbial growth and metabolism. However, since the concentration of $(NH_4)_2SO_4$ was optimized and fixed to 0.375%, the addition of mono-sugar might affect the carbon/nitrogen ratio in the fermentation broth which result in the nitrogen source becoming the limited factor for the amylase production. However, the addition of peptone could provide both the carbon source and nitrogen source to *B. velezensis* and thusly remained a relatively stable carbon/nitrogen ratio and enhanced the growth of microbe and the production of amylase. Therefore, the combination of the nitrogen source and various additives could be further optimized for enhancing the production of metabolites and enzymes.

3.3 The effect of fermentation conditions on amylase production

Besides the medium composition, the fermentation conditions such as initial medium pH (pH 6 to 9, Figure 2D), fermentation temperature (30 °C to 40 °C, Figure 2E), and fermentation time (24 hours to 120 hours) were also optimized for enhancing the production of amylase.

The results indicated that the initial medium pH of 8 and fermentation temperature of 35 °C would achieve the highest amylase production after 72 hours of fermentation (44.16 ± 2.873 U/mL) which was significantly higher than other initial medium pH and fermentation temperature ($p<0.05$). Interestingly, compared to our previous finding, the rice husk medium with initial medium pH of 6 was significantly higher than the starch medium with initial medium pH of 6 which was almost no amylase production detected. This could be due to the composition difference in the medium. The starch in the medium would be converted into glucose and acids during the fermentation which could significantly affect the medium pH and gene expression and inhibit amylase production (Chen et al., 2016; Fuertes-Perez et al., 2019). Moreover, the composition in the rice husk could also stabilize the medium pH and provide additional factors for enhancing amylase production (Thiyageshwari et al., 2018).

Table 1. Box-Behnken design (BBD) for optimizing amylase production

Run	Temp (X_1) / °C	pH (X_2)	Time (X_3) / Hours	Activity (U/mL)	Forecasted value
1	30 (-1)	8 (0)	96 (1)	29.884	29.528
2	30	8	48 (-1)	38.171	36.056
3	30	9 (1)	72 (0)	35.351	36.211
4	30	7 (-1)	72	32.626	34.235
5	35 (0)	9	96	32.548	32.042
6	35	7	96	31.073	29.82
7	35	8	72	47.291	44.161
8	35	8	72	41.642	44.161
9	35	8	72	43.551	44.161

10	35	9	48	32.791	34.044
11	35	7	48	35.819	36.326
12	40 (1)	8	96	20.417	22.532
13	40	8	48	24.157	24.512
14	40	9	72	26.544	24.935
15	40	7	72	27.832	26.971

3.4 The optimization of amylase production using RSM based on the BBD table

After the single-factor optimization, the highest amylase production (44.16 ± 2.873 U/mL) was achieved with the medium composed of 35% rice husk, 0.375% $(NH_4)_2SO_4$, 0.5% peptone, and cultured at 35 °C with initial medium pH 8 for 72 hours. To further optimize the amylase production, fermentation temperature (X_1: 30℃, 35℃, 40℃), initial medium pH (X_2: 7, 8, 9), and fermentation time (X_3: 48 h, 72 h, 96 h) were selected as the individual factors to optimize the amylase production via RSM. The BBD table with the experimental design and result, and the predicted value from RSM were listed in Table 1 and a second-order polynomial model equation was constructed to express the relationship between the amylase production and the individual factors and shown as following formula:

$$y = -9.237X_1^2 - 4.336X_2^2 - 6.767X_3^2 - 1.003X_1X_2 + 1.126X_2X_3 + 1.137X_1X_3 - 4.635X_1$$
$$- 0.015X_2 - 2.127X_3 + 44.161$$

The coefficients and statistical analysis of the RSM model were listed in Table S1. The squared multiple R was 0.952 which meant there would be 95% of the result could be explained by the constructed RSM model. Furthermore, the p-value of the regression and lack-of-fit were 0.008 and 0.601, respectively, which indicated that the model was reliable with a good fit with the data. The 2-D contour plots shown in Figure 3 suggested that the optimal amylase production was within the range of the model constructed and the interaction between the individual factors would affect the response value (amylase production) since all

the contour plots were shown as ellipses. Based on the equation and contour plots, the fermentation temperature would be the most important factor affecting amylase production, followed by fermentation time and medium pH. Interestingly, as mentioned above, the effect from medium pH was much lower compared to fermenting in the starch medium.
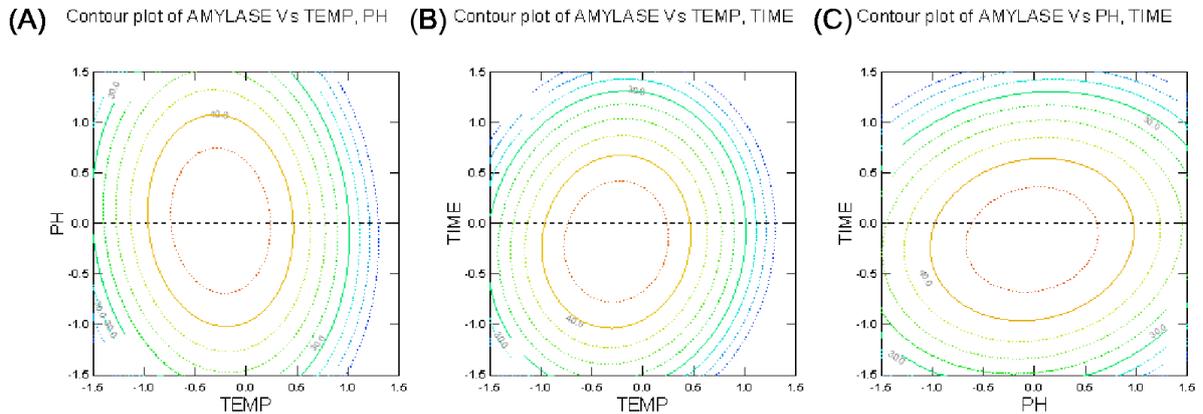
Figure 3. The 2-D counterplots showed the interaction between different individual factors: (A) pH vs Temperature, (B) Fermentation time vs Temperature, and (C) Fermentation time vs pH.

Based on the equation, the forecasted optimal amylase production would be 44.959 ± 5.473 U/mL (with a 95% confidence interval from 41.089 to 48.829) under the fermentation condition initial medium pH 8, fermentation temperature 33.69 ℃, and fermentation time 67.7 hours. A validation experiment was carried out to verify the optimal fermentation condition (Figure 4) with slight modification (initial medium pH 8, fermentation temperature 33.5 ℃, and fermentation time 68 hours). The result suggested that the amylase production under the optimal fermentation conditions was 43.94 ± 0.51 U/mL which was not significant difference compared to production after 72 hours of fermentation (44.13 ± 0.38 U/mL) and the optimal production before the RSM modeling (44.16 ± 2.873 U/mL). Therefore, the constructed RSM model could be considered reliable and used for simulating the amylase production changes within the tested range. Moreover, the optimal fermentation conditions for maximizing the amylase production would be relatively flexible in fermentation

temperature and fermentation time since there was no significant difference between the RSM optimal points and the points surrounding. This would be beneficial to industrial products since it would offer certain flexibility for the industry to adjust the production process.

3.5 The production of other biomolecules under the optimal culture condition
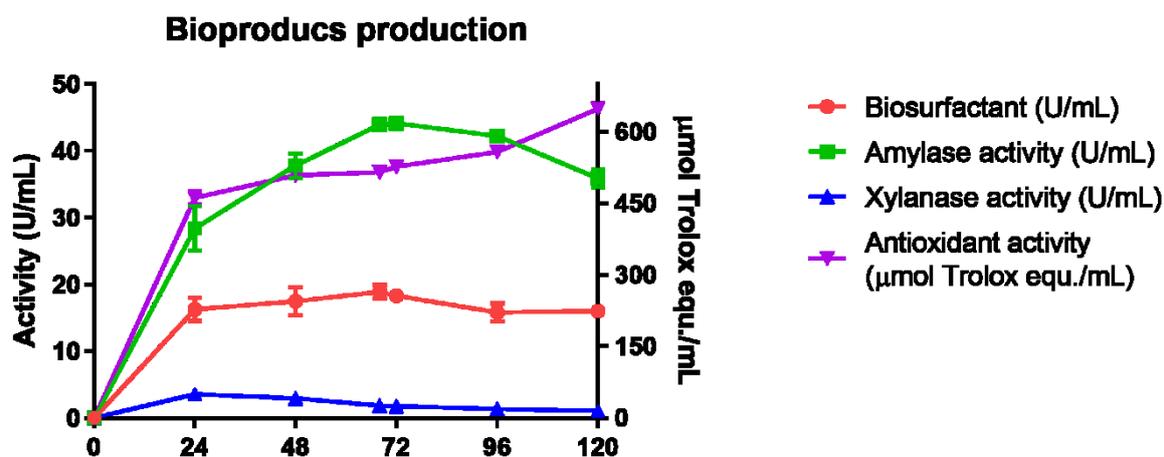
Figure 4. The production and activities of various biomolecules in the fermentation broth cultured at RSM-optimized conditions.

There were several other biomolecules produced during the fermentation with rice husk and their activities were shown in Figure 4. Similar to the fermentation in starch medium, the activity of CMCase was much lower compared to other data therefore it was not included in the figure. The results suggested that besides amylase, xylanase was also expressed during the fermentation but its production was much lower compared to amylase. Moreover, it was similar to the starch medium, the maximum xylanase production of 3.551 ± 0.037 U/mL was achieved after 24 hours of fermentation. Among the enzymes, biosurfactants and antioxidants were detected in the fermentation broth. The biosurfactant reached its highest emulsion

activity 18.91 ± 1.004 U/mL after 72 hours of incubation but no significant changes during the fermentation period (p<0.05) and it was lower than in the starch medium. Interestingly, the antioxidant activity was much higher compared to the starch medium meanwhile the activity was increasing until the end of the fermentation.

Xylanase was one of the major lignocellulases which were responsible for the degradation of xylan and it was one of the major enzymes involved in the degradation of agricultural waste biomass. Bibra et al. (2018) utilized corn stover as a carbon source and the bacterium *Geobacillus sp*. DUSELR13 could produce xylanase with activity 14.67 U/mL and the hydrolyzed corn stover would be easier converted into bioethanol. Geetha and Gunasekaran (2010) demonstrated that using wheat bran as a carbon source, *Bacillus pumilus* B20 would have the ability in producing xylanase with activity 313.3 U/mL. Therefore, the xylanase production in this study was not relatively high compared to other studies. Moreover, it could be the reason why the biosurfactant production would be lower than from the starch medium since the biosurfactant was mainly composed of lipopeptide, which required a significant amount of carbon for synthesis (Eras-Muñoz et al., 2022; Jumpathong et al., 2022). Interestingly, the antioxidant activity in the medium was much higher and it could be due to rich antioxidants in the rice husk (Lee et al., 2003). The study from Gao et al. (2018) indicated that there was 14.9 mg/g and 3.08 mg/g of total phenolic and flavonoid content in untreated rice husk   and the presence of xylanase and amylase could further break down the lignocellulose structure and further release the antioxidant compound from rice husk (Prabhu and Jayadeep, 2015; Tiwari et al., 2022; Wang et al., 2020). Besides antioxidants, extracted from rice husk also could be used as an anti-inflammation, antimicrobial, and anti-cancer agent (Gao et al., 2018; Risfaheri et al., 2018). However, the extraction of these bioactive compounds via the microbial route was seldom reported. Therefore, the application of lignocellulase in the extraction of rice husk could be a potential route for a green and

energy-saving route.

3.6 The characterization of biochar using hydrolyzed biomass and its potential in the application of adsorption
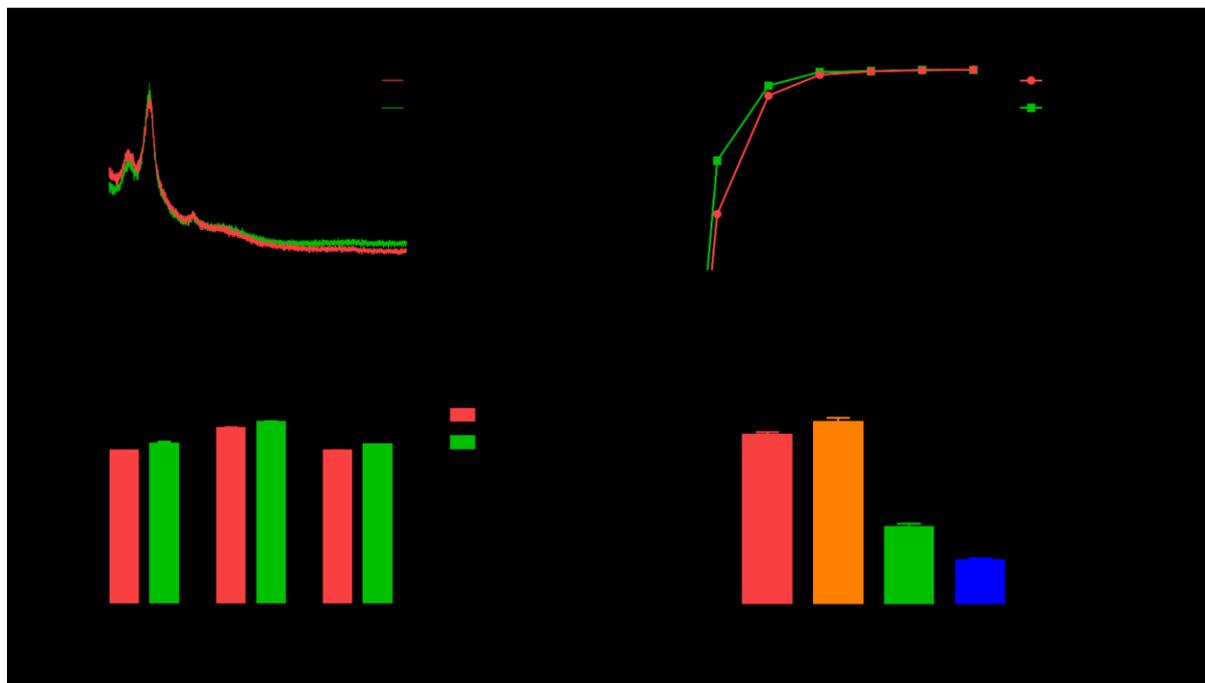
Figure 5. (A) XRD pattern of the rice husk and hydrolyzed rice husk from fermentation broth. (B) The adsorption of Congo red using BC and HBC. (C) The effect of solution pH in the adsorption activity. (D) The remaining activity after several times of reusing the immobilized xylanase.

After the fermentation, the fermented rice husk was partially hydrolyzed by xylanase, the remaining residue in the fermentation broth was collected and the rice husk incubated in the fermentation broth without bacterium inoculation was used as control. The collected residue was dried and scanned by XRD (Figure 5A). The XRD showed 3 clear peaks of cellulose at 2θ= 16.3°, 22.4°, and 34.5° with the highest intensity peak at 2θ= 22.4° and these peaks could be characterized as cellulose I allomorphism, referring to crystallographic planes 110,

200, and 004, respectively (Rashid and Dutta, 2020; Zhang et al., 2016). The XRD pattern of the control rice husk and hydrolyzed rice husk was similar, besides the CrI of rice husk (38.56%) was slightly lower than the CrI of hydrolyzed rice husk (39.71%). The rice husk control and hydrolyzed rice husk then were pyrolyzed and converted to biochar (BC) and (HBC) and their XRD pattern was shown in Figure S1. There were only 2 board peaks at $2\theta=23°$ and $43°$ and no sharp peak at all. Therefore, there was no crystalline structure detected which implied that both biochars were completely amorphous (Kim and Jung, 2023).

The potential of adsorbing industrial pollutants was evaluated based on the ability in adsorbing Congo red in water (Figure 5B). The result suggested that the HBC would have a significantly higher removal rate (86.14 ± 0.13, 98.81 ± 0.02, 99.41 ± 0.23 %) within the first 15 mins compared to the normal BC (78.27 ± 0.28, 95.69 ± 0.11, 97.41 ± 0.14 %). Both of the removal rates reached the maximum after 25 mins of incubation (99.5 ± 0.19 % and 99.54 ± 0.17 %, respectively, with no significant difference). The adsorption of Congo red under different pH was also evaluated and the result suggested the HBC would have slightly higher adsorption compared to the BC under different solution pH (pH 5, 7, and 9) (Figure 5C). Although the difference in the same pH solution was not significant ($p>0.05$), both biochars had significantly higher adsorption at pH 7 compared to pH 5 and 9. The potential of the biochar used for enzyme immobilization was also evaluated and the result suggested that the biochar can be used twice without any significant loss of activity. After that, the activity would drop to 45.5% when used for the third time and even drop to 25.89% when used for the fourth time.

CrI was one of the common indexes for evaluating the crystal structure of cellulose and the increase in the CrI usually referred to the higher ratio of the crystalline structure of cellulose, as lignocellulose (Trevorah et al., 2021). The increase of the CrI was mainly due to the decrease in crystallographic plane 110 and the increase in plane 200, which was

considered the amorphous region and crystalline region, respectively. It could be due to the expression of the xylanase which would hydrolyze the hemicellulose and the release of silica during the fermentation, which would result in lowering the amorphous region and reducing impurity of the cellulose and exposing a more crystalline region of the cellulose (Ezz et al., 2021; Kumari and Das, 2019). The study from Li et al. (2023) indicated that the biochar converted from cellulose with lower CrI would have higher thermal stability and hydrophobicity due to the higher aromatization compared to the higher CrI cellulose. Moreover, a study by Gray et al. (2014) indicated that higher hydrophobicity of the biochar would result in lowering its ability to water uptake and holding. Therefore, lowering the impurity and enhancing the crystallinity of the biomass could potentially enhance the water adsorption and pollutant removal (Figure 5B) of the biochar, and, thusly, the microbial pretreatment for biochar would be a potential approach for generating a more efficient adsorbent.

Using biochar as a support material was one of the promising immobilization strategies and studies had demonstrated its advantages and economic benefit compared to other immobilization approaches (Pandey et al., 2020). However, the reusability of the immobilized enzyme in this study was much lower compared to other studies. Ahmad and Khare (2018) showed that the cellulase immobilized on carbon nanotubes could be reused for up to 10 runs without much loss of activity. da Silva et al. (2022) demonstrated that the laccase immobilizing on avocado seed biochar could be reused up to 7 times and remained at more than 50% of activity. Therefore, improving the reusability of the immobilized enzyme would be one of the critical directions in this study and it could be achieved by further modifying or hydrolyzing the rice husk and improving the biochar production process.

4. Conclusion

In this study, various agricultural wastes were screened as a potential feedstock for producing amylase from *Bacillus velezensis* PhCL and the result suggested that rice husk would have the highest amylase production compared to other agricultural wastes. After that, the composition of the fermentation medium was optimized by single-factor experiments, and the fermentation conditions were further optimized by RSM. The result suggested that the optimal amylase production of 43.94 ± 0.51 U/mL could be achieved with fermentation temperature 33.5 °C, pH 8, and fermentation time 68 hours with a medium composed of 35% rice husk, 0.375% $(NH_4)_2SO_4$, and 0.5% of peptone. Furthermore, various biomolecules such as biosurfactants and antioxidants were detected in the fermentation broth. Moreover, the remaining rice husk residue was hydrolyzed by the xylanase and the biochar using hydrolyzed xylanase showed a faster adsorption compared to the control. However, the ability of enzyme immobilization needs to be improved since the reusability of the immobilized enzyme would be much lower than in other reported studies. The finding in this study would provide another approach for producing useful and functional biomolecules from *Bacillus velezensis* using low-cost feedstock and the further utilization of the residue after the fermentation.

Acknowledgement

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Table S1. The coefficients and statistical analysis of the RSM model

Estimates of the Regression Coefficients				
Effect	Coefficient	Standard Error	t	p-value
CONSTANT	44.161	1.550	28.495	0.000
TEMP	-4.635	0.949	-4.884	0.005
PH	-0.015	0.949	-0.015	0.988
TIME	-2.127	0.949	-2.241	0.075
TEMP*TEMP	-9.237	1.397	-6.612	0.001
PH*PH	-4.336	1.397	-3.104	0.027
TIME*TIME	-6.767	1.397	-4.844	0.005
TEMP*PH	-1.003	1.342	-0.747	0.489
PH*TIME	1.126	1.342	0.839	0.440
TEMP*TIME	1.137	1.342	0.847	0.436

| Analysis of Variance					
Source	df	Type I SS	Mean Squares	F-ratio	p-value
Regression	9	711.657	79.073	10.974	0.008
Linear	3	208.075	69.358	9.626	0.016
Quadratic	3	489.321	163.107	22.637	0.002
Interaction	3	14.261	4.754	0.660	0.611
Residual Error	5	36.027	7.205		
Total Error	14	747.684			
Lack of Fit Test					
Source	df	SS	Mean Squares	F-ratio	p-value
Lack of Fit	3	19.514	6.505	0.788	0.601
Pure Error	2	16.513	8.256		
Residual Error	5	36.027	7.205		
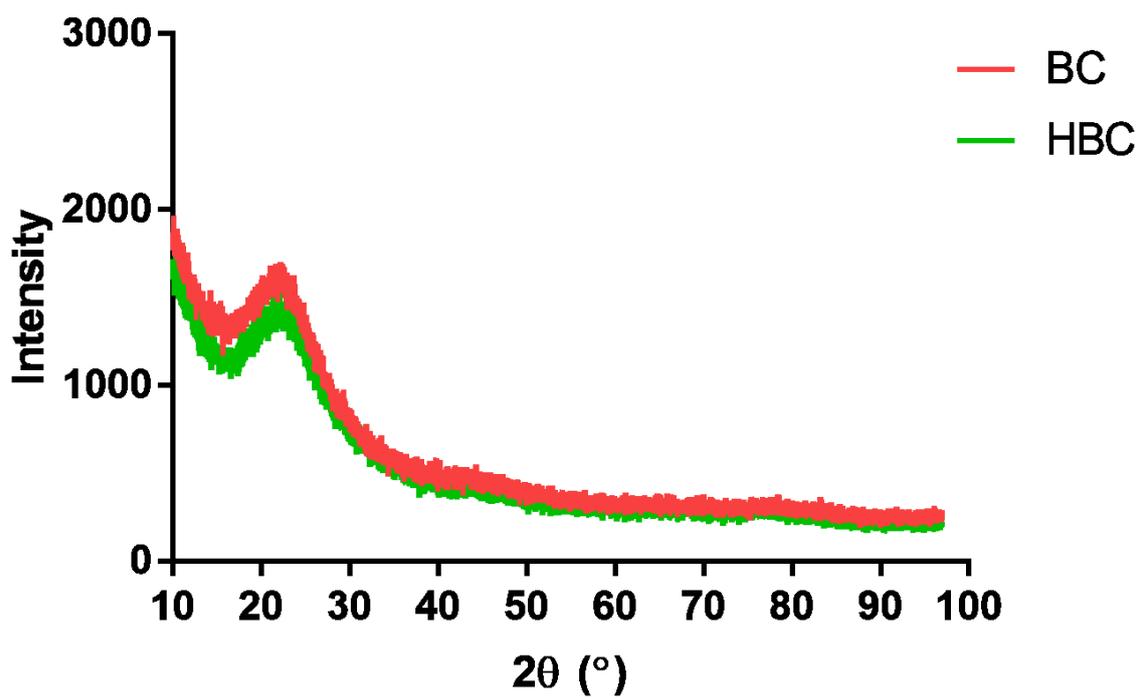
Figure S1. The XRD pattern of the hydrolyzed biochar and control biochar.

Chapter 4. Overexpression of a Thermostable GH11 Xylanase and Improving the Hydrolysis on Biomasses by Molecular Dynamic Simulation

Abstract:

GH 11 xylanase is one of the most well-studied enzymes among other xylanases. However, the application of xylanase in industrial applications was limited due to the low thermostability and was easily inhibited by various inhibitors. In this study, a thermostable GH 11 xylanase was cloned from *Bacillus sp.* P3 and its homologous protein structure was simulated by molecular dynamics. The flexible region of the protein structure was mutated and the wild-type and mutants were further characterized. The result suggested that mutant N159I was similar to the wild-type xylanase meanwhile had a higher substrate affinity and catalytic efficiency (Km= 5.84 mg*mL^{-1} and Kcat/Km = 19.58 mL*s^{-1}*mg^{-1}) compared to the wild-type (Km= 7.79 mg*mL^{-1} and Kcat/Km = 11.11 mL*s^{-1}*mg^{-1}). Besides, both wild-type and mutant N159I could remain at more than 80% of activity after incubating at 40 °C for 24 hours. Moreover, the mutant N159I could hydrolyze more reducing sugar from wheat straw (18.54 ± 0.43 mg/ g dried biomass) compared to the wild-type (12.34 ± 0.33 mg/ g dried biomass). Besides further exploring the potential of GH 11 xylanase in biomass hydrolysis for industrial application, this study also provided a further understanding of the structure-function relationship and showed the advance of computational technology in improving enzyme performance and biomass hydrolysis.

1. Introduction

For reducing the dependence on fossil fuel resources, an alternative and sustainable source for energy and chemical production has been one of the most popular research topics.

One of the promising futures is the transition from the fossil-based economy to the bio-economy (Kircher, 2012) and the usage of biofuel could reduce the emission of greenhouse gases and ozone layer depletion by up to 47% and 66%, respectively (Daylan and Ciliz, 2016). Biomass is considered a promising resource for producing second-generation biofuel and reducing the reliance on first-generation biofuel since the first-generation biofuel used sucrose and starch from foods and it could result in food-energy competition.

Lignocellulose is one of the major compositions in the plant biomass, which is mainly composed of cellulose (40-55%), hemicellulose (24-40%), and lignin (18-25%) which depended on the plant species (Howard et al., 2003b) and these components bound together via covalent and non-covalent interaction then forming a complicated 3D structure (Chio et al., 2019). Xylan is one of the dominant components in hemicellulose and it is a hetero-polysaccharide mainly composed of xylose which is linearly linked via β-1, 4 linkages. Xylan is highly available in nature environments since it is the second abundance polysaccharide in lignocellulose which could be regenerated through the carbon cycle, thus xylan, and its depolymerized product xylose could be considered a sustainable and renewable resource for industrial application (Walia et al., 2017).

Xylanase is generally referred to as the enzyme which was capable of breaking down the xylan (CE 3.2.1.X), especially breaking down the β-1, 4 linkages (EC 3.2.1.8). There are numerous research studies have shown that xylanase could be produced by various microorganisms including *Bacillus*, *Aspergillus*, *Streptomyces*, *Trichoderma*, plants, and even the rumen of higher animals (Collins et al., 2005; Hu et al., 2020; Javier et al., 2007). Xylanase can be classified into several glycoside hydrolase (GH) families including GH 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 52, and 62 (Bhardwaj et al., 2019b; Walia et al., 2017) and GH 10 and GH 11 are the most common and widely studied xylanase among other family members. Various studies have shown that xylanase has several potential and promising

industrial applications, including wood pulping process (Khonzue et al., 2011), biofuel production (Wang et al., 2019a), bio-bleaching (Azeri et al., 2010), sugar production (Kumar and Shukla, 2018), and animal feedstock additive (da Costa et al., 2019), etc.

One of the advantages of using enzymes and organisms for industrial applications is that their performances could be improved and modified using genetic engineering techniques. There are several tools could be used for improving the performance, for instance, $\Delta\Delta$ Gibbs free energy ($\Delta\Delta G$) and B-factor could be used for evaluating the stability of the enzyme after mutagenesis (Han et al., 2017a; Sun et al., 2019), modifying the substrate-binding site for the improving the enzymatic activity (Joo et al., 2011; Xiong et al., 2018), alignment with great performance enzyme and mutated specific residue, etc. Direct evolution is relatively straightforward. It only requires constructing a mutation library, screening an excessive amount of mutants, and identifying which mutants have better performance. Currently, there is some research combining 2 types of mutagenesis and using computational toolkit and machine-learning to perform the *in silico* direct evolution which could effectively reduce the experimental spending and enhance the effectiveness of the site-directed mutagenesis (Amrein et al., 2019; Cadet et al., 2018; Wu et al., 2019).

In this study, an endo-β-1, 4-xylanase gene (*xynA*) from a newly isolated *Bacillus* sp. P3 (Guo et al., 2017) was cloned and overexpressed in *Escherichia coli* BL21 (DE3). A homologous protein structure was constructed and its movement at high temperatures was simulated and analyzed using the molecular dynamics software GROMACS. Afterward, the highly flexible amino acids were selected for mutagenesis and the wild-type xylanase and its mutants were purified using the Ni^{2+}-NAT purification column, characterized, and discussed from the molecular aspects. Furthermore, the performance of the wild-type xylanase and best-preformed mutant on hydrolysis of various biomasses was further evaluated and discussed. The result from the mutagenesis and structural analysis could advance the

understanding of xylanase structure and provide more foundation for *in silico* direct evolution.

2. Materials and Methods

2.1 Microbial strains and reagents

Bacillus sp. P3 was previously isolated from forest soil (Thunder Bay, Ontario, Canada) and stored in our laboratory (Guo et al., 2017). The culture medium used for the growth of the *Bacillus* sp. and *E. coli* strains was Lenox Broth (LB) (Elbing and Brent, 2002). The purified beechwood xylan used as substrate was purchased from Cedarlane (Burlington, ON, Canada). *Pfu* DNA polymerase from Biobasic (Markham, ON, Canada) was used for polymerase chain reaction (PCR). The Plasmid Mini-Prep Kit and Isopropyl beta-D-1-thiogalactopyranoside (IPTG) used for plasmid extraction and inducing protein expression were purchased from Biobasic (Markham, ON, Canada). His-TALON Gravity Column Purification Kit used for enzyme purification was purchased from Clontech (Palo Alto, CA, USA). All other chemicals were purchased from Sigma or Thermo Fisher Scientific with analytic or ACS grade except specifically mentioned.

2.2 Construction of the *xynA* and its mutants' expression plasmid and the analysis of homologous protein structure

The genomic DNA of the Bacillus sp. P3 was extracted according to a freeze-thawing method (Chen et al., 2020) and the extracted genomic DNA was used as the template for PCR amplification. The *xynA* gene from *Bacillus subtilis* 168 (Accession ID: NC_000964) was selected as the template for designing primers for amplification and all the primers used in this study were listed in the supplementary material Table S1. The signal peptide cleavage site (Figure S1) was predicted by SignalP 5.0 (Almagro Armenteros et al., 2019) and the

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mutated *xynA* genes were obtained by overlap extension PCR (Urban et al., 1997). The PCR-amplified wild-type and mutated *xynA* genes were digested by NdeI and XhoI and ligated into pET21-a by T4 DNA ligase (Markham, ON, Canada), and the constructed plasmids were sent to Eurofins Genomics for Sanger sequencing.

The sequences obtained from Sanger sequencing were used for constructing the homologous protein structure using SWISS-MODEL (Waterhouse et al., 2018), and the wild-type sequence was uploaded to The National Center for Biotechnology Information (NCBI) with accession number OR059086. The active site and function were predicted and analyzed using I-TASSER (Roy et al., 2010) and PROSITE (Sigrist et al., 2012). The mutant's stability and the change of the change in Gibbs free energy ($\Delta\Delta G^{stability}$) were calculated using DynaMut2 (Rodrigues et al., 2018), and the protein structures were aligned and visualized using PyMOL open source (DeLano, 2002).

2.3 Molecular dynamic (MD) simulation by GROMACS and ligand-docking simulation by AutoDock4

The MD and structural changes of xylanase in NaCl solution at 80 °C were simulated using GROMACS ver. 5.1.1 (Han et al., 2017b; Lemkul, 2019). In short, the obtained homologous protein structures were solvated with the spc216 water model in a 0.8 nm octahedral box with the Amber ff99SB force field. Sodium and chloride were added with a concentration of 100 mM for neutralizing the system. After the energy minimization (< 1000 kJ/mol/nm) and system equilibration (5 ns in NPT and NVT, respectively), the protein was simulated at 80 °C with 1 bar pressure for 50 ns (2 fs/step). After the simulation, the root-mean-square deviation (RMSD), and root-mean-square fluctuation (RMSF) were analyzed using the built-in function of GROMACS.

The binding affinity and docking properties between the ligand and xylanase were

simulated and analyzed by AutoDock4.2 (Morris et al., 2009). In short, the structure of xylanase was set as a rigid molecule, and xylan composed of 2 xylose molecules was set as a flexible ligand molecule. The enzyme structure and ligand molecule were energy minimized by GROMACS and Chem3D (PerkinElmer Informatics, USA) with a minimum RMS gradient of 0.01, respectively. The maximum evaluation was set as "Long" with 2500000 steps and the Lamarckian genetic algorithm was used for the ligand-protein docking

2.4 Heterologous expression, harvesting, and purification of *xynA* in *E. coli* BL21 (DE3)

The expression plasmid *xynA*-pET21a was transformed into *E. coli* BL21 (DE3) via heat shock at 42 °C for 90 sec. The positive clones were selected using an LB agar plate containing 100 μg/mL of ampicillin (Amp). The positive clones were picked up from the plate and inoculated into 5 mL LB medium in a test tube and shaken at 37°C with the rotation speed at 250 rpm overnight. The overnight cultured *E. coli* BL21 (DE3) was inoculated into a fresh 50 mL LB medium containing 100 μg/mL Amp at the ratio 1:100. IPTG was added into the medium at the final concentration was 0.5 mM when the OD_{600} of the medium reached 0.6. After adding the IPTG, the *E. coli* BL21 (DE3) was cultured at 25 °C with a rotation speed of 250 rpm.

After 24 h of incubation, the cells were collected by centrifugation at 12, 000 g for 15 min. The cell pellets were re-suspended in the 5 mL of 50 mM potassium phosphate buffer (pH 7) on ice and ultrasonicated at a resonance that operated 10 s intervals of sonicating and cooling. The soluble protein was obtained by centrifugation at 12, 000 g for 10 min, and the supernatant was kept at 4°C until purification. The expressed soluble xylanase was purified using the His-TALON Gravity Column Purification Kit according to the manufactory's manual and the eluted protein was further desalted by the PD-10 Desalting column. The purified enzyme was stored at 4°C and further used for activity assay and downstream

process. The concentration of the purified enzyme was measured according to Bradford's reagent (Bradford, 1976) using bovine serum albumin as the standard curve.

2.5 Characterization of the wild-type and mutated xylanase, their thermostability, and catalytic kinetics

The xylanase activity was measured under various reaction conditions using beechwood xylan as substrate and referred to our lab method (Wang et al., 2019a). Briefly, 10 µL of the diluted purified enzyme was mixed with the 20 µL of 1% substrate solution in each well of the 96-well microplate. The microplate would be placed into the water bath at the desired temperature for 10 min, then immediately cool down and add 60 µL of the 3, 5-dinitrosalicylic acid reagent (DNS reagent, prepared according to (Miller, 1959a)) and heat in the boiling water for 5 min. The absorbance at 540 nm (A_{540}) of the reaction mixture was measured using an Epoch Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA), and the amount of released reducing sugar was calculated by the standard curve using xylose as standard. The enzyme activity was expressed in International Units (IU), as the amount of enzyme required for releasing 1 µmol of xylose in 1 min.

The effect of pH on the recombinant xylanase activity was measured in various buffers (pH 3 to 10) at 60°C and the optimal reaction temperature was determined in 50 mM pH 5 citrate buffer at a range of temperatures (30 °C to 80 °C). Furthermore, various chloride salts (dissolved in 50 mM Tris-HCl pH 7 buffer and with) were added to the reaction with a final concentration of 2.5, 5, and 10 mM for investigating the effect of metal ions on the xylanase activity. The thermostability was evaluated by incubating the xylanase at 40 °C for 24 hours and the activity was measured under the optimal reaction condition. Various concentrations of xylan were used for the determination of the Km and v_{max} of the wild-type and mutated xylanase under optimal reaction conditions.

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2.6 Biomass hydrolysis by wild-type and mutated xylanase

Wheat straw and oat straw obtained from local farms were used as substrates for evaluating the xylanase biomass hydrolysis ability. The biomass hydrolysis test was referred to Wang et al. (2019a) with minor modifications. In short, the biomasses with sizes between 20 to 40 meshes were washed with 50 ℃ hot water twice and then 90% ethanol once for washing out the background sugars and potential inhibitors. The washed biomasses were dried in a chemical fume hood for around 2 days until the weight was constant. The overexpressed xylanase was mixed with the dried biomasses in a centrifuge tube at the ratio of 300 U/g biomass and the tube was incubated at a 40 ℃ incubator with a rotation speed of 250 rpm for 24 hours. The supernatant was collected and centrifuged at 12, 000 g for 5 min to obtain a particle-free solution and the released reducing sugar in the solution was determined by the DNS method as mentioned above.

2.7 Statistic analysis and data processing

All the experiments were conducted with at least triplicated and the data was presented as mean ± SD. The one-way ANOVA and Tukey's multiple comparisons test in GraphPad Prism 7.0 were used for the significant test and comparative analysis. The group with a p-value less than 0.05 was considered a statistically significant difference.

3. Result and Discussion

3.1 The sequence and homologous protein structure of *Bacillus* sp. P3 *xynA* gene and its mutagenesis

The *xynA* gene from this *Bacillus* sp. P3 contained an open reading frame of 639 bp which encoded 213 amino acids with a theoretical molecular weight of 23.24 kDa and

theoretical isoelectric point of 9.42. The prediction indicated that the 28th amino acid would be the signal peptide cleavage site (Figure S1) and thus the remaining 185 amino acids were used for overexpression with a theoretical molecular weight of 20.22 kDa and theoretical isoelectric point of 9.03. The amino acid sequence was aligned with other reported xylanase amino acid sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST). The result suggested that the *xynA* gene encoded a highly identical (99%) xylanase compared to other reported *Bacillus* xylanase such as *Bacillus velezensis* FZB42 (accession number WP251257227), *Bacillus amyloliquefaciens* (accession number WP109567268), *Bacillus subtilis* (accession number AGO02727), etc. Based on the wild-type *xynA* gene sequence, a homologous protein structure was constructed using xylanase (SMTL ID: 1xnb.1) as the template with 95.14% sequence identity and GMQE score of 0.99 which indicated that the constructed homologous protein structure would be relatively reliable. The analysis from PROSITE and I-TASSER suggested that the expressed xylanase would belong to GH 11 family and the catalytic domain (with active site E78 and E172) was located in the palm and finger of the structure (Törrönen et al., 1994).
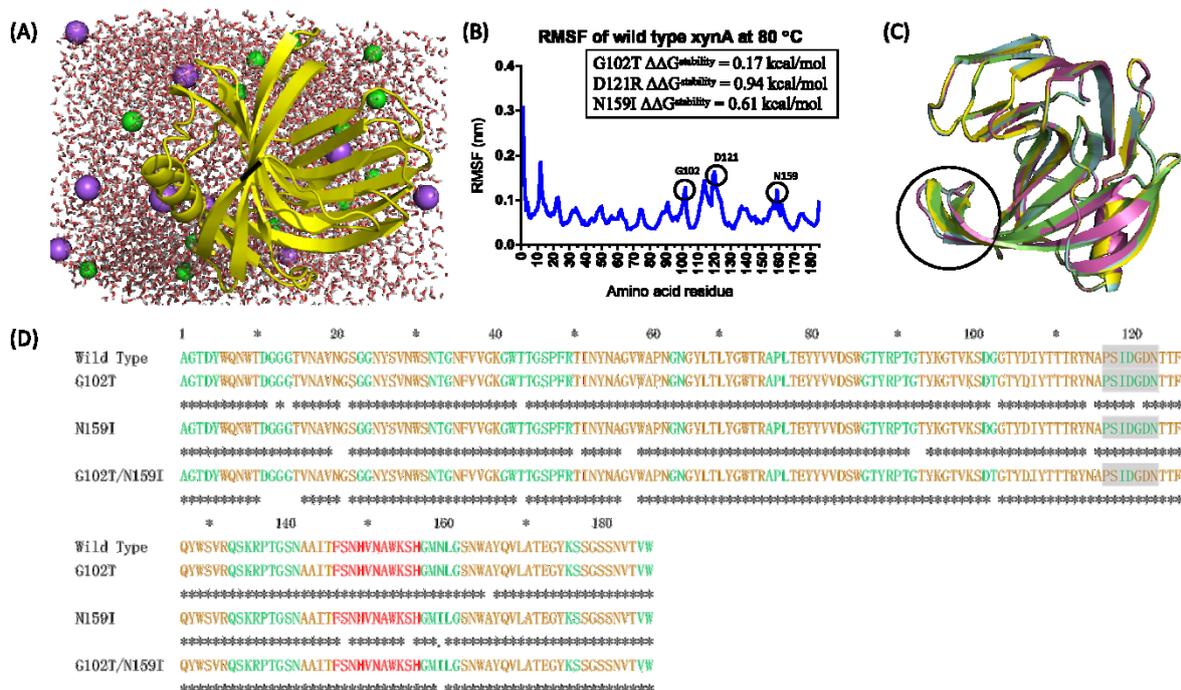
Figure 1. Bioinformatic analysis of expressed protein. (A) The homologous protein structure energy was minimized in 100 mM NaCl solution. The purple ball was Na+ and the green ball was Cl-. (B) The RMSF analysis of the dynamic simulation result and the most stable mutation of the potential residue based on the ΔΔGstability. (C) Overall of the structure overlaps wild type (yellow), G102T (pink), N159I (cyan), and G102T/N159I (green), and the cycle indicated the thumb region. (D) The structure alignment of 4 xylanases. All the structures were aligned to the wild-type structure and the "*" indicated identical and "." indicated a similar position.

The constructed protein structure was input into GROMACS and the structural changes in 100 mM NaCl solution at 80 °C were simulated (Figure 1A). The RMSF analysis indicated that amino acids G102, D121, and N159 had the highest individual residue flexibility (Figure 1B) and thus were chosen for potential residues for site mutagenesis. These residues were *in silico* screened by replacing them with various amino acids and the mutants were evaluated based on the changes in the stability ($\Delta\Delta G^{stability}$). The result indicated that the mutant G102T, D121R, and N159I would provide the highest stability in each residue with $\Delta\Delta G^{stability}$ 0.17 kcal/mol, 0.94 kcal/mol, and 0.61 kcal/mol, respectively (Figure 1B and Table S1). Therefore, mutant G102T, D121R, and N159I were constructed and overexpressed in *E. coli* BL21. Since mutant D121R had lost most of the activity (data not shown), only mutant G102T, N159I, and G102T/N159I were further characterized and analyzed.

The secondary structures of energy-minimized homologous mutants' structure were compared to the wild-type structure and the overall secondary structures were similar except for the mutant G102 and N159I which did not form the β-sheet structure at the thumb-shape loop (Figure 1C). Furthermore, besides the secondary structure, the structure of the mutants was aligned with the wild-type structure and the result indicated that even though most of the

secondary structures were similar, the exact distance and location of the amino acid could be various and thusly result in different enzymatic properties (Figure 1D).

The homologous protein structure of the expressed xylanase was shown as a typical "right-hand" shape which contains a highly conserved β-jellyroll fold structure and it could be further divided as thumb, finger, and palm regions which were playing different roles in xylanase activity. Studies indicated that the dynamic of the thumb region would significantly affect the catalytic efficiency of the xylanase (Mhlongo et al., 2015; Vieira and Ward, 2012) and Marneth et al. (2021) indicated that the flexibility of the thumb region would also transit the reaction of xylanase from hydrolysis to transglycosylation. Therefore, the significant changes in the thumb region could result in distinguished enzymatic properties among mutants.

As mentioned above, there were several strategies for improving the enzymatic performance and one of the most common approaches was modifying the enzyme flexibility using site mutagenesis. There were several common indexes could be used for evaluating the flexibility or stability of the enzymes such as RMSF (Li et al., 2022), B-factor (Sun et al., 2019), Gibbs free energy ΔΔG (Rajabi et al., 2022; Yin et al., 2007), etc. Lai et al. (2021) indicated that modifying the flexibility in the finger and palm region of the xylanase structure could result in enhancing the catalytic efficiency and thermostability. Moreover, Liu et al. (2022a) indicated that the flexibility of the thumb and finger region would also significantly affect the binding between the xylanase and its substrates and inhibitors.

3.2 Effect of the reaction temperatures, pHs, and metal ions on the wild-type and mutated xylanases activity

The activities of wild-type and mutated xylanases were measured under different reaction temperatures (Figure 2A) and various pH (Figure 2B). All the expressed xylanase

had the optimal reaction temperature at 60 °C and most of the xylanase had remained at least 50% of activity under all the tested temperatures except mutant G102T. The ability in adapting to a broad range of reaction temperatures implied that these xylanases could be adapted for multiple industrial applications. Furthermore, mutant N159I had a higher relative activity compared to the wild-type under high reaction temperatures (70 °C and 80 °C) which implied that mutant N159I might have a higher thermostability compared to the wild-type xylanase. All the xylanases had an optimal reaction pH of 5 except mutant G102T which had optimal pH at 7.   Moreover, even though the wild-type xylanase had a higher tolerance at different pH compared to the mutants, wild-type xylanase, mutant N159I, and G102T/N159I could remain active (remaining more than 50% activity) under a board range of pH (3 to 11). Similar to a board range of reaction temperature, the board range of reaction pH also further expanded the potential applications of the expressed xylanase such as improving feedstock digestibility and pulp bleaching (Kallel et al., 2016; Taneja et al., 2002).
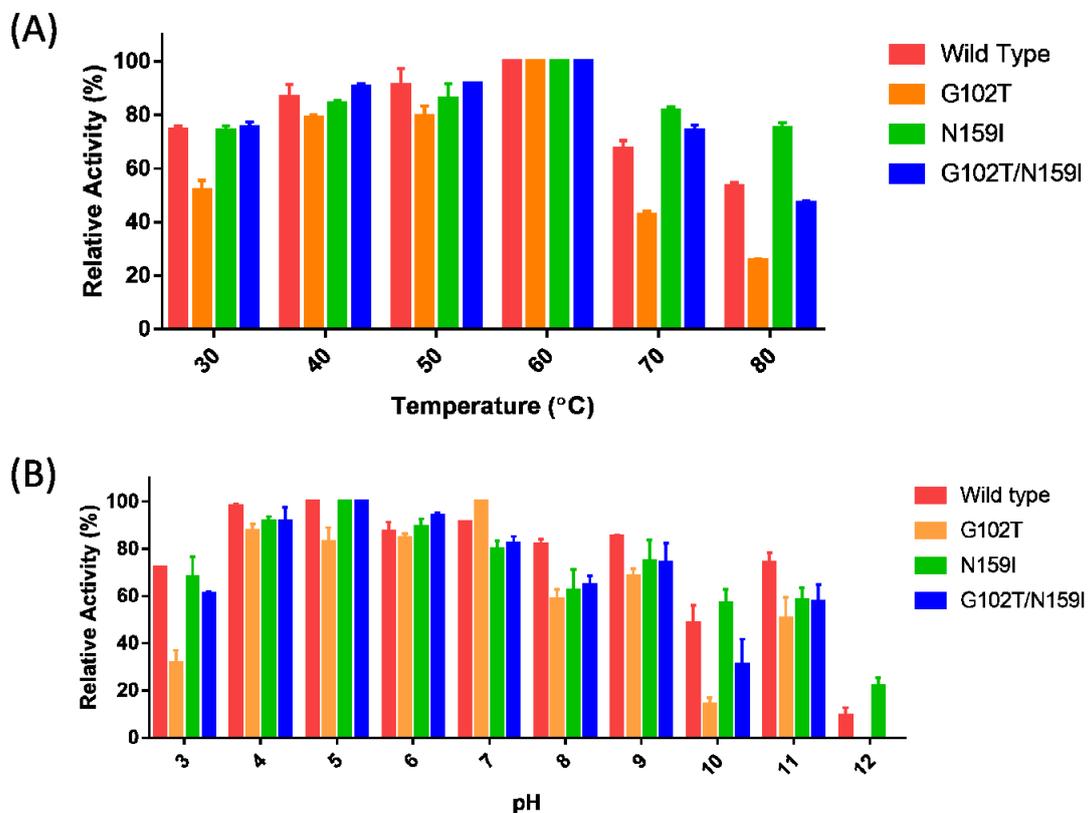
Figure 2. (A) The effect of reaction temperature on the expressed xylanase activity. (B) The effect of reaction pH on the expressed xylanase activity.

The expressed wild-type and mutated xylanase had a similar optimal reaction condition as other GH 11 xylanase from other *Bacillus* species. Cao et al. (2021) and Paës et al. (2012) summarized various studies regarding GH 11 xylanase and these studies indicated that GH 11 would have an optimal reaction pH ranging from 5 to 8 and optimal reaction temperature ranging from 40 °C to 60 °C. However, compared to other studies, the expressed xylanase in this study had a better potential for industrial application due to its higher ability in adapting to various reaction conditions. Takita et al. (2019) expressed a GH 11 xylanase from *Bacillus sp*. 41 M-1 and it only remained high activity within 50 °C to 65 °C and pH 5 to 10. A study from Wang et al. (2023) also expressed and characterized a GH 11 xylanase from *Bacillus amyloliquefaciens* with high activity (> 50% of highest activity) temperature ranging from 40 °C to 55 °C and pH ranging from 6 to 9. The expressed GH 11 xylanase in the study from Li et al. (2018a) also only remain its high activity within the temperature ranging from 50 °C to 70 °C and pH ranging from 5 to 9. Therefore, even though the expressed xylanase had similar optimal reaction conditions as other reported GH 11 xylanase, the expressed wild-type xylanase and mutant N159I were able to remain a relatively high activity in a boarder range of reaction temperature (30 °C to 80 °C) and pH (3 to 11).

Besides reaction temperature and pH, the effect of the various metal ions with different concentrations (2.5, 5, 10 mM) on wild-type and mutant activity was investigated (Figure 3). Among all the ions, Cd^{2+}, Co^{2+}, Cr^{3+}, and Fe^{3+} had significant effects on the activity of both wild-type and mutant ($p<0.05$). In specific, Cd^{2+}, Co^{2+}, and Cr^{3+} inhibited all the expressed xylanase activity and the inhibition was stronger along with increasing the ions concentration. Moreover, a low concentration (2.5 mM) of Fe^{3+} significantly enhanced the expressed

xylanase activity but the improvement turned into inhibition when the Fe^{3+} concentration increased. Interestingly, Ni^{2+} significantly enhanced the activity of mutant G102T and G102T/N159I while Zn^{2+} significantly inhibited the activity of both mutants but had no significant effect on the wild-type and mutant N159I. Other tested ions did not have a significant effect on enzyme activity or at least remain at 80% of the control (without ions) condition.
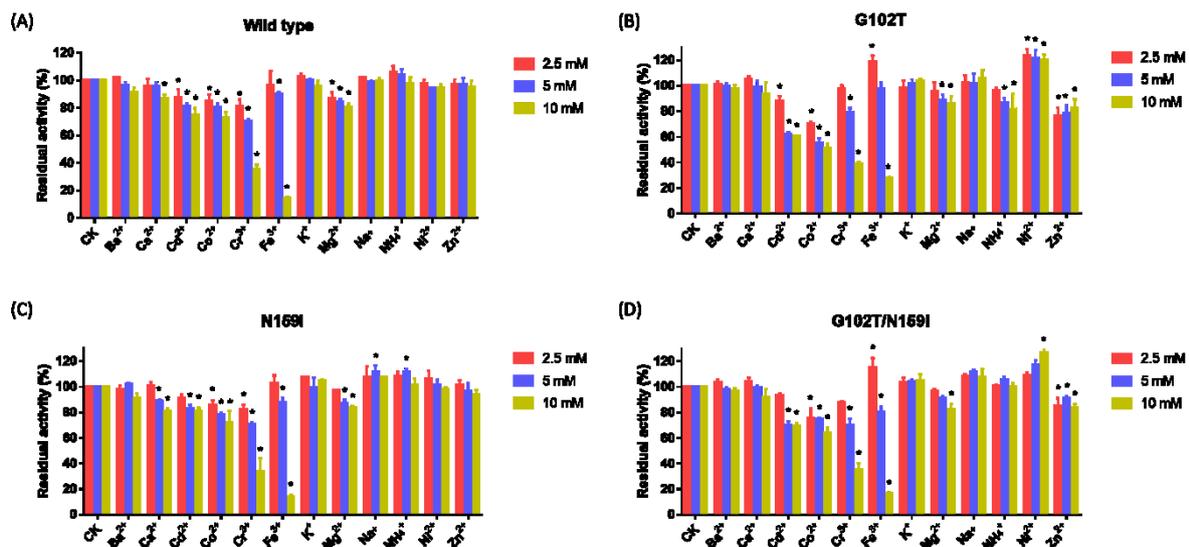
Figure 3. The effect of various metal ions (as chloride salt) on expressed xylanase (A) wild type, (B) G102T, (C) N159I), and (D) G102T/N159I. The "*" indicated that it was significantly different compared to the control which was without any metal ions (p<0.05).

The interaction between the metal ions and enzymatic activity was complicated since metal ions could not only directly affect the conformation of the protein structure (Hou et al., 2023), but also affect the properties of the substrate and result in affecting the formation of the protein-substrate complex (Marasinghe et al., 2021). Therefore, due to the complexity and diversity of the enzyme structure, there were few absolute conclusions on the relationship between ions and GH 11 xylanase activity. However, de Cassia Pereira et al. (2017) summarized some of the ions which generally inhibited xylanase activity. The inhibition of

Cd^{2+} and Co^{2+} was generally observed in various xylanases, including GH 10, 11, and arabinofuranosidases (Amel et al., 2016; de Cassia Pereira et al., 2017; Khasin et al., 1993; Seemakram et al., 2020).

Interestingly, even though Zn^{2+} is also considered a general inhibitor for xylanase activity (de Cassia Pereira et al., 2017; Marasinghe et al., 2021; Ratanakhanokchai et al., 1999), it did not significantly inhibit the activity of wild-type and mutant N159I. Based on the metal ion binding analysis shown in Figure S2 (Lu et al., 2022), the thumb region of the expressed xylanase would be the highest potential region binding with Zn^{2+} and mutant G102T and G102/N159I would have a higher binding potential compared to wild-type and mutant N159I. As mentioned above, since the flexibility and movement of the thumb region would significantly affect the catalytic efficiency of the GH 11 xylanase, the binding with Zn^{2+} in that region would also significantly affect the enzyme properties.

In contrast, Ni^{2+} improved the activity of mutant G102T and G102/N159I and also had no significant effect on wild-type and mutant N159I. Ni^{2+} was reported that it could inhibit or have no effect on the activity (Adiguzel et al., 2019; Bai et al., 2012; Vikramathithan et al., 2012; Wu et al., 2006), but relatively few reports indicated that Ni^{2+} could enhance GH 11 xylanase activity (Chithra and Muralikrishna, 2008; Rajagopalan et al., 2013). Interestingly, Heinen et al. (2018) characterized xylanase from *Aspergillus tamarii* Kita and its xylanase had a similar profile on metal ions affection compared to mutant G102T. This could be further investigated to understand the mechanism of Ni^{2+} in inhibiting and improving catalytic efficiency.

3.3 Characterization of thermostability and the catalytic kinetics of the wild-type and mutated xylanases
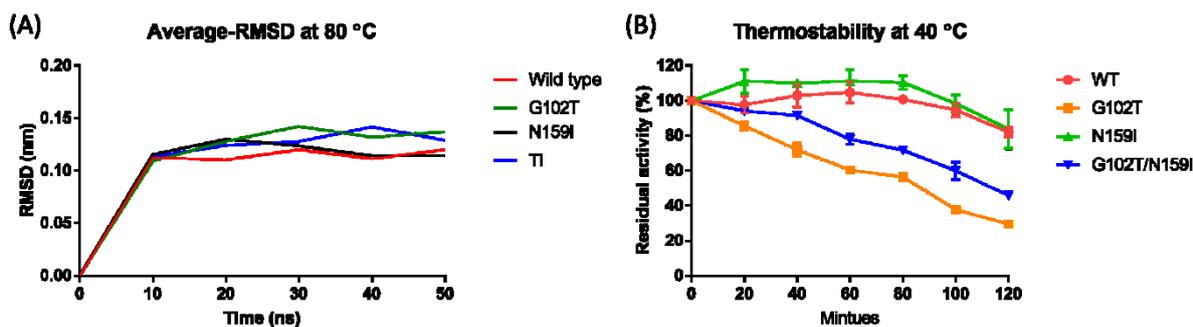
Figure 4. (A) The RMSD of the molecular dynamics simulation and the result was presented as average every 10 ns. (B) The thermostability of the expressed xylanases at 40 ℃.

The RMSD and thermostability of the expressed xylanases at 40 ℃ were evaluated and analyzed (Figure 4). The RMSD (Figure 4A) indicated that wild-type (0.96 Å on average) and mutant N159I (0.99 Å on average) would have lower flexibility, or higher stability in other words, compared to mutant G102T (1.08 Å on average) and G102/N159I (1.06 Å in average) at 80 ℃ simulations. The experimental result also supported the simulation outcome. The wild-type and mutant N159I remained more than 80% of the activity after incubating at 120 mins at 40 ℃ but only 29.55 % and 45.83 % remained in mutant G102T and G102/N159I (Figure 4B), respectively. Moreover, the thermostability of wild-type and mutant N159I had no significant difference most of the time (p<0.05) which was corresponding to the result from the RMSD analysis.

As mentioned above, RMSD was one of the common indexes for evaluating protein flexibility and studies had indicated that a lower RMSD value would refer to a more rigid protein movement, and result in better thermostability (Miao et al., 2022; Ning et al., 2018; You et al., 2019). Therefore, the advance in molecular dynamics would provide a deeper and more dynamic understanding of the changes in protein conformation and protein movement. Thermostability is one of the critical factors affecting the application of enzymes and one of the important applications of xylanase is hydrolyzing lignocellulose for the pre-treatment of

biogases and bioethanol production. Since the fermentation usually would be mild temperature (30 °C to 40 °C), the evaluation of thermostability and biomass-hydrolysis ability of the expressed xylanase was conducted under 40 °C (Feng et al., 2021; Prasertsan et al., 2017).

The wild-type xylanase and mutant N159I had remained at more than 80% of activity after incubating at 40 °C for 120 mins which had shown a better thermostability than other reported GH 11 xylanase. The study from Xiong et al. (2019) indicated that the xylanase from *Penicillium janthinellum* MA21601 only remained at around 20% activity after incubating at 45 °C for 30 mins. de Amo et al. (2019) had shown that xylanase from *Myceliophthora heterothallica* F.2.1.4 remained at 50% activity after incubating at 40°C for 60 mins. Xylanase from *Bacillus sp.* was characterized by Safitri et al. (2021) and the result indicated that the xylanase would remain around 60% activity after incubating at 40°C for 120 mins. Therefore, the expressed wild-type xylanase and mutant N159I could be appropriate for biomass hydrolysis pretreatment based on thermostability.
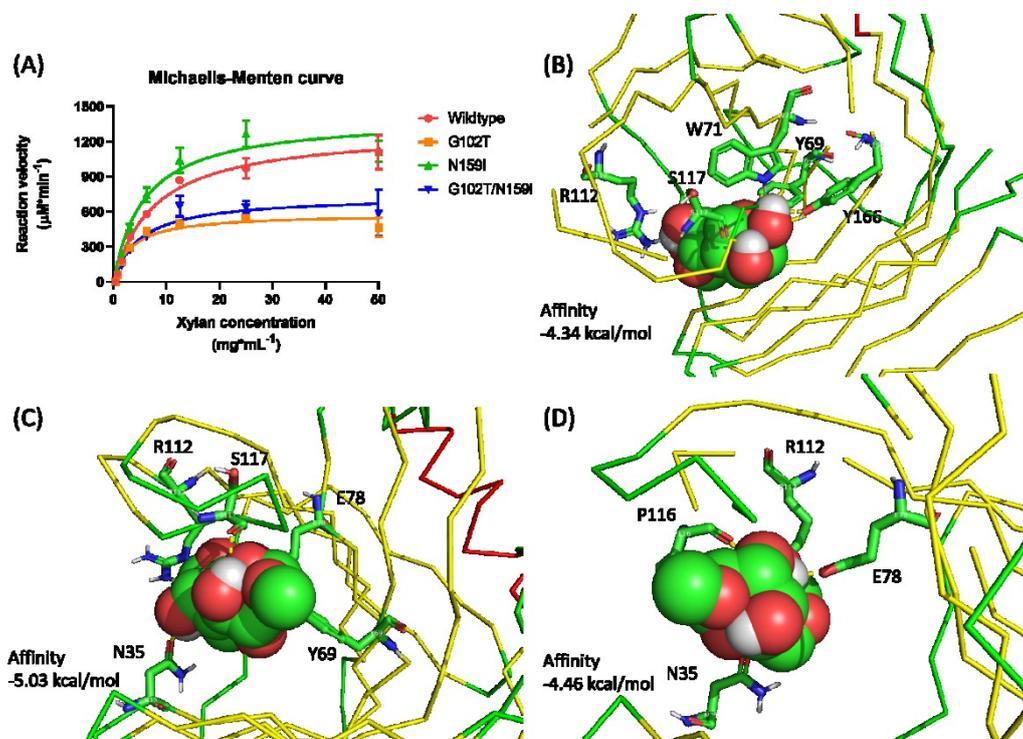

Figure 5. (A) The catalytic kinetics of the expressed xylanases based on the

Michaelis-Menten curve and the ligand-docking model of the expressed xylanase (B) Wild

type, (C) G102T, and (D) N159I.

Besides the thermostability, the catalytic kinetics of the expressed xylanase were also

determined (Figure 5 and Table 1). Moreover, the docking between the substrate and protein

structure was also analyzed from a molecular aspect.   Overall, mutant N159I was a better

version of the wild-type with a higher catalytic efficiency and a lower Km (Km= 5.84

mg*mL^{-1} and Kcat/Km = 19.58 mL*s^{-1}*mg^{-1}) compared to wild-type (Km= 7.79 mg*mL^{-1}

and Kcat/Km = 11.11 mL*s^{-1}*mg^{-1}). Moreover, the docking simulation also supported the

experimental result by showing that the mutant N159I would have a stronger substrate

affinity compared to the wild-type (Figure 5B, D). Interestingly, mutant G102T would have

the lowest Km which implied that it would have the highest affinity between the enzyme and

substrate. Furthermore, the docking analysis also suggested the same result that mutant

G102T would have the strongest affinity between the enzyme and substrate among all the

expressed xylanase (Figure 5C). However, the v_{max} of G102T was much lower compared to

the wild-type and other mutants. Therefore, overall speaking, mutant N159I could be a better

candidate for biomass hydrolysis compared to the wild-type.

Table 1. The catalytic kinetics of the expressed xylanase and predicted substrate affinity

based on the docking result.

Xylanase	Activity$_{max}$ (μmol*min^{-1} mg^{-1})	v_{max} (μM*min^{-1})	Km (mg*mL^{-1})	Affinity$_{Predicted}$ (kcal*mol^{-1})	Kcat (s^{-1})	Kcat/Km (mL*s^{-1}*mg^{-1})
Wild-type	234.2	1305	7.79	-4.34	86.56	11.11
G102T	95.4	582	3.28	-5.03	34.65	10.56
N159I	330.6	1409	5.84	-4.46	114.36	19.58
G102T/ N159I	175	734	5.02	-4.62	55.81	11.12

Enhancing the substrate binding affinity was also one of the strategies for improving

enzymatic hydrolysis (Li et al., 2018a; Yang et al., 2020). The docking models of the expressed xylanases were similar to other reported studies: The substrate would be located in the hydrophobic space within the palm and finger region and several amino acids such as tryptophan (W), tyrosine (Y), proline (P), asparagine (N), and arginine (R) would be involved in the binding with the xylan backbone (Pollet et al., 2009; Saleem et al., 2021; Vardakou et al., 2008). Moreover, the sugar ring in the substrate would be covered and stacked with the aromatic or cyclic amino acids with hydrophobic interaction and these amino acids would be involved in the substrate specificity and recognization (Fujimoto et al., 2021; Paës et al., 2012; Yang et al., 2020; Zhang et al., 2015b). The molecular dynamic analysis and docking simulation would provide further information on the conformation changes and substrate binding process.

3.4 The performances of wild-type and mutant N159I in biomass hydrolysis

After the characterization of the wild-type xylanase and its mutants, wild-type and mutant N159I were used for hydrolysis agricultural wastes, wheat straw and oat straw, and results were listed in Table 2. The result suggested that after pre-washing, no reducing sugar would be released after 24 hours of incubating at 40 °C when no xylanase was added into the buffer. When the same unit of xylanase was added into the buffer, both wild-type and mutant N159I could hydrolyze a similar amount of reducing sugar from oat straw (37.75 ± 0.82 mg/g biomass and 38.51 ± 0.73 mg/g biomass, respectively) with no significant difference ($p>0.05$). Interestingly, mutant N159I could significantly hydrolyze more reducing sugar from wheat straw (18.54 ± 0.43 mg/g biomass) compared to the wild-type xylanase (12.34 ± 0.33 mg/g biomass), even though yields were lower than hydrolyzed from oat straw.

Table 2. The yield of the reducing sugar after hydrolyzing wheat straw and oat straw at 40 °C for 24 hours. The "*" indicated a significant difference between the data ($p<0.05$).

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	Yield of reducing sugar (mg/ g dried biomass)		
	Control (no enzyme)	Wild type	N159I
Wheat straw	0	12.34 ± 0.33*	18.54 ± 0.43*
Oat straw	0	37.75 ± 0.82	38.51 ± 0.73

The differences between wheat straw and oat straw could be due to the presence of the wheat xylanases inhibitors such as *Triticum aestivum* xylanase inhibitors (TAXIs) and xylanase inhibitor proteins (XIPs) (Tundo et al., 2020). The study from Gebruers et al. (2010) indicated that the level of TAXIs and XIPs would be significantly higher in common wheat rather than in barley and oat and, thusly GH 11 xylanase would have much lower hydrolysis ability in wheat compared to oat. Payan et al. (2004) indicated that the XIP from wheat would target the thumb and palm region of the GH 11 xylanase by interacting with the curved β sheet which results in blocking the active site of the GH 11 xylanase. Therefore, the conformation changes in mutant N159I could affect the interaction between the mutant and various XIPs, even TAXIs, and result in a higher yield in wheat straw hydrolysis.

Mutagenesis for improving xylanase performance in biomass hydrolysis had been studied for decades. Song et al. (2012) have combined direct evolution and site-mutagenesis for improving the hydrolysis performance of GH 11 xylanase from *Thermobacillus xylanilyticus* in hydrolyzing wheat straw and the mutants with higher catalytic efficiency and tolerance to inhibitors had increased the yield of xylose from 16.7% to 18.6%. Damis et al. (2019). mutated a GH 11 xylanase from *Aspergillus fumigatus* RT-1 via direct evolution and the mutants with higher catalytic efficiency improved the releasing sugar from kenaf With the advanced computer science technology and understanding of protein structure-function relationship, more options would be available for further improving the performance of enzymatic hydrolysis via mutagenesis or synthesis biology, such as machine-learning

direct-design mutagenesis and *in silico* direct evolution (Ariaeenejad et al., 2022; Broom et al., 2020).

4. Conclusion

In this study, a GH 11 xylanase gene from *Bacillus sp.* P3 was amplified and overexpressed in *E. coli* BL21. Furthermore, the homologous protein structure of the expressed protein was simulated by molecular dynamics, and the flexibility of the structure was analyzed. The residues with high flexibility were mutated and the wild-type and mutants (G102T, N159I, and G102T/N159I) were characterized. The result suggested that the characteristic of mutant N159I was similar to the wild-type with a higher catalytic efficiency and stronger substrate affinity. Furthermore, the hydrolysis of wheat straw using mutant N159I would result in a higher yield of reducing sugar compared to the wild-type. This study provided another approach for designing mutagenesis and a step forward in the understanding of the structure-function relationship for advancing biomass hydrolysis.

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Figure S1. The signal peptide analysis of the cloned full-length *xynA*. The result indicated that the potential cleavage site would be A28.

Table S1. Primer used in this study. The underlined sequence was the cut site.

xynA_signal_F (NdeI)	AAATTT<u>CATATG</u>ATGTTTAAGTTTAAAAAGAATTTCTTAGTTGG
xynA_F (NdeI)	AAATTT<u>CATATG</u>GCCGGCACAGATTACTGGC
xynA_R (XhoI)	AAA<u>CTCGAG</u>CCACACTGTTACGTTAGAACTTC
G102T_F	TGTAAAGAGTGATACAGGTACATATGACA
G102T_R	TGTCATATGTACCTGTATCACTCTTTACA
D121R_F	CCATTGATGGCGATAACACTACG
D121R_R	CGTAGTGTTATCGCCATCAATGG
N159I_F	TCATGGAATGAATCTGGGCAGT
N159I_R	ACTGCCCAGATTCATTCCATGA

Table S2. The screening table of the potential stable mutants based on the $\Delta\Delta G^{stability}$

| Mutation	$\Delta\Delta G^{stability}$ (kcal/mol)	Mutation	$\Delta\Delta G^{stability}$ (kcal/mol)	Mutation	$\Delta\Delta G^{stability}$ (kcal/mol)
G102T	0.17	D121R	0.94	N159I	0.61
G102A	-0.01	D121Q	0.82	N159S	0.58
G102R	-0.06	D121T	0.75	N159M	0.54
G102P	-0.06	D121N	0.75	N159L	0.53
G102H	-0.11	D121S	0.73	N159G	0.49
G102S	-0.24	D121K	0.55	N159C	0.48
G102N	-0.27	D121H	0.52	N159T	0.46
G102Q	-0.28	D121P	0.34	N159R	0.43
G102E	-0.38	D121G	0.34	N159D	0.34
G102D	-0.39	D121C	0.34	N159E	0.33
G102K	-0.44	D121L	0.31	N159V	0.29
G102C	-0.52	D121I	0.24	N159K	0.15
G102F	-0.66	D121M	0.17	N159P	0.12
G102M	-0.71	D121Y	0.16	N159Q	-0.04
G102Y	-0.74	D121E	0.12	N159H	-0.14
G102W	-0.82	D121V	0.07	N159A	-0.21
G102L	-0.84	D121A	0.07	N159Y	-0.34
G102I	-0.89	D121F	-0.09	N159F	-0.46
G102V	-1.21	D121W	-0.1	N159W	-0.51
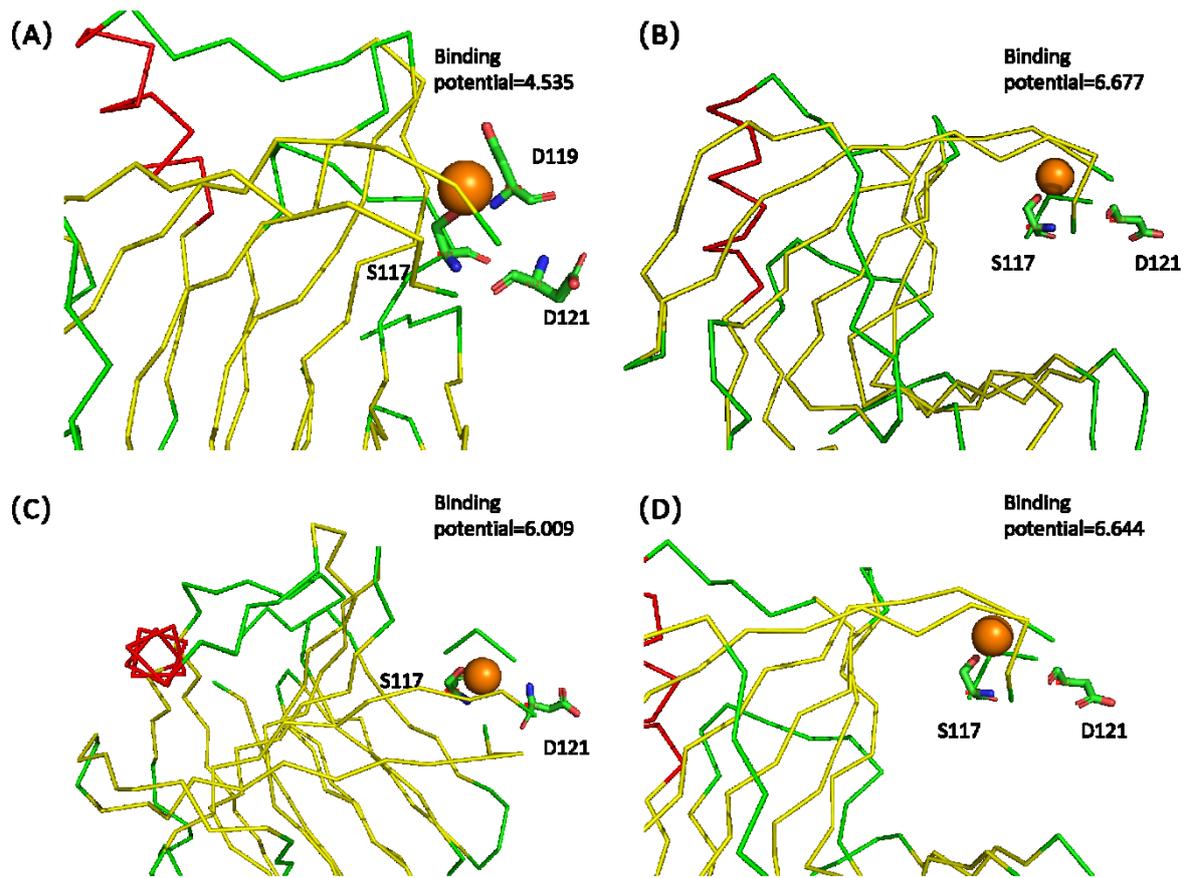
Figure S2. The potential binding site of Zn^{2+} (orange ball) in the expressed xylanases, (A) Wild type, (B) G102T, (C) N159I, (D) G102T/N159I.

Chapter 5. Conclusion and future recommendations

1. Conclusion

In this study, for advancing the utilization of agricultural waste biomass and *Bacillus velezensis*, a newly isolated *Bacillus velezensis* PhCL was characterized and its production of various useful biomolecules was discussed. The amylase production was optimized via response surface methodology and the optimal production of 35.626 ± 0.87 U/mL could be achieved at medium pH 8, temperature 40 °C, and fermentation time 72 hours. The statistical analysis also revealed that the medium pH was important for the amylase production using the starch medium. Moreover, various biomolecules were purified using sequential precipitation and solvent extraction. The result suggested that the yield and purity of biosurfactant from ammonium sulfate precipitation would be lower than acid precipitation, but the enzyme could be purified and recovered from the ammonium sulfate precipitation which could lower the production cost. Furthermore, the purified crude biosurfactant exhibited a better performance in the bioremediation of oil-contaminated soil compared to commercial surfactant, although it did not enhance the solubilization of polyaromatic hydrocarbon. The findings in this study could further step forward in the utilization of the useful bioproducts from *Bacillus velezensis* for environmental bioremediation and industrial application and development of an environmentally and economically sustainable bioproduction.

Moreover, various agricultural wastes were used as feedstock for producing the biomolecules from *B. velezensis* PhCL. After the screening of the biomass and optimizing the fermentation via response surface methodology, the optimal amylase production of 43.94 ± 0.51 U/mL could be achieved with a fermentation temperature of 33.5 °C, pH 8, and fermentation time of 68 hours with a medium composed of 35% rice husk, 0.375% $(NH_4)_2SO_4$, and 0.5% of peptone. Moreover, using rice husk as feedstock can significantly

enhance the antioxidant activity in the medium which could be due to the release of phenolic compound in rice husk during the fermentation. Moreover, the remaining rice husk in the fermentation broth was used for producing biochar. The biochar produced by the hydrolyzed rice husk showed faster adsorption compared to the control and it could be due to the hydrolysis of the microbe and thusly increase the purity of the cellulose which resulted in a more hydrophilic biochar. The finding in this study would provide another approach for producing useful and functional biomolecules from *Bacillus velezensis* using low-cost feedstock and the further utilization of the residue after the fermentation.

Finally, a GH 11 xylanase gene from *Bacillus sp.* P3 was overexpressed in *E. coli* BL21. To further improve its performance in hydrolysis, the constructed homologous protein structure was analyzed using molecular dynamics and the result suggested that residue G102, D121, and N159 had high flexibility which might account for the instability of the enzyme. Therefore, wild-type xylanase and mutant G102T, N159I, and G102T/N159I were overexpressed, purified, and characterized. The result suggested that mutant N159I and wild-type shared a similar characteristic profile, except N159I had a higher catalytic efficiency and stronger substrate affinity. G102T exhibited an interesting interaction with various metal ions. Moreover, mutant N159I hydrolyzed more reducing sugar from wheat straw compared to wild-type xylanase. This could be due to the presence of the xylanase inhibitor in wheat straw which could significantly inhibit the wild-type xylanase, meanwhile, the structural changes in the thumb region of mutant N159I could prevent the inhibitory effect. This study provided another approach for designing mutagenesis and a step forward in the understanding of the structure-function relationship for advancing biomass hydrolysis.

2. Future recommendations

There would be some future recommendations and ongoing side-projects regarding the

study:

(1) Using lignin as the carbon source for producing polyols and antimicrobial substance

As mentioned in Chapter 2, *Bacillus velezensis* PhCL was isolated due to the ability in utilizing lignin as the sole carbon source, however, the efficiency of utilizing lignin was much lower compared to other studies (Mei et al., 2020; Verma et al., 2020; Zhu et al., 2017). However, the absorbance at 280 nm (A_{280}) was increasing during the fermentation (Figure 1A). Even though a decrease of A_{280} generally referred to the degradation of lignin due to the degradation of phenolic compounds, Shin and Lee (1999) and Ulmer et al. (1983) indicated that the increase of A_{280} would be related to the degradation of the large lignin molecules to small molecular-weight molecules which resulted in the changes of the absorption coefficient of medium.
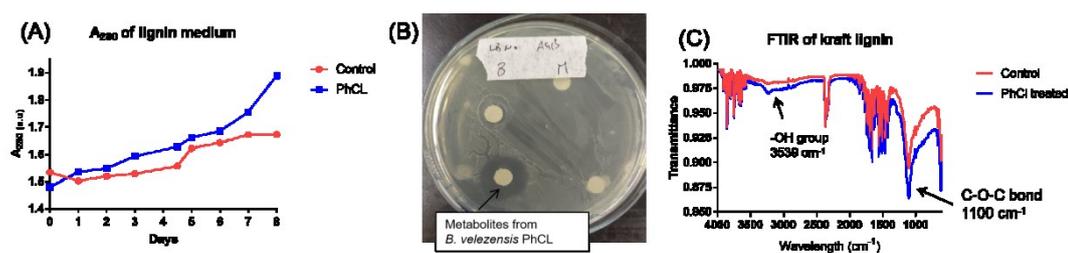

Figure 1. The changes of lignin after fermentation and the antimicrobial activity of the Bacillus velezensis PhCL. (A) The changes of A280 during the fermentation, (B) the antimicrobial activity of metabolites from Bacillus velezensis PhCL against Agrobacterium tumefaciens. (C) The changes of FTIR from different kraft lignin samples,

Moreover, the gel permeation chromatography (GPC) analysis (Table 1) suggested that the number average molecular weight (Mn) was increased from 1712 Da to 2020 Da, meanwhile, the weight average molecular weight (Mw) was increased from 4289 Da to 4396 Da, and thusly the polydispersity index (PDI) was decreased from 2.504 to 2.176 after the fermentation and it implied that the fermentation of *B. velezensis* PhCL could consume or

convert the low-molecular weight lignin molecules and unify the molecular size distribution of the lignin molecules in the medium which would be beneficial to use it as raw material for other polymer syntheses (Danaei et al., 2018).

Moreover, as mentioned in Chapter 1, the metabolites produced by *B. velezensis* would be able to inhibit the growth of several plant pathogens. Our results suggested that the metabolites from *B. velezensis* PhCL in the starch medium would significantly inhibit the growth of *Agrobacterium tumefaciens* (Figure 1B) which is a potential plant pathogen and the result was consistent with the result from Abdallah et al. (2018). Interestingly, based on the result from LC/MS/MS analysis, *B. velezensis* PhCL could produce a significantly higher amount of bacillaene, an antibiotic produced by *Bacillus* species, in the lignin medium compared to the glucose medium. However, the analysis of transcriptome and quantitative PCR had not finished yet, thus further confirmation would be necessary.

Table 1. The GPC result of the lignin molecules in after the B. velezensis fermentation.

Sample	Mw (Da)	Mn (Da)	PDI (Mw/Mn)
Control	4289	1712	2.504
B. velezensis treated	4396	2020	2.176

Furthermore, instead of using commercial pure lignin powder, black liquor from Resolute Forest Products, Thunder Bay was also used as the carbon source for the fermentation and the kraft lignin was extracted after the fermentation. The result from Fourier-transform infrared spectroscopy showed that the absorbance around 1100 cm^{-1} and 3539 cm^{-1} were increased after the fermentation (Figure 1C), which indicated that the amount of C-O-C (ether bond) and –Oh (hydroxyl group), respectively, was increased after the bacterial treatment.

Therefore, by combining the above findings, *B. velezensis* PhCL could be utilizing black

liquor, a waste from pulp and paper industries, for producing various metabolites and modified lignin molecules and these molecules could be separated using the purification strategy in Chapter 2. The low PDI lignin molecules with more ether bonds and hydroxyl groups could be used as the feedstock chemicals for the synthesis of polyurethane foam, another promising application of lignin molecules.

(2) Overexpression and characterization of a thermostable amylase and mutagenesis for improving its metal ions-tolerances

Since the amylase production was optimized in the study, the gene coding the amylase was cloned from the *B. velezensis* PhCL genome and overexpressed in *E. coli* BL21. The gene was sequenced and aligned with other sequences in the NCBI database. The gene sequenced and expressed was annotated as "starch-binding hydrolase" and "α-amylase". It contained a starch recognizing-domain similar to the carbohydrate-binding modules of GH 25 and GH 26, which was without the calcium-binding pocket (Boraston et al., 2006). Therefore, this could be the potential reason why the calcium ions would not enhance the amylase activity in Chapter 2.
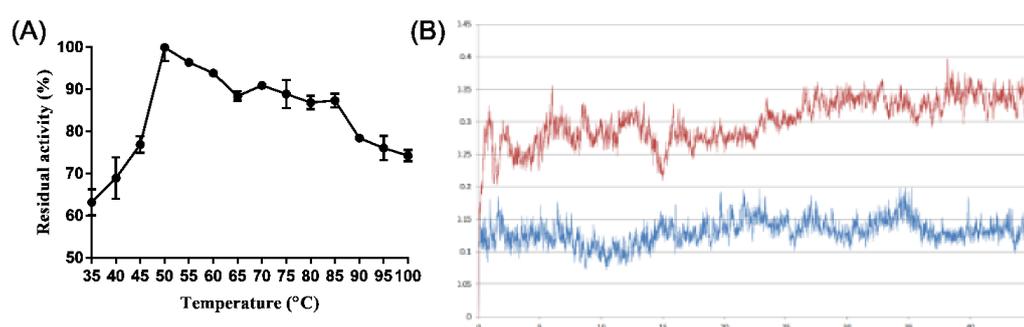

Figure 2. The optimal reaction temperature of the expressed amylase (A) and its RMSD analysis (B). The red line indicated that RMSD value of amylase with signal peptide and the blue line indicated the value of amylase without signal peptide.

Further characterization suggested that this amylase would have an optimal reaction temperature of 50 °C and it could remain at more than 70% of its optimal activity even if the reaction is at 100 °C (Figure 2A). Interestingly, the same coding DNA-sequence amylase was overexpressed by Zhang et al. (2021) but their result suggested that the optimal reaction temperature was 70 °C and it would drop to 56.2 % of optimal activity at 75 °C and decrease when increasing temperature. This could be due to the study from Zhang et al. (2021) expressing the protein with its signal peptide, but not in our study. Based on the molecular dynamic simulation, the existence of the signal peptide could significantly increase the RMSD, flexibility in other words, of the expressed enzyme which would significantly affect their stability at high temperatures (Figure 2B). Therefore, the effect of signal peptide and polyhistidine-tag on the protein catalytic efficiency and thermostability would be further investigated and discussed.

Table 2. The Michaelis-Menten kitnetics equation of the expressed amylase

Enzyme	Vmax (U/mg)	Km (mg/mL)
Control (no ions)	24.21	10.942
Cadmium chloride (5mM)	10.194	9.011

Furthermore, since the overexpressed amylase was significantly inhibited by cadmium ions, site-mutagenesis was performed to enhance the tolerance to cadmium ions. Based on the Michaelis-Menten kinetics equation, the Km of the amylase was not affected by the cadmium ions but the ions lower the v_{max} of the enzyme (Table 2). It implied that the cadmium could be inhibiting the amylase activity via non-competition inhibition. Based on the metal-ion binding prediction, mutant S254M and S256E was constructed for disrupting the cadmium binding pocket. However, the expression result indicated that there was a significant loss of activity in S254M, although the tolerance to cadmium ions was enhanced. The structure of

the mutant could be further analyzed to understand the reason for changes in the enzyme properties. However, from the aspect of improving the performance in industrial applications, direct evolution would be more suitable in the case of involving metal ions, since most of the simulations were not performing well in predicting ions-protein interaction, especially involved charges or pH changes in the solution (Aho et al., 2022).

Therefore, for improving the performance of the amylase, a combined strategy could be used: direct evolution was applied for enhancing the tolerance of cadmium ions and iodine screening was used for screening the mutant library (Bessler et al., 2003). After that, the catalytic efficiency or other drawbacks could be further improved by the site-mutagenesis based on the result from molecular simulation.

Expanding the point from this, advancing in the structure-function relationship could further improve the molecular dynamics simulation. One of the critical challenges of direct evolution is the screening methodology, how to quickly and relatively accurately find the positive result from a mutation library (Wang et al., 2021)? For example, the enzyme that could not be detected by color formation or did not have significant morphological changes was extremely difficult to quickly identify from a large number of colonies. One of the potential solutions was *in silico* direct evolution with the advancing bioinformatics technologies. AlphaFold had shown its potential in predicting protein structural (Jumper et al., 2021; Jumper et al., 2020), and machine learning and artificial intelligence were also applied for *in silico* screening, reactions that could not be easily observed or measured and now could be screened by computational calculation (Broom et al., 2020; Wittmann et al., 2021). Further understanding of the structure-function relationship could provide more fundamental factors and data for constructing the machine learning or deep learning model, which would eventually be beneficial to protein engineering.

(3) The genetic engineering of the *B. velezensis* PhCL for improving its ability in utilizing agricultural waste and its potential application in bioremediation

As mentioned in Chapter 3, the xylanase activity of *B. velezensis* PhCL was lower compared to other studies which might hinder its other potential applications. Therefore, for enhancing the production of lignocellulase, the insertion of heterogeneous lignocellulase would be one of the potential options. A recombination gene cassette was constructed. Mutated xylanase with higher catalytic efficiency (xylanase N159I in Chapter 4) was ligated with the selection marker, ampicillin-resistance gene (AmpR) since the *B. velezensis* PhCL was sensitive to ampicillin, and a xylose-inducible promoter P_{xyl}, identified based on the conserved region with other *Bacillus* species (Kim et al., 1996; Rygus et al., 1991) and amplified from the genome of the *B. velezensis* PhCL. The gene encoding catechol 2, 3-dioxygenase (*catE*) was selected as the recombination location since Fan et al. (2019) indicated that the knock-out of the *catE* would not result in a fatal effect on the growth and the gene encoding the starching-binding hydrolase mentioned above was also selected as the potential insertion location. It was expected that the insertion of the additional lignocellulase could enhance the hydrolysis of the agricultural biomasses and have a higher yield of other biomolecules as well. Furthermore, the highly active enzyme also could be used for lowering the production cost meanwhile the hydrolyzed biomass could have a higher specific surface area which could be further used for producing biochar or non-charred bioabsorbent. However,

Overall, *B. velezensis* PhCL would have a strong potential in various applications including enzyme production, bioremediation, etc. and the production cost could be further lowered using agricultural wastes biomasses as feedstock. Moreover, some potentials were hindered which needed further exploration and the exploration could result in an advancement in developing a sustainable and green economy.

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