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Article

Novel α-Amylase Inhibitor Hemi-pyocyanin Produced by Microbial Conversion of Chitinous Discards

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Abstract: α-amylase inhibitors (aAIs) have been proved efficient for the management of type 2 diabetes. This study aimed to search the potential aAIs produced by microbial fermentation. Among various bacterial strains, *Pseudomonas aeruginosa* TUN03 was found as a potential aAI - producing strain, and shrimp heads powder (SHP) was screened as the most suitable C/N source for fermentation. *P. aeruginosa* TUN03 exhibited the highest aAIs productivity (3100 U/mL) in the medium containing 1.5% SHP with the initial pH of 7-7.5, and fermentation was performed at 27.5 °C in 2 days. Further, aAIs compounds were investigated for scale-up production in a 14 L – bioreactor system, and the results highlighted high yield (4200 U/mL) in much shorter fermentation time (12 h) compared to fermentation in flasks. The bioactivity-guided purification resulted in the isolation of one major target compound. This active compound was identified as hemi-pyocyanin (HPC) via using gas chromatography-mass spectrometry and nuclear magnetic resonance, and its purity was analyzed by high-performance liquid chromatography. Notably, HPC demonstrated potent activity comparable to acarbose, a commercial antidiabetic drug; this is the first-ever report of aAI activity of HPC. The docking study indicated that HPC inhibits α-amylase via binding to amino acid Arg421 at the biding site on enzyme α-amylase with good binding energy (-9.3 kcal/mol) and creating two linkages of H-acceptor.

Keywords: α-amylase inhibitors; diabetes; hemi-pyocyanin; marine discards; microbial conversion

1. Introduction

The fishery processing industry has been producing increasingly large amounts of by-products, with the globally reported accessible waste of 27.85 million tons per year [1]. Among various fishery wastes, marine chitinous wastes (MCWs), including crab shells, squid pens, shrimp shells, and shrimp heads abundantly obtained as fishery by-products, have been extensively used for the extraction and bioproduction of many active compounds as a recently emerging trend [2]. MCWs have been utilized as carbon/nitrogen (C/N) sources for fermentation to produce various added valuable compounds such as enzymes, exopolysaccharide, chitosan oligomer, and *N*-acetyl-D-glucosamine [3,4], antioxidant, anti-NO, and anti-cancer compounds [5-7], biofertilizers [8], insecticidal materials [9], dye, and biosorbents [10]. MCWs have also been used for the extraction of astaxanthin, β-carotene esters, and essential amino acids [11-12]. Recently, MCWs were extensively used for the bioproduction of α-glucosidase inhibitors. In this work, MCWs

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were investigated for the production of α-amylase inhibitors (aAIs; anti-diabetic compounds) via microbial fermentation.

Diabetes mellitus is a global health issue that significantly reduces the quality of life and health of people [13]. Among the two major types of diabetes mellitus, type 2 diabetes (T2D) accounts for 90% of diabetic cases [14]. In recent years, natural carbohydrate digestive enzyme inhibitors (CDEIs), including α-glucosidase inhibitors and aAIs, have been proved as a potentially effective therapy for the management of T2D [15-18]. Though some commercial inhibitors such as acarbose, voglibose, and miglitol have been available, the use of these commercial drugs may cause some side effects; thus, new inhibitors from natural sources are needed [18].

CDEIs may be obtained from various natural sources and wildly isolated from medicinal plants [16,18,19]. However, it is difficult to produce a large scale of inhibitor compounds from herbal sources [20-22]. Microbial fermentation is another alternatively utilized approach to produce natural compounds, including CDEIs [2,3,5,17,20]. In our previous works, we reported the production and purification of α-glucosidase inhibitors from microbes [17,22] and extensively used MCWs as C/N sources for the production of α-glucosidase inhibitors via fermentation [7,24-29]. With our research direction of recycling organic wastes into value-added anti-diabetic compounds, we investigated the use of CDEIs for the cost-effective production of aAIs via microbial fermentation in this study. The production of aAIs was further scaled up in a 14 L - bioreactor system, and the major active compound was isolated and identified. Then, a docking study was performed to elucidate the interaction of the inhibitors toward the target enzyme.

2. Results and Discussion

2.1. Screening Active α-Amylase Inhibitors Producing Strain

More than 100 bacterial strains obtained from our previous studies were assessed by fermentation to produce aAIs. Of these, the supernatants of seven strains showed positive anti-α-amylase activity (Table 1), with the inhibition ranging from 47-89%. Three strains included *Bacillus cereus* RB.DS.05, *Pseudomonas aeruginosa* TUN03, and *B. atrophaeus* H10 demonstrated the highest inhibition values (approximately 90%). For further confirmation of the results, the activities of the supernatants were tested at various dilutions and expressed as productivity (U/mL). *P. aeruginosa* TUN03 produced the highest activity in the supernatant at 2430 U/mL. Thus, this strain was chosen for further investigation.

Table 1. The α-amylase inhibitory activity of tested bacterial strains.

Bacterial strains	α-Amylase inhibitory activity	
	Inhibition (%)	Productivity (U/mL)
Bacillus megaterium CC05	71±2.1	-
Acinetobacter baumannii CC11	47±0.9	-
Bacillus marisflavi BMT2	63±1.3	-
Bacillus cereus RB.DS.05	89±3.2	1750±87.1
***Pseudomonas aeruginosa* TUN03**	**88**±2.7	**2430±106**
Bacillus acidicola B14	56±2.2	-
Bacillus atrophaeus H10	89±4.3	1856±112

The shrimp head powder was used as a C/N source for fermentation in 4 days at 30 ^{0}C. The supernatant was harvested by centrifugated at 12000 rpm for 10 min and used for detection of activity. (-): not determined.

Pseudomonas aeruginosa has been reported as valuable for its utilization in agriculture [30-33], environment management [34-35], and in the industry for the production of some secondary metabolites such as rhamnolipid, vanillin, enzymes, pigments, and plant promoting compounds having their wide range of applications [36-41]. Based on our recent review of published studies, there is no report on using this genus for the production of aAIs via fermentation. Thus, the new finding of α-amylase inhibitory activity of this strain enriches the bioactivity catalog of *P. aeruginosa.*

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2.2. Production of α-Amylase Inhibitors via P. aeruginosa TUN03 Fermentation

To establish the fermentation process to produce aAIs with higher productivity, some factors, including the effect of C/N sources and culture conditions, were examined, then aAIs were scaled-up for production in a 14-L bioreactor system.

- *The effect of C/N sources on the productivity aAIs produced by P. aeruginosa TUN03:* Various marine chitinous materials such as squid pen powder (SPP), shrimp head powder (SHP), demineralized shrimp shell powder (de-SSP), demineralized crab shell powder (de-CSP), and a commercial medium nutrient broth (NB) were used as the sole C/N source for TUN03 fermentation and comparison of aAIs production. As shown in Figure 1a, the medium containing SHP, SPP, and NB showed the highest activity on day 2 of fermentation with respective inhibition values of 88, 85, and 72%. For better clarity of the results, the activity was also calculated and expressed as U/mL. The result presented in Figure 1b indicates that SHP was the most suitable C/N source and provided the highest productivity (2448 U/mL). Therefore, this substrate was chosen for the cost-effective production of aAIs in further experiments. The bacterial growth was also assessed but showed no correlation with aAIs production (data not shown); thus, this factor was also not presented in the next experiments. Shrimp industrial wastes have been used for the production of chitin, protein, carotenoids, dye adsorbents, enzymes, and acetylcholinesterase inhibitor compounds [2,42-44]. Shrimp head was also used as a fish meal [45] and in the diets [46]. Recently, shrimp head was extensively investigated as the sole C/N source for the production of α-glucosidase inhibitors [27-29]. Different from the previous studies, in this work, we highlight here the novel and potential use of this low-cost material as the sole C/N source for the production of aAIs via microbial fermentation.

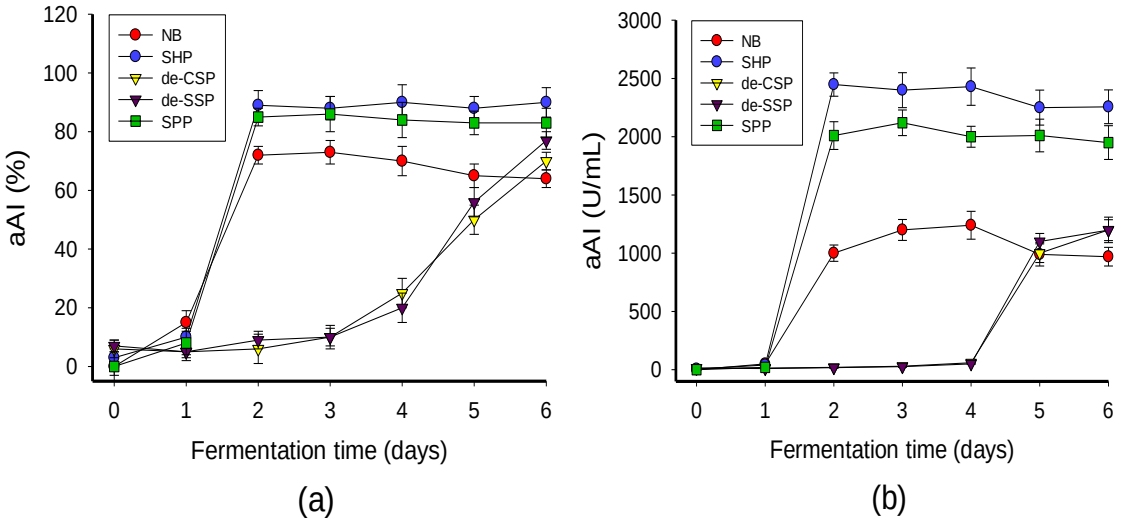

Figure 1. Screening of suitable C/N sources for fermentation. The C/N sources were used at 1% for fermentation at 30 ^{0}C with 150 rpm shaking speed for 6 days. The α-amylase inhibitory activity of the fermented culture broth was tested per day and expressed as % (**a**) or U/mL (**b**).

- *The effect of culture conditions on aAIs production by P. aeruginosa TUN03*: To enhance aAIs productivity, some culture conditions such as concentration of SHP, culture temperature, initial pH of culture medium, and cultivation medium volume were investigated for their effect on fermentation (Figure 2). As presented in Figure 2a, the suitable concentrations of SHP were determined to be at 1.5 – 2 %. Considering saving input material in fermentation, the 1.5 % SHP was chosen for the next experiments. The data in Figure 2b indicates that *P. aeruginosa* TUN03 gave the highest aAIs yield (2785-2850 U/mL) with cultivation temperature in the range of 27.5 – 32.5 ^{0}C, and 27.5 ^{0}C was set as the optimum

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cultivation temperature for saving energy. *P. aeruginosa* TUN03 was found to produce aAIs with high productivity in the initial pH of the culture medium of 7-7.5 (Figure 2c). The initial pH of the culture medium of 7 was set for the further investigation of cultivation medium volume. The result in Figure 2d shows aAIs production by *P. aeruginosa* TUN03 on a small scale (20-30 mL of medium in a 100 mL flask). Overall, *P. aeruginosa* TUN03 could give the highest productivity of aAIs when a 30 mL medium containing 1.5 % SHP was used with initial pH 7-7.75 in a 100-mL flask and fermented at 27.5 ^{0}C for 2 days.

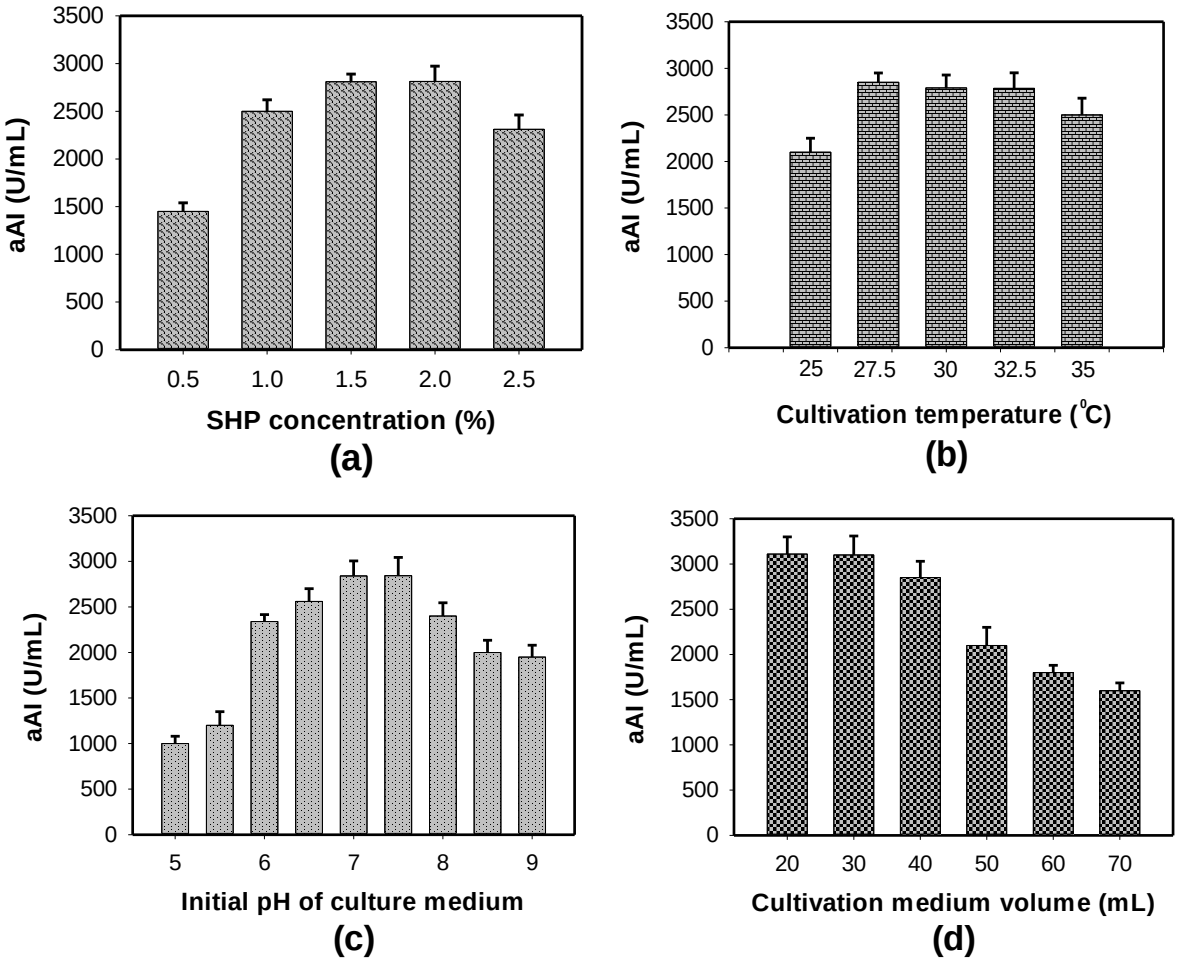

Figure 2. The effect of SHP concentration (a), cultivation temperature (b), initial pH of culture medium (c), and cultivation medium volume (d) on aAIs productivity in *P. aeruginosa* TUN03 fermentation.

- *Scale-up of production of α-amylase inhibitors using a 14-L bioreactor system:* In microbial technology, reactor systems are considered as a strong tool for scaling-up bioproduction of active secondary metabolites as well as a significant reduction of cultivation time [2,49-50]. In this work, we utilized a 14 L-bioreactor system for fermentation to scale up the production of aAIs. As shown in Figure 3, aAIs were produced from 4 h of fermentation and reached the highest productivity of 4200 U/mL at 12 h. These experimental data indicated that the scale-up in a bioreactor system gives significantly higher aAIs productivity in a much shorter time than that in a flask. Up till now, several studies reported the production of antidiabetic compounds via microbial fermentation [24-29]. However, in most of these previous works, antidiabetic compounds were produced on small scales in flasks. In this work, we attempted large-scale production of antidiabetic compounds in a 14-L bioreactor system and got positive outcomes.

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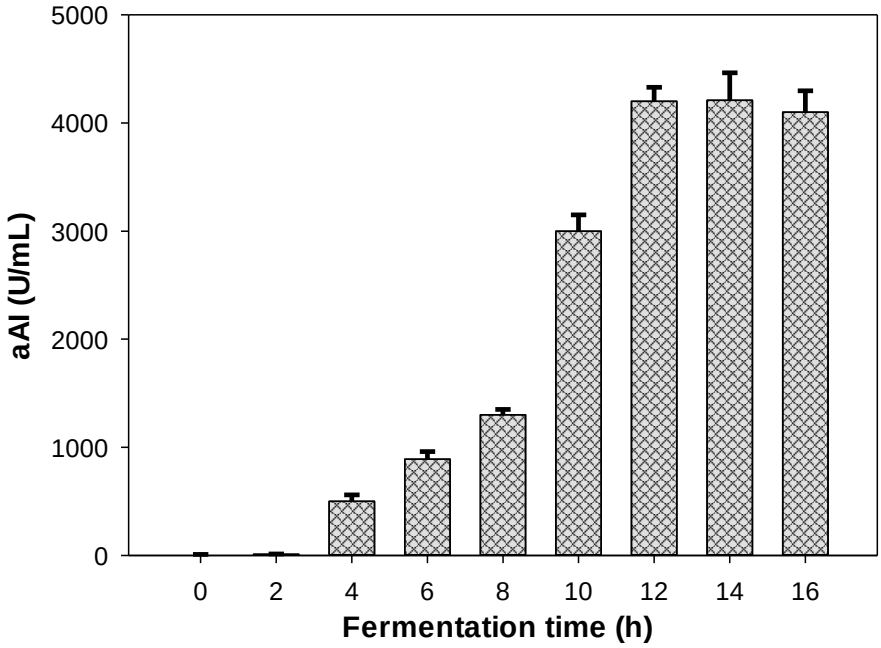

Figure 3. Scale-up production of aAIs in a 14 L-bioreactor system. Standard errors (SE) are shown as error bars.

2.3. Determination and Isolation of Major Active Compound from the Culture Broth

Phenazine compounds are majorly produced by *P. aeruginosa*. Thus, these compounds may play an important role in the bioactivities of *P. aeruginosa*; we found that the density of these pigments seemed to increase along with aAIs production during the *P. aeruginosa* TUN03 fermentation. On the other hand, earlier studies reported microbial aAIs as proteins [51-52]. Thus, to quickly predict the most active component, the supernatant was used to prepare some samples, including the crude pigment phenazines (the chloroform layer, named as CPP), the residue water layer (named as RWL), the crude protein (the supernatant was precipitated by ethanol, named CP) and the crude sample (the supernatant was vaporized to dried powder, named CS) to detect aAIs activity. The processes of preparation, purification, and identification of target compounds are summarized in Figure 4.

As shown in the Figure 5a, the protein potion and the residue water layer showed no activity, while the pigment phenazines displayed high activity with a maximum inhibition of 89% (at a low concentration of 1 mg/mL) which is much higher than that of a crude sample with max inhibition of 88% (at high concentration of 4mg/mL). The results indicated that pigment phenazines were the major α-amylase inhibitor compounds existing in the sample. Thus, this portion with pigments was further separated using an open silica column, and seven sub-fractions (SF-1, SF-2, SF-3, SF-4, SF-5, SF-6, and SF-7) were obtained. Then the activity of these substrates was tested and presented in Figure 5b. The component SF-1 was found to exhibit the strongest activity (91%), while the components SF-2, SF-3, and SF-4 displayed inhibition values under 26%, SF-6 and SF-7 demonstrated very weak activity. The active phenazine compound SF-1 obtained in the yellow form was identified based on analysis of gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR, incuding ^{1}H-NMR, ^{13}C-NMR, DEPT 135, DEPT 90, and DEPT 45) (the spectra were presented in the supplementary materials, including Figure S1, S2, S3, S4, S5, and S6), and further confirmed to possess a high grade of purity using high-performance liquid chromatography (HPLC) profiles (Figure 6).

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The 14-L Bioreactor system containing 4 L medium after 12 h of fermentation

The culture broth was centrifugated at 12000 rpm in 10 min

Supernatant solution

Liquid layer by chloroform

Precipitation of the supernatant by 70% ethanol solution

The supernatant was vaporized at 50 °C to form dried powder

Chloroform layer (Crude pigment phenazines)

Residue water layer

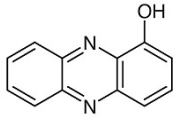
Crude protein

Crude sample

Loaded on to an opened silica column and eluted with the gradient solvent system chloroform/methanol (100/0 – 80/20)

SF-1 | **SF-2** | **SF-3** | **SF-4** | **SF-5** | **SF-6** | **SF-7**

Identification of the target compound by GCMS, NMR, HPLC



Hemi-pyocyanin

Figure 4. The process of preparation, purification, and identification of target compounds (α-amylase inhibitors).

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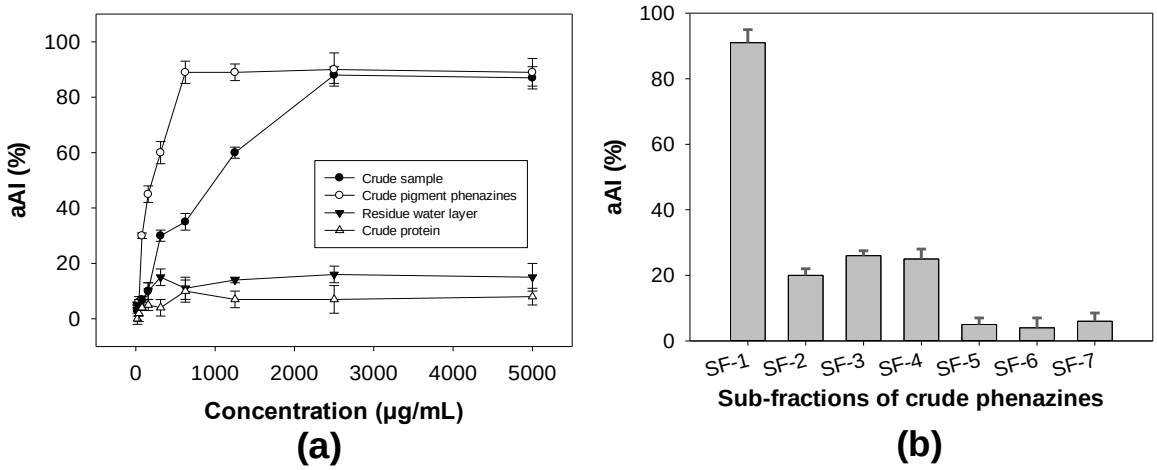

Figure 5. The bioactivity-guided extraction process of α-amylase inhibitor compound. The α-amylase inhibitory activity of some major portions extracted from crude sample tested at various concentrations (**a**) and sub-fractions of the crude pigment phenazines portion tested at the concentration of 150 µg/mL (**b**).

As shown in Figure S1A, one major peak appeared at the retention time of 13.09 of the GC profile of the purified component SF-1. For further analysis, the mass of this peak was compared to those of known compounds in the chemicals bank of the GCMS system (Figure S1B), resulting in the prediction of this compound as hydroxyphenazine with chemical formula as $C_{12}H_8N_2O$. This compound was further identified its chemical structure based NMR spectra, and the analysis of ^{1}H-NMR and ^{13}C-NMR was as follows: ^{1}H-NMR (600 MHz, $CDCl_3$) δH: 8.25 (1H, m) δH: 8.21-8.25 (2H, m), δH: 7.76-7.89 (4H, m), δH: 7.24-7.26 (1H, m) (Figure S2). ^{13}C-NMR (151 MHz, $CDCl_3$) δC: 151.7; 144.3; 144.5; 141.2; 134.7; 131.9; 130.8; 130.5; 129.7; 129.2; 119.9; 108.9 (Figure S3).

The ^{1}H-NMR chemical shirft indicated that 8 H atoms with δH from 7.0 to 8.5 ppm should be in the aromatic rings. Of these the H atom with δH: 8.25 should be assigned to be the H atom of hydroxyl group while other 7 H atoms located in the aromatic rings at 2, 3, 4, 6, 7, 8, 9 with δH: 8.21-8.25 (2H, m), δH: 7.76-7.89 (4H, m), δH: 7.24-7.26 (1H, m), respectively.

The ^{13}C-NMR provided the signals of 12 C. Of these, the C atom (δC: 151.7) was assigned correspond to C of hydroxyl group (C-OH) in the aromatic rings, and other C atoms are assigned to be located in the aromatic rings at 2, 3, 4, 4a, 5a, 6, 7, 8, 9, 9a, 10a with δC: 144.3; 144.5; 141.2; 134.7; 131.9; 130.8; 130.5; 129.7; 129.2; 119.9; 108.9, respectively. In addition, the ^{13}C-NMR chemical shirt copoupling with spectra of DEPT 135, DEPT 90, and DEPT 45 (Figures S4, S5, and S6) also indicated that this compound containing 7 groups of CH and 5 C. The above evidences resulted in identification of this compound as hemi-pyocyanin (other name: 1-hydroxyphenazine), and the NMR spectra of this isolated compound are corresponding to those of 1-hydrophenazinedentified in the earlier report [53].

For checking purity, the HPLC was performed, and the purified HPC (SF-1) also appeared with one clear peak (Figure 6). This data confirmed the high purity of HPC purified in this work. This was then further tested in detail for aAI activity (Figure 7). For comparison, this active compound and acarbose were tested at various concentrations. HPC showed potential α-amylase inhibition with the max inhibition and IC50 values of 92.2% and 3.1 µg/mL, respectively (Figure 7). The activity of this compound was comparable to that of acarbose, a commercial antidiabetic drug with the max inhibition and IC50 values of 91% and 4.2 µg/mL, respectively. HPC was reported with potential medicinal effects, such as anticancerous against the cell lines 1321N1 and A549 [54,55], antibacterial activity against various human pathogenic bacterial strains *Candida albicans*, *Aspergillus fumigatus*, *Escherichia coli*, *Xanthomonas campestris* pv. *vesicatoria* [55-57], anti-inflammatory effect [58]. HPC has also reported application in agriculture with the anti-effects against various pathogens fungal and bacterial strains [59-60]. However, there is no report of the α-amylase inhibitory activity of HPC, to the best of our knowledge, and

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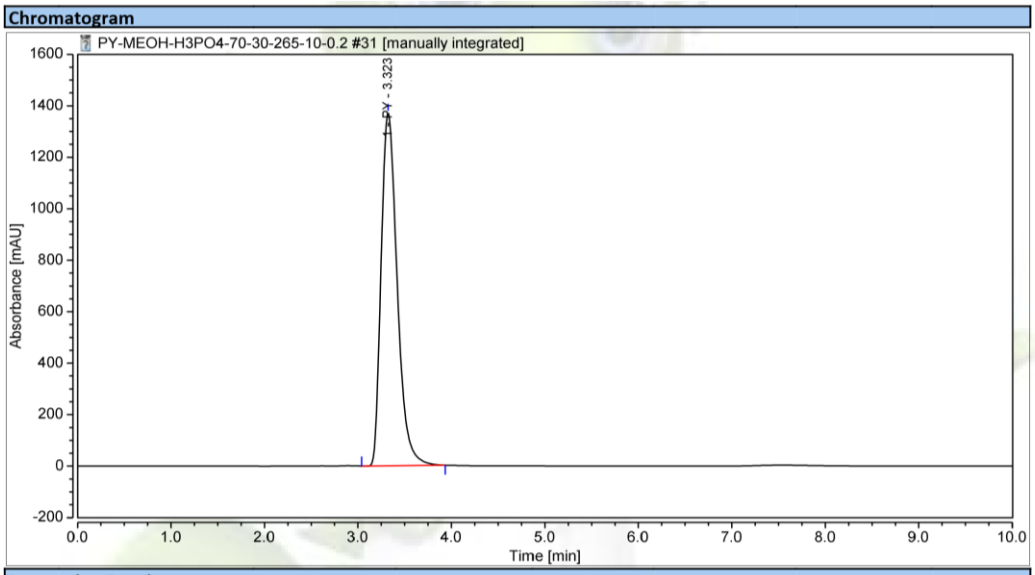
as per our review of the literature. Thus, the new finding in this work may provide the novel use of HPC in medicines.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	PY	3.323	272.456	1369.878	100.00	100.00	n.a.
Total:			**272.456**	**1369.878**	**100.00**	**100.00**	

Figure 6. The HPLC profile of the purified compound, hemi-pyocyanin.

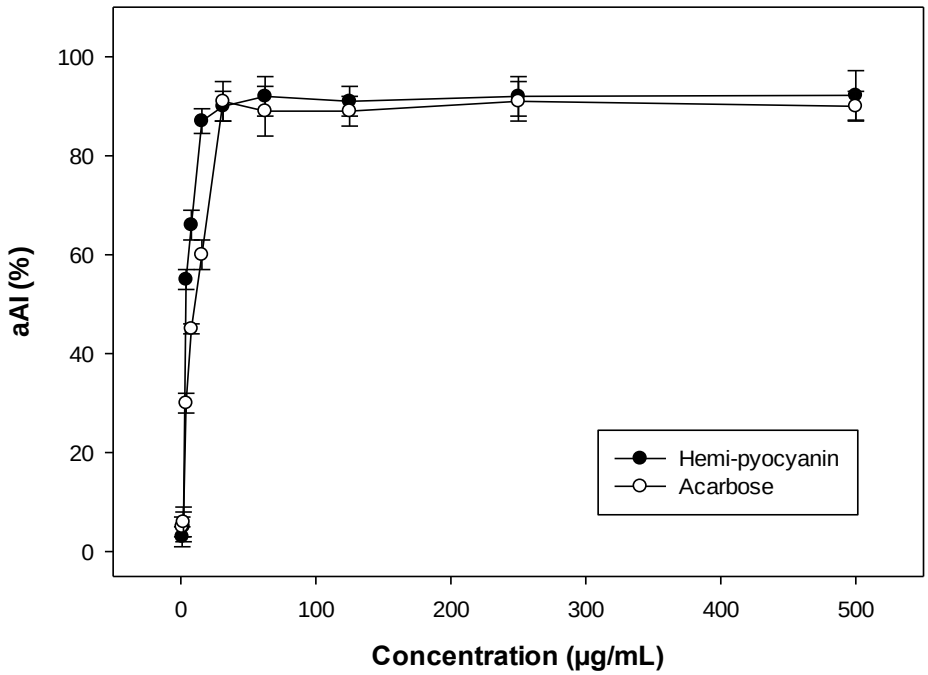


Figure 7. The α-amylase inhibitory activity of the purified hemi-pyocyanin and acarbose

2.4. The Interaction of The Inhibitor Hemi-pyocyanin at The Binding Site of α-Amylase via Docking Study

The docking study was performed to explore the interactions of ligands (HPC and acarbose) and the targeting enzyme α-amylase. Before docking, the structures of ligands and protein enzymes were prepared using ChemBioOffice 2018 software and MOE-2015.10 software presented in Figure 8.

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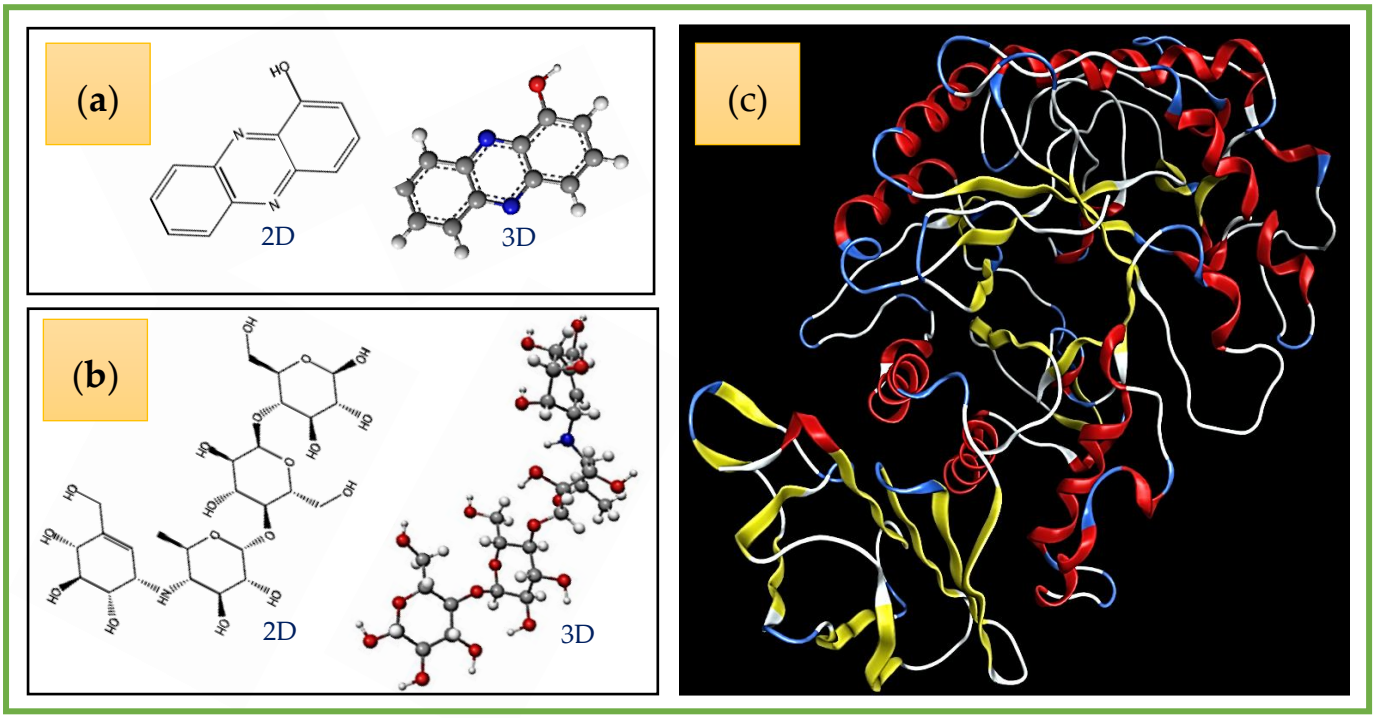

Figure 8. The 2-D and 3-D structures of hemi-pyocyanin (**a**), acarbose (**b**), and the crystal structure of α-amylase (**c**).

The active zone of the ligands on the target protein was determined using the site finder function in MOE. The out-put data of MOE indicated that there are the four most active zones (AZ-1, AZ-2, AZ-3, and AZ-4) of ligands on the enzyme α-amylase (Figure 9). Of these, the inhibitor HPC was found to be the most active on the active site AZ-2, while acarbose demonstrated potential inhibition against α-amylase at the active site AZ-1. AZ-1 contains up to 36 amino acids (Val49 Val50 Val51 Thr52 Asn53 Trp58 Trp59 Tyr62 Gln63 His101 Gly104 Ser105 Gly106 Ala107 Ala108 Tyr151 Leu162 Val163 Gly164 Leu165 Arg195 Asp197 Ala198 Lys200 His201 Glu233 Val234 Ile235 Glu240 His299 Asp300 Arg303 Gly304 His305 Gly306 Asp356). AZ-2 contains up to 28 amino acids (Ala3 Pro4 Gln5 Thr6 Gln7 Ser8 Gly9 Arg10 Thr11 Arg92 Trp221 Phe222 Pro223 Arg252 Ser289 Asp290 Pro332 Tyr333 Gly334 Phe335 Thr336 Arg398 Val401 Asp402 Gly403 Gln404 Pro405 Arg421). Thus, these active zones were chosen for assessing the docking performance of acarbose and hemi-pyocyanin, respectively.

In docking simulation, the value of RMSD (Root Mean Square Deviation) was used to define whether the interactions between ligand and the targeting protein enzyme are accepted or not. When RMSD reaches a value higher than 3.0 Å, the interaction was considered as not significant biding. The interaction is widely accepted when this value is under 2.0 Å [61]. As shown in Table 2, both HPC and acarbose are bound with the target enzyme with the RMSD values of 1.68 and 1.59, indicating that these two ligands successfully bound to the protein enzyme. For determination of the potential inhibition of the ligands towards the targeting enzyme, a docking score (DS) was used. The ligand is defined as a potent inhibitor when it has the binding energy value under −3.20 kcal/mol [62]. In this work, HPC and acarbose were found to bind with α-amylase to generate DS values of -9.3 and -12.1 kcal/mol, respectively, which are much lower than −3.20 kcal/mol. Thus, this result confirmed that both these compounds are potential α-amylase inhibitors.

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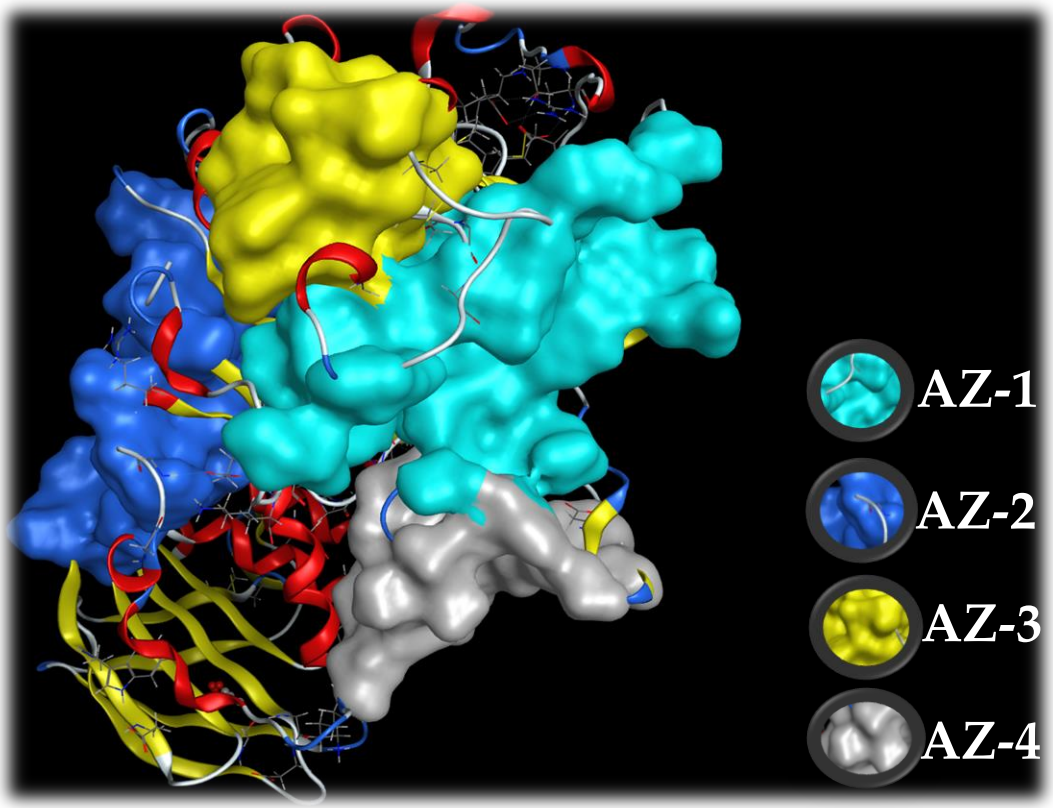

Figure 9. The four active zones (AZ) on the targeting enzyme α-amylase based on the out-put data of MOE using the site finder function.

Table 2. The docking simulation results of ligands binding with target enzyme α-amylase (aA)

Ligands (Inhibitors)	**Symbol (Ligand-protein)**	**RMSD (Å)**	**DS (kcal/mol)**	**Number of linkages**	**Amino acids interacting with the ligand** [Distance (Å) / E (kcal/mol)/linkage type]
Hemi-pyocyanin (HPC)	HPC-aA	1.68	-9.3	2 linkages (H-acceptor)	Arg421 (3.20/-0.7/H-acceptor) Arg421 (3.10/-1.7/H-acceptor)
Acarbose (AB)	AB-aA	1.59	-12.1	4 linkages (1 H-donor and 3 H-acceptor)	Asp300 (2.65/-2.8/H-donor) Lys200 (3.37/-1.9/H-acceptor) Gln63 (3.05/-1.1/H-acceptor) Lys200 (3.04/-2.1/H-acceptor)

To understand the interactions of ligands towards the targeting enzyme, the simultaneous interactions were examined and the details are presented in Figure 10. HPC interacts with α-amylase at the active zone via creating two linkages H-acceptor with amino acid Arg421. These two linkages are formed by the connection of the receptor group –NH2 of Arg421 to O1 and N2 of HPC with the distance and energy binding of these linkages of 3.20 Å, -0.7 kcal/mol, and 3.1 Å, -1.7 kcal/mol, respectively. The positive compound acarbose was found binding at the active zone on α-amylase via forming four linkages, including one H-donor and three H-acceptor linkages were formed. The amino acid Asp300 and Gln63 in the active zone were found to connect with O6 and O10 of acarbose to form one linkage H-donor and one linkage H-acceptor with their recorded distances and energy binding of 2.65 Å, -2.8 kcal/mol, and 3.05 Å, -1.1 kcal/mol, respectively, while the amino acid Lys200 connected with O8 and O16 of acarbose to generate two H-acceptor linkages with the distance and energy binding of 3.37 Å, -1.9 kcal/mol, and 3.04 Å, -2.1 kcal/mol, respectively.

Based on the in vitro activity and docking study of the inhibition against α-amylase, the biosynthesized and purified active compound HPC in this work is a potential α-amylase inhibitor with the activity comparable to acarbose, as such it may be suggested as a potent candidate antidiabetic drug. However, further studies in various animal models and clinical studies are required for the development of this potential compound into an antidiabetic drug.

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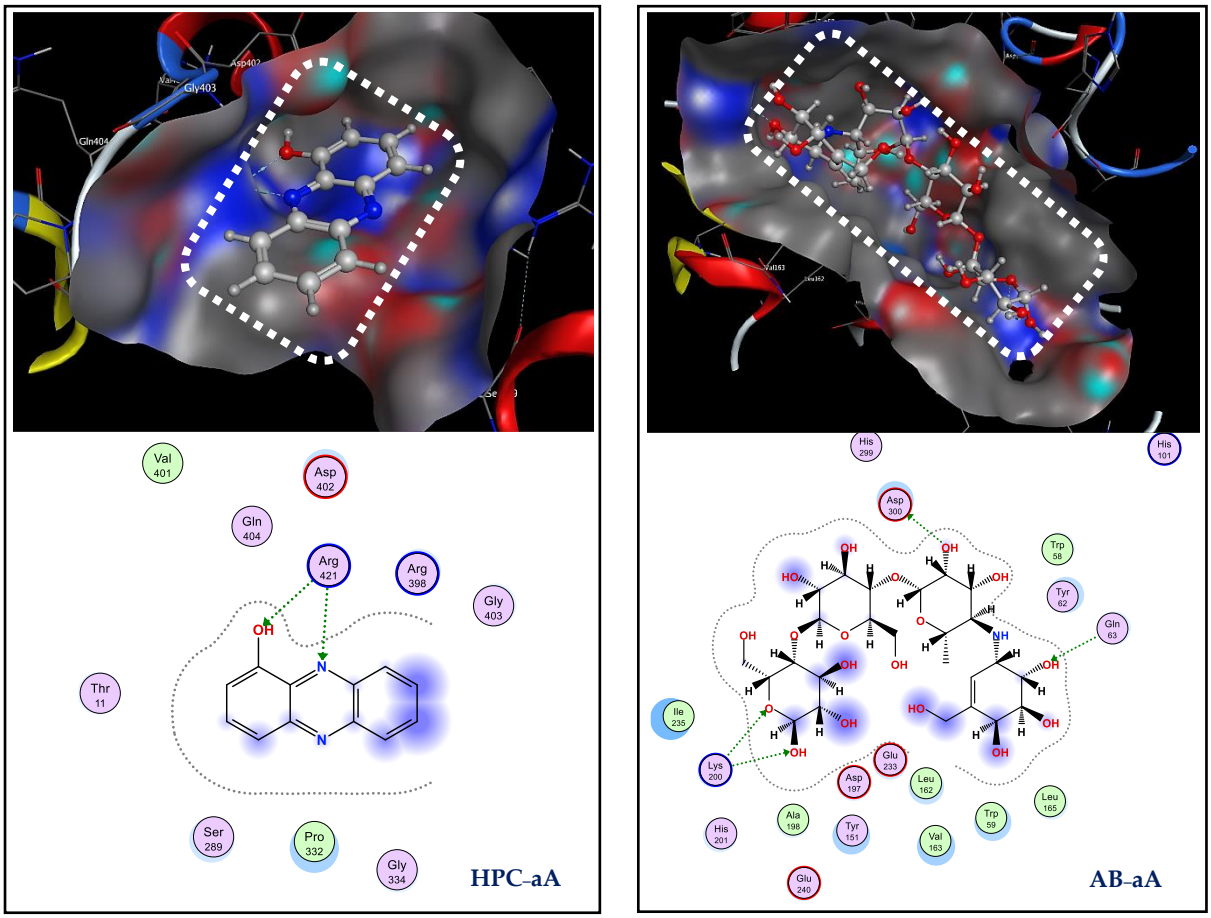

Figure 10. The performance of docking study of hemi-pyocyanin (HPC) and acarbose with the α-amylase enzyme. The interactions and binding of the ligands HPC and acarbose with the α-amylase enzyme are labeled as HPC–aA (**a**) and AB–aA (**b**), respectively.

3. Materials and Methods

2.1 Materials

The bacterial strains were obtained from our previous works [41,63]. The marine chitinous discards such as shrimp shells, shrimp heads, crab shells, and squid pens were required from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). Porcine pancreatic α-amylase (type VI-B) and acarbose were purchased from Sigma Aldrich (St. Louis City, MO, USA). Shrimp shells and crab shells were demineralized as per the method presented in our previous study [64]. Silica gel (Geduran® Si 60, size: 0.040-0.063 mm) was purchased from Merck Sigma Chemical Co. (St. Louis City, MO, USA). The nutrient broth was purchased from Creative Life Science Co., Taipei, Taiwan, and some solvents used in this work were from Sigma Aldrich (St. Louis City, MO, USA).

2.2. Methods

2.2.1. α-Amylase Inhibitors Production via Microbial Fermentation Experiments

- Screening of active α-amylase inhibitors producing strain and suitable marine chitinous discards for fermentation

The bacterial strains were obtained from our previous works were examined for fermentation of SHP. A liquid medium of 30 mL (initial pH 6.8) containing 1% SHP, 0.1% K2HPO4, and 0.05% MgSO4·7H2O in a 100 mL-Erlenmeyer flask was fermented by different strains at 30 ^{0}C with shaking speed of 150 rpm, in 4 days. Then the supernatant was harvested by centrifuged at 12000 rpm for 10 min and used for detection of α-amylase inhibitory activity. The most active strain *P.s aeruginosa* TUN03 was used for

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further experiments of screening the most suitable C/N sources. A total of four marine chitinous discards, including SPP, SHP, de-SSP, and de-CSP, and a commercial medium nutrient broth (NB) were used as sole C/N sources. These C/N sources were used at 1% in the basal salt medium of 0.1% K2HPO4 and 0.05% MgSO4·7H2O. The fermentation was performed at 30 ^{0}C with a shaking speed of 150 rpm for 6 days. The supernatant was harvested per day and used for the detection of activity. SHP was found as the most suitable material for fermentation and used for our nest investigation.

- The effect of culture conditions on aAIs production by P. aeruginosa TUN03

To approach a higher aAIs productivity, some culture conditions were tested. A 30 mL liquid medium containing different concentrations of SHP (0.5, 1, 1.5, 2, and 2.5%), 0.1% K2HPO4, and 0.05% MgSO4·7H2O in a 100 mL-Erlenmeyer flask was fermented by *P.s aeruginosa* TUN03 at 30 ^{0}C with a shaking speed of 150 rpm, and the activity of the supernatant was tested after 2 days of fermentation. SHP (1.5%) was chosen for further experimentation to investigate the effect of culture temperature (25, 27.5, 30, 32.5, and 35 ^{0}C), initial pH of culture medium (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9), and cultivation medium volume (20, 30, 40, 50, 60, and 70 mL). The following experiments were designed based on the optimal conditions
achieved from previous experiments.

- Scale-up of production of α-amylase inhibitors using a 14-L bioreactor system

The optimal culture conditions investigated from all the above-mentioned experimental results were applied for scale-up of production of aAIs in a 14-L bioreactor system (the 14-L BioFlo/CelliGen 115 bioreactor system - Eppendorf North America, Enfield, Connecticut, US). Bacterial seeds with 500 mL were pre-incubated in some 250-mL flasks at 30 °C for 2 days and then transferred to the fermenter containing 4.5 L of medium containing 1.5% SHP, 0.1% K2HPO4, 0.05% MgSO4·7H2O with initial pH 7. The fermentation was performed at 2.75 ^{0}C at a shaking speed of 150 rpm, with a dissolved oxygen content of 1.0 vvm for 16 h. Sampling was done every 2 h. The supernatant was harvested by centrifugation of the sample at 12000 rpm in 10 min and used for detection of aAI activity.

2.2.2. α-Amylase Inhibitory Activity Assay

The α-amylase inhibitory activity was estimated as per the method of Bernfeld [65] with modifications. One hundred and fifty microliters of the α-amylase solution (0.25 U/mL) was mixed with 50 µL sample (supernatants or compounds at different concentrations) and kept at 37 ^{0}C for 10 min, and then 200 µL soluble starch (0.25%) was added to start the reaction which was further kept at 37 ^{0}C for 20 min. The amount of reducing sugar produced by the action of the enzyme was measured at OD_{540nm}. The enzymatic inhibitory activity was estimated using the below-mentioned equation:

α-amylase inhibitory activity (%) = (C-E)/C x 100,

where E is the optical density of the reaction containing sample (inhibitor) and enzyme, while C is the optical density of the reaction containing enzyme and the same volume of distilled water instead of the sample [48]. The IC50 (µg/mL) and productivity (U/mL) were defined and calculated per the method presented by Nguyen et al. (2016) [47]. In this assay, the enzyme (0.25 U/mL) and substrate (0.25 % w/v) were both prepared in 10 mM CaCl2 solution in 20 mM Tris–HCl buffer at pH 7 before use.

2.2.3. Extraction and Identification, and Purity Confirmation of α-Amylase Inhibitors

Extraction of α-amylase inhibitors: The culture broth obtained from the fermentation in the 14-L bioreactor system was centrifuged at 12000 rpm for 10 min to harvest the supernatant, which was further used to prepare some samples. The supernatant was separated by the chloroform layer to obtain the crude pigment phenazines (the chloroform layer) and the residue water layer. The crude protein contained in the supernatant was obtained by precipitating with 70% ethanol solution. The supernatant was also vaporized at 50 ^{0}C to dried powder (crude sample). The crude pigment phenazines exhibited the most activity and were thus, further used to isolate the target components using an

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opened silica column (Geduran® Si 60, size: 0.040-0.063 mm, 30 × 2 cm) with gradient solvent system chloroform/methanol (100/0 – 80/20) to obtain seven sub-fractions: SF-1, SF-2, and SF-3, (eluted at chloroform/methanol of 100/0), SF-4 (eluted at chloroform/methanol of 100/5), SF-5 and SF-6 (eluted at chloroform/methanol of 100/10), and SF-7 (eluted at chloroform/methanol of 100/20). Component SF-1 was found to be active and identified by using GCMS and NMR. Its purity was further confirmed by HPLC analysis.

Identification of α-amylase inhibitors: GC-MS was conducted to identify the presence of active phenazine compounds. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min, and 1µL of an injection volume was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 250 °C, the oven temperature was programmed to 70 °C (isothermal for 1 min), with an increase of 15 °C/min to 200 °C, ending with a 2 min isothermal at 200 °C. MS data were acquired at 70 eV, a scanning interval of 0.5 s, and fragments from 50 to 650 Da.

Purity confirmation of α-amylase inhibitors using HPLC: The compound was dissolved in methanol then filtered through a 0.22 µm membrane. The sample solution (2 µL) was injected into the HPLC systems (Thermo-Ultimate 3000 UPLC system - ThermoScientific, USA) and separated by a column (Hypersil GOLD aQ C18 column, 150mm x 2.1 mm, particle size 3 µm) using the solvent systems of methanol/acidified 0.1% H3PO4 (70/30 v/v) with the flow rate of 0.2 mL/min. The compound was detected at 265 nm.

2.2.1. Docking Study

The docking study was performed following the steps described in our earlier reports [2,23].

- *Preparation of targeting protein:* The structural data of the protein enzyme (α-amylase) was obtained from Worldwide Protein Data Bank. The 3-D structure was produced using MOE QuickPrep based on the positions of the ligand within 4.5 Å and the presence of important amino acids. The active zones were found via using the site finder in MOE, and all of the water molecules were removed before the recreation of enzymic action zones.

- *Preparation of ligands:* The structures (2D and 3D) of HPC and acarbose were prepared using the ChemBioOffice 2018 software, and optimized using the MOE-2015.10 software with parameters of Force field MMFF94x; R-Field 1: 80; cutoff, Rigid water molecules, space group p1, cell size 10, 10, 10; cell shape 90, 90, 90; and gradient 0.01 RMS kcal.mol−1A−2.

- *Docking Performance:* The docking was performed on the ligands of HPC and acarbose towards the targeting enzyme α-amylase using MOE-2015.10 software. The output data, including energy biding (docking score, DS), RMSD, linkages types, amino acid compositions, the distances, and the energy biding of each linkage, were recorded.

4. Conclusions

P. aeruginosa TUN03 was selected as a potential aAIs - producing strain, and SHP was found to be the most suitable C/N source for the production of aAIs via fermentation, and high productivity of aAIs was achieved. Further scale-up production of aAIs with higher yield and much shorter fermentation time via the utilization of a 14 L – bioreactor system was also attempted. HPC was found as a major active compound and novel potential α-amylase inhibitor produced by *P. aeruginosa* TUN03. Finally, the docking study was also conducted to elucidate the interaction of this inhibitor towards the targeting enzyme α-amylase. HPC was found to bind to amino acid Arg421 at the biding site on enzyme α-amylase with good binding energy (-9.3 kcal/mol) and create 2 linkages of H-acceptor. The results of this work indicated that shrimp heads discard is a valuable source for cost-effective bioproduction of novel potential α-amylase inhibitor HPC which may be suggested as a good candidate to be developed as an antidiabetic drug.

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