Aspergillus sclerotiorum Whole Cell Biocatalysis: A Sustainable Approach to Produce 3-Hydroxy-Phenazine 1-Carboxylic Acid from Phenazine 1-Carboxylic Acid

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Article

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Abstract: In green chemistry, filamentous fungi are regarded as a kind of robust microorganism for the biotransformation of natural products. Nonetheless, the screening of microorganisms is crucial for the effective biotransformation of natural products such as phenazine compounds. The precursor metabolite of most phenazine derivatives in *Pseudomonas* spp. is phenazine-1-carboxylic acid (PCA), the key constituent of shenqinmycin, widely used to control rice sheath blight in southern China. In this study, a new fungus strain *Aspergillus sclerotiorum* was isolated, which can efficiently convert PCA into 3-hydroxy-phenazine 1-carboxylic acid (3-OH-PCA). Moreover, an effective whole cells biotransformation system was constructed by screening optimal reaction conditions and carbon sources. Hence, *Aspergillus sclerotiorum* exhibited desirable adaptation by consumption of different carbon sources and maximum whole cell biomass (10.6 g/L DCW) was obtained as a biocatalyst from glucose. Optimal conditions for whole-cell biocatalysis of PCA were evaluated, including PCA concentration of 1120 mg/L, pH 7.0, temperature of 25°C, rotation rate 200 rpm and dry cell weight 15 g/L for 60 h, thus 1060 mg/L of 3-OH-PCA was obtained and the conversion efficiency of PCA was 94%. Hence, the results of repeated batch mood revealed that the biotransformation efficiency of fungus pellets reduced with each subsequent cycle but remained stable in all five cycles with the provision of glucose supplement. These findings open the prospective of using filamentous fungi for the whole cell biocatalysis of phenazine in enormous amount and efficient production of 3-OH-PCA. Moreover, these results laid the foundation for further research to disclose the genetic based mechanism of the strain responsible for PCA biotransformation.

Keywords: *Aspergillus sclerotiorum*; phenazine 1-carboxylic acid; biotransformation; 3-hydroxy phenazine 1-carboxylic acid; green chemistry

1. Introduction

Biotransformation is a powerful tool for obtaining valuable products in industrial processes [1,2].The most impressive advantage of biotransformation over other traditional synthesis techniques is the respective enzyme's selectivity, which enantiomerically generates pure products and reduces the cost of purification. In the biotransformation reaction, purified enzymes or whole microbial cells can accomplish the reduction, oxidation, isomerization, and hydroxylation of natural products to produce novel derivatives. This process is mild, less expensive and doesn't use toxic reagents or solvents, making it environmentally friendly [3–5]. Chemical catalysis shows too less potent beneficial effect due to the production of toxic byproducts and required unfavorable and undesirable extreme conditions [6] Specifically, whole-cell biocatalysts are more industrially favorable for the production of chiral alcohols than purified enzymes [7]. The whole cell's confined environment contains the

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enzymes and required cofactors such as NADH/NADPH. However, the whole cells regenerate the cofactors, but need to be added to the pure enzyme for the continuation of biotransformation process, which is quite expensive. [8–10]. Thus, whole-cell biocatalysts were considered a kind of robust and most effective way for the biocatalytic production of challenging and fine chemicals [11]. Various organisms were used to carry out the biotransformation of different natural compounds [12]. In all of them, the microbial system is more significant because of the short doubling time of biomass and can be genetically modified through well-defined techniques [13].

However, for the successful biotransformation process, the screening of microorganisms is a key step as they widen the chances of drug discovery from the industrial valuable natural products, such as phenazines compounds. Phenazines are three-ringed nitrogen-containing secreted metabolites found in environmental, industrial and clinical settings. The precursor metabolite of mostly phenazine derivatives in *Pseudomonas* spp. is phenazine-1-carboxylic acid (PCA; $C_{13}H_8N_2O_2$) and already showed great application in the agriculture sector to control various fungal diseases [14–17]. Hence, the physiological function, biosynthesis and regulation of PCA are well understood, but its nature of microbial biotransformation is largely unknown. The biotransformation and modification of phenazine compounds are deemed risky and make agricultural settings susceptible to various diseases. Thus, the precursor metabolite PCA is the target of various studies to screen and identify the microbes capable of its biotransformation. Fortunately, different bacterial strains have been reported and identified that can transform PCA by growing cells such as *Sphingomonas* sp. DP58 [18,19], *Nocardia* sp. LAM0056 and *Rhodococcus* sp. JVH1, *Mycobacterium* sp. DNK1213, *Mycobacterium* sp. CT6 and *Mycobacterium* sp. ATCC6841[20,21]. In addition to bacterial biotransformation, some studies demonstrated PCA modification by growing cells of filamentous fungi [22,23]. However, the derivatives of PCA portrayed more specific antimicrobial activities than the parental compound which encourages us to isolate and identify filamentous fungus strain that are capable of PCA modification with unique functional features [24]. In green chemistry, filamentous fungus is considered an effective microorganism due to their excellent efficiency in conducting biotransformation of various substrates [25]. The previous study explored that, the strain *Aspergillus sclerotiorum* CBMAI 849 act as a whole cell biocatalyst and modify the potent antioxidant 2-hydroxychalcone into hydroxydihydrochalcone [26]. In addition, the 2,5-furandicarboxylic acid (FDCA) is an important bio-based source for biodegradable polyesters production and identified as a top opportunity for the chemical industry in the future by US Department of energy (US DOE) [27]. Thus, the precursor metabolite 5-hydroxymethyl furfural (HMF) oxidized by filamentous fungus strains *A. flavus* and *A. niger* (NRRL 567) and produce 2,5-furandicarboxylic acid (FDCA) [28]. According to the best of our knowledge, there is no report regarding the whole cells biocatalysis of PCA by filamentous fungus *Aspergillus sclerotiorum*. The present study aims to explore the potential of *Aspergillus sclerotiorum* as a biotransformation agent for the production of 3-hydroxy phenazine 1-carboxylic acid (3-OH-PCA) through whole-cell biotransformation of PCA. The fungus strain was initially identified from red spots that appeared as a contaminant in the cultured plate of PCA-producing bacterial strain *P. chlororaphis* GP72. The study subsequently screened favorable carbon sources for the production of *Aspergillus sclerotiorum* pellets and optimized the physicochemical parameters to construct an efficient whole-cell biocatalysis system for the production of 3-OH-PCA. Furthermore, the biocatalytic potential of the fungal pellets was explored in cycling mode in the fed-batch fermentation. These findings contribute to the knowledge that the strain *Aspergillus sclerotiorum* displays the potential to become a significant biotransformation agent. In the future, this research could lead to the development of more efficient and sustainable biotransformation processes that utilize fungal strains for the production of valuable metabolites.

2. Materials and Methods

2.1. Selection of Chemical and Media

The principal organisms studied, *Aspergillus sclerotiorum*, were isolated as contaminants and identified in our laboratory. Stock culture of newly isolated fungus strain (*Aspergillus sclerotiorum*) was maintained on Yeast peptone dextrose (YPD) medium. YPD medium contained (g L-1): Glucose

30.0, Peptone 10.0, yeast extract 5.0. Phenazine-1-carboxylic acid (purity ≥ 98%) was purchased from Nonglebio Co., Ltd., Shanghai, China. The composition of mineral salts medium (MSM) contained NH4Cl 2.0 g/L, K2HPO4 5.2 g/L, KH2PO4 3.7 g/L, Na2SO4 1.0 g/L, MgSO4 0.1 g/L. However, the composition of trace elements solution is composed of 35 mg/L CuSO4. 5H2O, 50 mg/L H3BO3, 40 mg/L MnSO4. 7H2O, 40 mg/L ZnSO4. 7H2O, 20mg/L CO(NO3)2. 6H2O, 30 mg/L (NH4)6 Mo7O24. 4H2O mg/L. The Czapek-Dox mineral salt medium with the following composition (g L-1) was used to isolate fungal strain: MgSO4 .7H2O 0.5, K2HPO4 0.5, NaNO3 2.0, KCl 0.5, FeS04.7H2O 0.01, KH2PO4 0.5 and 30 g glucose. The solid medium was made by mixing 12 g/L agar with the proper liquid media. The initial pH of the medium was maintained at 7.0 and sterilized for 15 minutes at 121°C. YPD medium supplemented with various carbon sources such as glucose, glycerol and xylose to screen the most suitable carbon source for obtaining of whole cell biomass mass of *A. sclerotiorum*. All of the chemicals used in this experiment were of analytical grade.

2.2. Isolation and Identification of Fungus Strain

Czapek-Dox mineral salt medium and the broad-spectrum antibiotics gentamycin (50mg/ml) were employed and the fungus strain was isolated from the red spot of contaminated petri dishes of *P. chlororaphis* GP72. Fungus strain was repeatedly subcultured on Czapek-Dox agar medium plates to ensure pure culture and obtained conidiospores. To further view the colony morphology and texture, fungi were inoculated on modified Czapek Yeast Agar (CYA) plates and grown at 28°C for 14 days as previously reported [29]. The genomic DNA (*Aspergillus sclerotiorum*) was extracted using Takara (Takara BioInc., Shiga, Japan) genomic DNA extraction kit. The internal transcribed spacer (ITS) region was selected for fungal strain identification due to its small size and practicality, and mycologists endorsed it as the official barcode for fungal identification[30]. The PCR for the entire internal transcribed spacer (ITS) region (ITS1, 5.8S, ITS2) amplification was carried out using the Universal oligonucleotide primers provided in Table 1. The thermocycling parameters utilized for the amplification were typically 95 °C for 5 minutes of denaturation, 35 cycles at 94 °C for 30 seconds of annealing, 52 °C for 30 seconds of annealing, and 72°C for 8 minutes of extension, as described in previous studies [31]. Following that, the PCR results were run on an ethidium bromide-stained 1% agarose gel with a 1kb DNA ladder to determine the most probable size of the amplified DNA fragment (550 to 650 bp). The amplified products were purified using a PCR purification kit (Takara Bio, Dalian, China). DNA fragments were subsequently sequenced by Sunny Biotech Co., Ltd (Shanghai, China). The Basic Local Alignment Search Tool program in the National Center for Biotechnology (NCBI) was used and performed sequence alignment. To determine the evolutionary homology of a fungus with other organisms, a bootstrap consensus phylogenetic tree was constructed by comparing multiple sequences of 14 strains using the neighbor-joining (NJ) method and the molecular evolutionary genetics analysis tool MEGA-X[32].

Table 1. Primers for PCR amplification.

Sequence	Primers name	Primers sequence	Reference
ITS	IT1F	CTTGGTCATTTAGAGGAAGTAA	[30]
	ITS4	TCCTCCGCTTATTGATATGC	

2.3. Whole-Cell Biotransformation of PCA by Aspergillus sclerotiorum

A two-stage culture model was utilized for the whole cell biocatalysis of PCA. In the first stage, fungal spores were inoculated into 250 mL Erlenmeyer flask contained 50 mL YPD medium at a concentration of 1×109 mL-1 and shaken at 200 rpm under 28°C for 60 h. Hence, several distinct well-defined morphological pellets formed in the flasks. For the biotransformation reaction, 60 h old pellets of *A. sclerotiorum* were collected and washed three times with PBS solution. Thus, in the second stage, the fungus pellets of each pre-cultured flask were re-suspended into 250 mL Erlenmeyer flask containing 50 mL MSM medium with 336mg/L PCA (Nongle Co., Ltd., Shanghai, China). The

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Erlenmeyer flask culture was incubated for 36 h at 28°C, 200 rpm. Each sample was collected in triplicate at every 6 h of interval and further evaluated for PCA biotransformation. Moreover, fungal pellets were harvested and washed by vacuum filtration with 66.7% acetic acid solution with various pH 3.0, pH 4.0, pH 5.0, pH 6.0, pH 7.0 and released the adsorbed compound and analyzed as reported previously [33].

2.4. Isolation and Extraction of the Target Compound

The biotransformation broth containing whole cell biomass was collected and acidified with acetic acid with pH 2.0 and extracted with ethyl acetate by shaking vigorously before being static for phase separation. The extraction was collected, evaporated and concentrated under reduced pressure at 33 ◦C. The obtained crude extracts were loaded on Lab-scale glass chromatographic column (4 cm × 45 cm) containing Macroporous resin Diaion HP-20 (M-Chemical Co., Ltd., Tokyo, Japan). Further, we eluted the column with different ratios of methanol solutions (100%-water, 20% methanol, 50% methanol, 100% methanol) and optimized the condition of 3-OH-PCA purification. The collected samples were further analyzed through HPLC (Agilent series 1200 system, Agilent Technologies Group, Santa Clara, USA) equipped with a C-18 reversed-phase column (4.6 ×250 mm, 5 mm; Agilent Technologies) and UV detector (Agilent, Santa Clara, USA) to disclose the best pioneer solution for 3-OH-PCA separation and purification. Moreover, UPLC-MS analysis was carried out on UPLC Acquity/Q-TOF MS Premier (Waters Co., Ltd., Milford, USA) for the structure elucidation of the target compound.

2.5. The Effect of Various Factors on the Whole-Cells Biotransformation of PCA

Unless specified, the MSM medium with pH value of 6.0, PCA concentration 336 mg/L, 200 rpm and Dry cell weight 10 g/L was incubated at 28 °C. Firstly, the fungus pellets were added to MSM medium and sample were collected at a specific interval to assess the biotransformation performance by measuring concentration of PCA and its derivative 3-OH-PCA. Various factors such as PCA concentration, pH, temperature, rotatory rates and different fungus biomass concentration were investigated in order to optimize conditions for enhancing PCA biotransformation capabilities of *A. sclerotiorum*. Subsequently, the effect of various factors on the biotransformation performance, single factor tests were set with 50mL reaction mixture in 250 mL Erlenmeyer flasks as follows. 1) PCA concentrations 4 mM (896 mg/L), 5 mM (1120 mg/L), 6 mM (1344 mg/L), 7 mM (1568 mg/L) and 8 mM(1792 mg/L; 2) pH levels: 3.0, 5.0, 7.0, 12.0; 3) Temperatures: 25, 30, 33, 37, 42°C; 4) Rotatory rates: 0, 50, 100, 200 rpm; 5) Fungus biomass(DCW): 20, 15, 10, 5 g/L. Samples were drawn after each 12 h and total incubation time was 96 h. The same optimized conditions were used for subsequent experiments.

2.6. Time Course of Whole Cell Biotransformation of PCA and the Production of 3-OH-PCA

The Whole cells biocatalysis reaction was assessed under the optimal conditions of 15 g/L DCW, 1120 mg/L PCA, pH 7.0. The flasks containing 50 mL reaction mixtures were incubated in the shaker at 200 rpm and 25 °C. Hence, the samples were collected each 12 h of intervals. After 60 h of incubation the reaction was stopped by adding 66.7% acetic acid and extracted by ethyl acetate. After vigorous shaking and centrifugation for 5 min, the organic phase was separated and evaporated and resuspended in acetonitrile for HPLC analysis.

2.7. The Reusability of Fungus Pellets for Fed-batch Fermentation

The biotransformation efficiency of *A. sclerotiorum* pellets were investigated in repeated- batch mood with glucose supplement and without glucose supplement separately, where MSM medium with optimized PCA concentration (1120mg/L) was replenished at the end of each cycle and the fungus pellets were recycled accordingly. In addition, the glucose supplement (20g/L) was provided at the end of each cycles parallel with no glucose supplement group to uncover the sustainability of biotransformation potential of fungal pellets in parallel conditions. The step of reutilization of fungus pellets were repeated five times with each resident time 60 h. After 60 h of incubation, the fungus

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pellets were harvested, centrifuged, cleaned, and inoculated in the new flask of MSM media with the same concentration of PCA (1120 mg/L) for the next cycle of biocatalysis.

2.8. Analytical Methods for PCA Biotransformation

In order to measure the concentration of newly produced PCA deravatives,1mL fermentation broth was collected, centrifuged, and adjusted its pH 2.0 and then 2 mL ethyl acetate was added and vigorously shaken for 5 min before being static for phase separation. The organic layer was collected, vacuum dried, re-suspended in 1.0 mL acetonitrile and filtered through a 0.22 μm filter for HPLC. The mobile phase composed of acetonitrile and 0.1% formic acid with gradient profile as follow: 0-5 min, 20% acetonitrile; 5.01-15 min, 40% acetonitrile; and 15-20 min, 20% acetonitrile. The wave length used for the detection of newly accumulated compound was 254 nm with 1 mL/min flow rate by infusing a 20 μL sample via an auto-sampler eluting under isocratic conditions. Fungus biomass was separated and dried at 50 ◦C. Later on the dried cell weight was calculated as previously reported [34]. All obtained values are the means of three replicates as a standard deviation. Moreover, the ultra-performance liquid chromatography & mass spectrometry (UPLC-MS) was carried out using UPLC Acquity/Q-TOF MS Premier (Waters Co., Ltd., Milford). The mass spectrometer was run in positive ion detection mode set to scan between 50 and 1000 m/z.

2.9. Statistical Analysis

The experiments performed in this study run in triplicates, and values presented here were the average of triplicates considering all values standard deviation.

3. Results and Discussion

3.1. Fungus Isolate is Responsible for Red Pigment Production

Pseudomonas chlororaphis had previously been investigated in our lab for the synthesis of different phenazine derivatives such as PCA, 2-hydroxyphenazine (2-OH-PHZ) and 2-hydroxyphenazine 1-carboxylic acid (2-OH-PCA) [35,36]. In many instances, spontaneous strikingly red pigments have been observed as a contaminant in the LB cultured plate of *P. chlororaphis* GP72 incubated at 28°C as shown in the Figure 1A. Thus in the current study, the fungus strain was screened from the red spots of the contaminated plate of *P. chlororaphis* GP72 and repeatedly sub-cultured on Czapek-Dox mineral salt medium with broad spectrum antibiotic gentamycin (50 mg/ml), but no red spots have been observed as illustrated in Figure 1B. We detected the recurrence of the red color pigments by re-inoculating the fungal strain into plates containing *P. chlororaphis* GP72 and validated it by repeating the experiment multiple times and portrayed in Figure 1C. In addition, white colony of pure fungus culture was obtained on CYA media as shown in the Figure 1D. The red pigments were thoroughly studied, revealing that they were caused by fungal strain and modify the structure of pre-existing PCA into another derivative in the culture plate.

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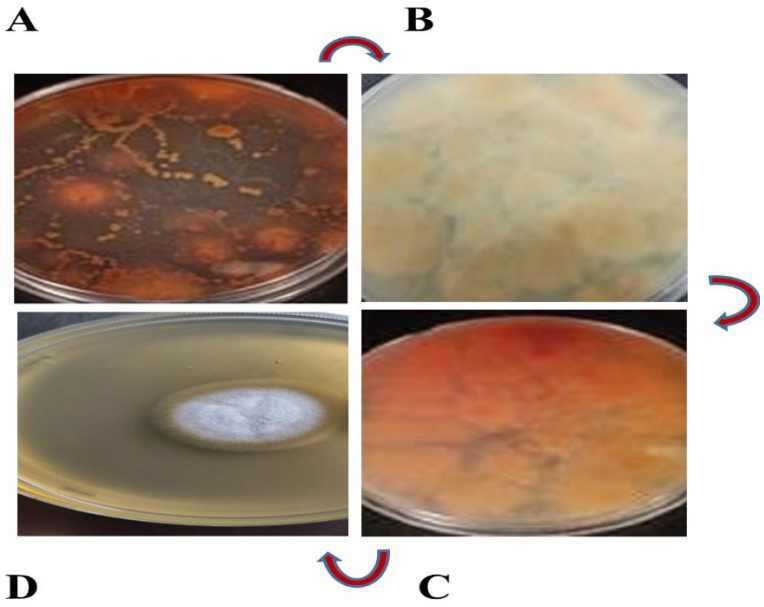

Figure 1. Fungus strain was screened and obtained the pure culture. (A) Old contaminated LB plate of *P. chlororaphis* GP72 showing red spots. (B) Isolated fungal strain devoid from red spots on Czapec Agar medium. (C) The fungus plate was re-inoculated with *P. chlororaphis GP72* and red color appeared. (D) Colony of the pure fungus culture on CYA medium.

3.2. Identification of Fungus Strain as Aspergillus sclerotiorum

In order to explore the identity of fungus strain responsible for the production of red pigments in the culture plate of *P. chlororaphis* GP72. Therefore, the fungus ITS sequence was determined and based on the blast analysis by using validated reference sequences (National Centers for Biotechnology Information), the sequence of the entire internal transcribed spacer (ITS) (ITS1, 5.8S, ITS2) exhibited the greatest identity with the genus *Aspergillus* (Gene bank accession no., MW386817.1). When the sequence identity of the fungal internal transcribed spacer (ITS) region (ITS1, 5.8S, ITS2) was compared to pre-existing sequences in the Gene Bank database, the newly isolated strain was classified as *Aspergillus sclerotiorum*. Additionally, our claim was supported by an evolutionary framework in the phylogenetic tree (Figure 2A). Multiple sequence alignment of the ITS sequence (ITS1, 5.8S, ITS2) of 14 strains using the neighbor-joining (NJ) method with the molecular evolutionary genetics analysis tool MEGA-X resulted in a bootstrap consensus phylogenetic tree. Hence, the results of ITS sequence comparison suggested that, the newly isolated fungus strain exhibited 100% homology with *Aspergillus sclerotiorum*. Macroscopically study of *Aspergillus sclerotiorum* onto Czapek Yeast Agar (CYA) medium showed white mycelia and had liquid colony exudate, while the spore was beige in color (Figure 2B) as reported previously [37]. Thus, fungal strain, *A. sclerotiorum* was validated following extensive analysis using morphology and culture characterization, coupled with molecular identification.

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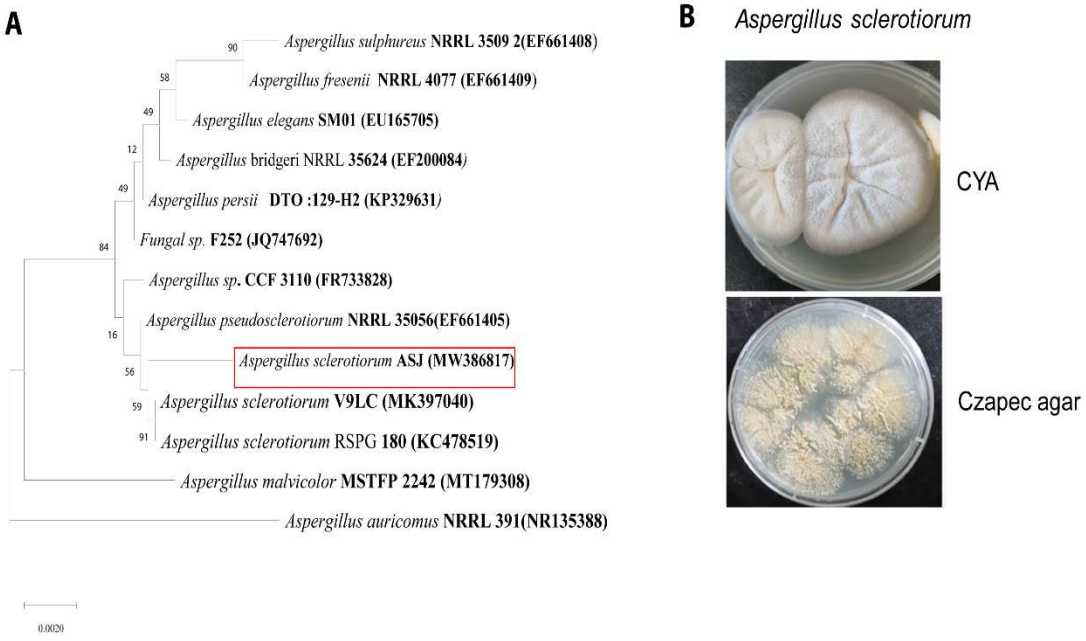

Figure 2. (**A**) Phylogenetic tree of *Aspergillus sclerotiorum* (MW386817) and related species. Based on the ITS sequence analysis the newly isolated strain relies in the same clades with *Aspergillus sclerotiorum* as portrayed in the evolutionary tree. (**B**) The fungus strain onto Czapek Yeast Agar (CYA) medium showed white mycelia and had liquid colony exudate, but the spore was beige in color.

3.3. Whole-Cell Biocatalysis of Phenazine 1-carboxylic Acid by Aspergillus sclerotiorum

Currently, various genera of microorganisms capable of biotransformation of PCA have been reported, such as *Nocardia*, *Mycobacterium*, *Sphingomonas* and *Streptomyces*[18–21,38]. In addition to bacteria, some studies demonstrated PCA modification by growing cells of filamentous fungi [22,23]. But the strain *Aspergillus sclerotiorum* has never been reported to carried out whole cell biocatalysis of PCA. Hence, the current work explored PCA modification by filamentous fungal strain *A. sclerotiorum* and portrayed outstanding efficiency of biotransformation via whole-cell biocatalysis processes. In order to explore the biocatalytic potential of *Aspergillus sclerotiorum*, the biomass obtained from YPD medium and incubated in MSM containing 336 mg/L of PCA for 36 h. As shown in Figure 3A, there was a time varying reduction of PCA during the incubation while the control group still remained the original concentration. To further determine the direction of PCA flux, we searched the HPLC profiles of PCA feeding experimental samples (Figure 3B). A new peak (retention time: 11 min) with a time varying increase of peak area was detected, which confirmed that *Aspergillus sclerotiorum* strain is able to transform PCA to new compound and later on confirmed 3-OH-PCA. As the results shows the complete depletion of PCA (336 mg/L) in 30th hours and the accumulation of 280 mg/L (Figure 3C).

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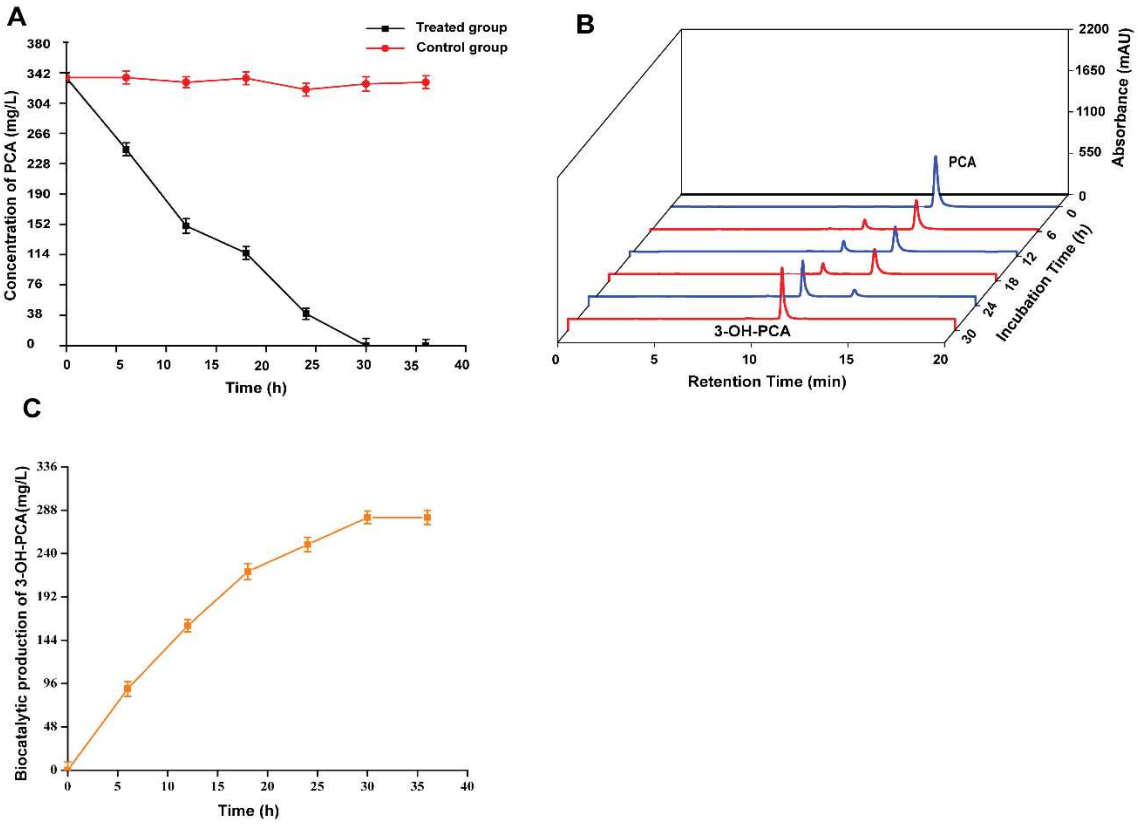

Figure 3. (**A**) Biotransformation of PCA by *A. sclerotiorum* (Treated group and control group). (**B**) HPLC chromatogram of new compound(3-OH-PCA) and substrate PCA, peaks at 11.2, and 14 min respectively. (**C**) Concentration of the newly accumulated compound (3-OH-PCA).

We further explored that, the pH of the culture broth rises from 6.0 to 7.0 during 30 h of incubation period that may affect the solubility and distribution of newly accumulated compounds as shown in Figure 4A. Hence, the newly formed compounds sensitive and change its color with pH change which may affect its distribution as shown in Figure 4D. In addition, the fungus pellets provided quite enough surface area for extracellular metabolites to be adsorbed. Thus we have collected the fungus pellet and washed with vacuumed filtration by different concentration of Acetic Acid solution (pH 3.0, pH 4.0, pH 5.0, pH 6.0, pH 7.0) and released the trapped compound. As shown in the Figure 4B, the solution with pH 3.0, showed comparatively good effect (20 mg/L) than pH 4.0, 5.0, while the removal effect of neutral solution pH 6.0 and 7.0 could not show good effect. In brief, a relatively strong acidic solution was better than a weak one on the effect of 3-OH-PCA removal. So the total concentration of 3-OH-PCA reached up to 300 mg/L, in which 6% located on the pellet while 94% accumulated in the filtrate as illustrated in Figure 4C.

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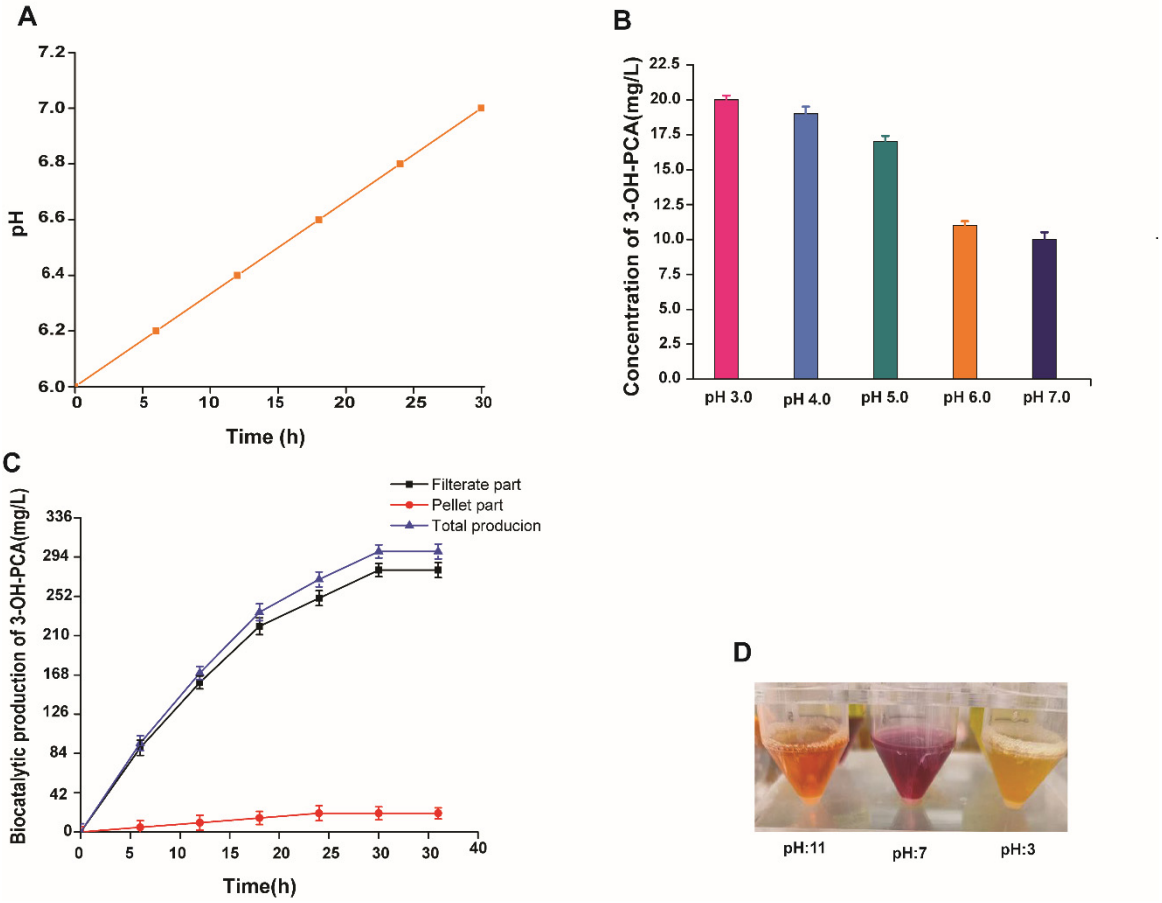

Figure 4. (**A**) Time courses of broth pH (**B**) Effect of various washing solution on the removal of 3-OH-PCA from the fungal pellets. (**C**) Distribution of newly produced compound (3-OH-PCA) in the cultured of *Aspergillus sclerotiorum* in shake flask. (**D**) The newly formed compound are sensitive and change its color with pH change.

3.4. Purification and Structure Elucidation of the Biotransformation Product

After the confirmation that, a new product was accumulated through whole cell biocatalysis of PCA, thus, a large scale(4L) biotransformation experiment was conducted and the accumulated product was purified by ethyl acetate extraction and silica gel column chromatography as shown in Figure 5A. After the purification process, total 1g new metabolite (3-OH-PCA) was obtained from 1.3 g of PCA. The high resolution mass was determined by UPLC-MS analysis Figure 5B. The high-resolution mass of the new compound from [M+H]+ peak was m/z 241.0539 (calculated for $C_{13}H_8N_2O_3$, 241.0539) suggesting that the molecular formula is $C_{13}H_8N_2O_3$ as reported previously [22].

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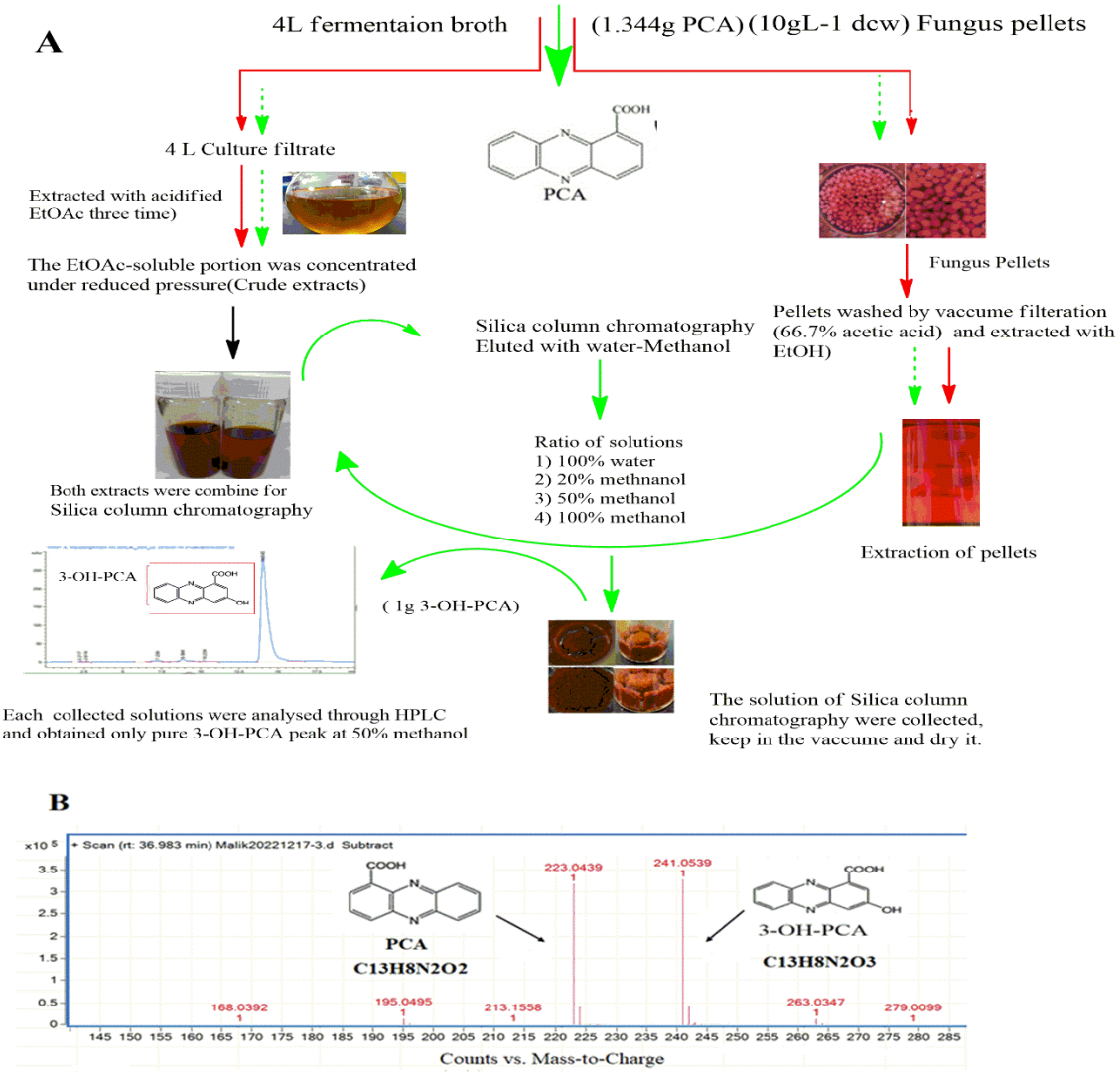

Figure 5. (**A**) Flow chart of the extractions and purification of phenazine metabolite 3-OH-PCA. (**B**) UPLC-MS spectrum of the newly accumulated compound. The molecular mass of the new compound was calculated to be 241.0539 (m/z 241.0539).

3.5. Whole Cell Biomass Preparation of A. sclerotiorum from Glucose

Since the 3-OH-PCA showed strikingly red condense color and most possibly act as coloring agent, there is dire need to search a low cost way for the production of fungus pellets. Thus, a most proficient whole cells biotransformation system was developed since the production of PCA 10 g/L has been reported [39]. Rich medium is the best option to prepare whole cell biomass of fungal strain *A. sclerotiorum*, subsequently we have used YPD medium following by biotransformation reaction in mineral salt medium. Firstly, the growth of fungus strain *A. sclerotiorum* was investigated in YPD medium containing 20 g/L different carbon sources including glycerol, glucose and xylose. As illustrated in the Figure 6A, the strain *A. sclerotiorum* can grow in YPD medium with the above mentioned carbon sources. The dry cell weight (DCW) reached up to 10.6 g/L after the strain was incubated for 60 h in YPD medium containing glucose and glycerol as a carbon sources, which was higher than that obtained in YPD medium containing Xylose. In the first 36 h and 48 h, the growth efficiency of the fungal strain was higher on the medium containing glycerol as a carbon source, while after 60 h of incubation the dry cell weight of the fungal strain obtained from the medium containing glucose was similar to glycerol. Glucose is the major carbon source in microbial fermentation and widely produced through natural process photosynthesis. Thus, the strain *A. sclerotiorum* was further cultivated used different concentration of glucose as a carbon source in YPD medium. As shown in the Figure 6B, the dry cell weight of the fungal strain increased initially with the added glucose concentration

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and reached up to 10 g/L, but further enhancement in the dry cell weight of the fungal biomass was negligible, when the concentration of glucose was higher than 20 g/L. Thus, the YPD medium containing 20 g/L was selected to prepare fungal biomass for whole cell biocatalysis of PCA.

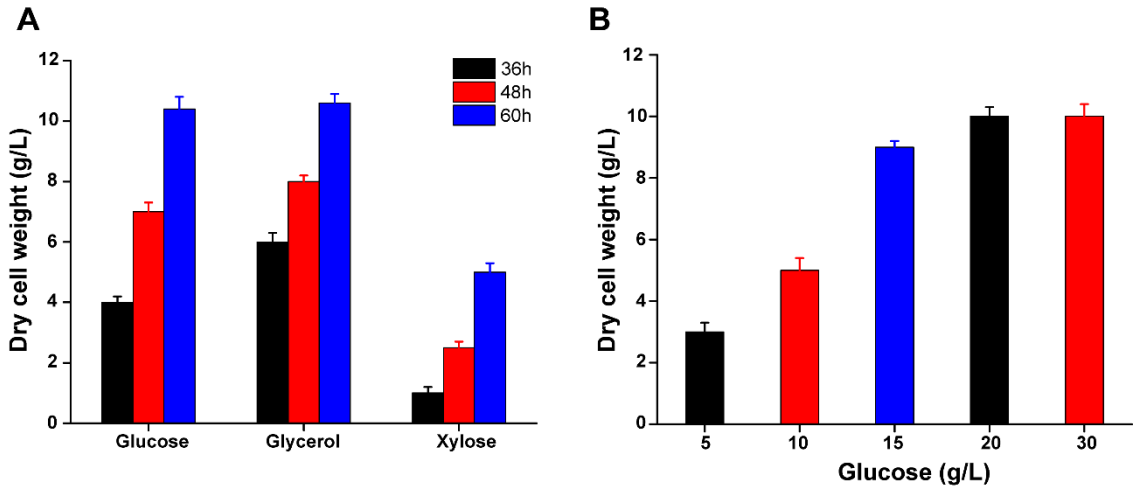

Figure 6. Screening carbon sources for the preparation of *A. sclerotiorum* whole cells. (**A**) Dry cell weight (DCW) of *A. sclerotiorum* cultivated in (YPD) medium supplemented with different carbon sources. (**B**) The DCW and biomass production using different concentrations of glucose.

3.6. Biocatalytic Production of 3-OH-PCA Under Optimized Conditions

The biocatalytic production of a new metabolite from PCA catalyzed by *A. sclerotiorum* was assessed for critical optimization factors such as substrate concentration, rotatory rate, pH, and temperature. In terms of enzyme overproduction, altering the culture conditions could be more helpful than genetic alteration [40,41]. Therefore, culture conditions were optimized in the current study using a one-variable-at-a-time procedure

3.7. The Effect of PCA Concentration on the Biocatalytic Production of 3-OH-PCA

The substrate concentration is an essential variable in biocatalysis reactions, and it requires thorough research to reveal the toxicity of the substrate. Different PCA concentrations 4 mM (896 mg/L), 5mM(1120 mg/L), 6mM(1344 mg/L), 7mM (1568 mg/L), and 8 mM(1792 mg/L) were tested to determine its effect on *A. sclerotiorum* biotransformation efficiency. As demonstrated in Figure 7A and 7B, the strain *A. sclerotiorum* could completely biotrasform PCA into a new metabolite(3-OH-PCA) within 60 and 72 hours as PCA concentration were 896 and 1120 mg/L respectively. The maximum accumulated concentration of new formed compound (3-OH-PCA) were 850 and 1030 mg/L respectively. The production rate of newly formed compound were 14.16 and 15.5 mg/L/h as the initial concentration of PCA were 896 and 1120 mg/L respectively. PCA could not be entirely biotrasform as the initial PCA concentration was higher than 1344 mg/L. The conversion efficiency of PCA was 94.8% and 92% and resulted 850,1030 mg/L of newly formed compound. So according to the obtained results, the bioconversion rate of PCA and the net accumulation of 3-OH-PCA are higher, when the concentration of PCA is 1120 mg/L. Thus, we have used this concentration for the following experiments.

Consistent with our study, substrate concentration positively influences the bioconversion process of 2-methyl-1-phenyl propane-1-one up to 33.7 Mm (5.0 g), but has an inhibitory impact as its concentration increases. This suggested that the enzyme activity decreased with increasing concentration, resulting in substrate toxicity [42,43]. Another research emphasized the relevance of substrate concentration in the whole-cell biocatalysis process, and comparable characteristics were observed in whole cells of the fungus *Aspergillus flavus*. When the substrate soybean saponin concentration was increased from 2%, the biotransformation rate reduced along with inhibition of Soyasapogenol B synthesis[42]. Several studies have revealed the same phenomenon during the chiral chemicals

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production in the whole cells biocatalysis reaction of recombinant strain *E. coli* [44]. Likewise, the biocatalytic potential of fungal strain *Aspergillus sydowii* CBMAI 934 reduced with increasing phenyl acetonitrile concentration from 20.0 µL, and no substrate conversion into the corresponding carboxylic acid at 40.0 and 60.0 µL nitrile [45]. Therefore, the results of the current study explored that the substrate concentration is critical in precisely determining the capacity of the whole-cell biocatalysis process.

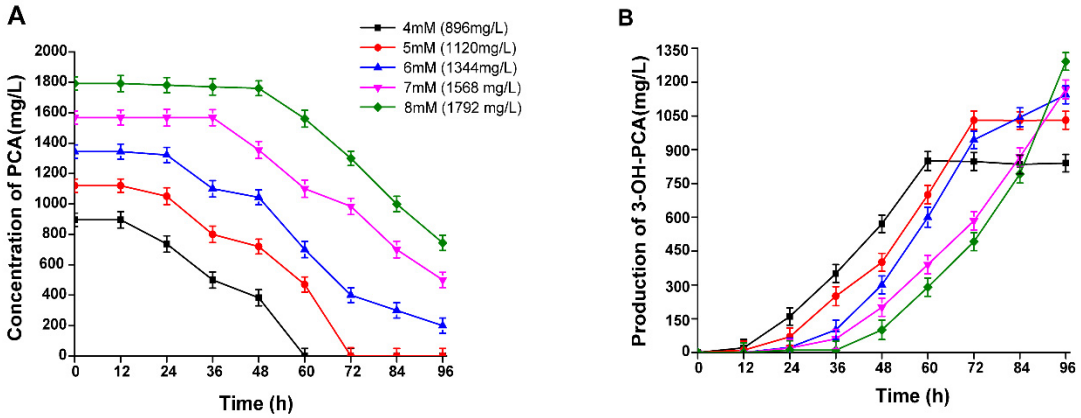

Figure 7. Biotransformation of various PCA concentrations into 3-OH-PCA using strain *A. sclerotiorum.* (**A**) concentration of PCA, (**B**) production of 3-OH-PCA.

3.8. Effect of Initial pH on the Biotransformation of PCA

The pH had a significant impact on the overall efficiency of the biocatalysis process in the cell. In the current study, biotransformation of PCA was performed at various pH values (3.0, 5.0, 7.0, 9.0 and 12.0) using strain *A. sclerotiorum*. When the initial pH values were 5.0 and 7.0, strain *A. sclerotiorum* could completely biotrasform PCA within 72 h, respectively. Hence, comparing the biocatalytic potential of the strain *A. sclerotiorum* between pH 5.0 and 7.0, the biotransformation rate of PCA was the highest at pH 7.0 between 24 h and 60 h. As a result, 3-OHP-PCA was significantly accumulated and its concentration were 1045 and 990 mg/L respectively. Although, the PCA biotransformation at pH 9.0 was not good and the residual PCA concentration maintained stable up to the end of fermentation as shown in the Figure 8A, 8B. The PCA biotransformation efficiency was 62.5% and 700 mg/L 3-OH-PCA was obtained at the initial pH value 9.0. According to the obtained results, the PCA was well biotransformed under neutral condition. The ionic state of enzymes, substrates, and products modified by pH can directly or indirectly affect enzyme activity and significantly impact substrate-enzyme active site interaction [46]. Furthermore, the substrate's solubility and ionic state, which fluctuate with pH, influence the enzyme's selectivity and active site [47,48]. Consequently, pH altered the enzyme's structure and active site, resulting in a distinct interaction of the active site with the substrate, which impacted the rate of biocatalysis in *A. sclerotiorum*. Hence, a neutral pH of 7.0 was employed for biocatalytic production of 3-OH-PCA.

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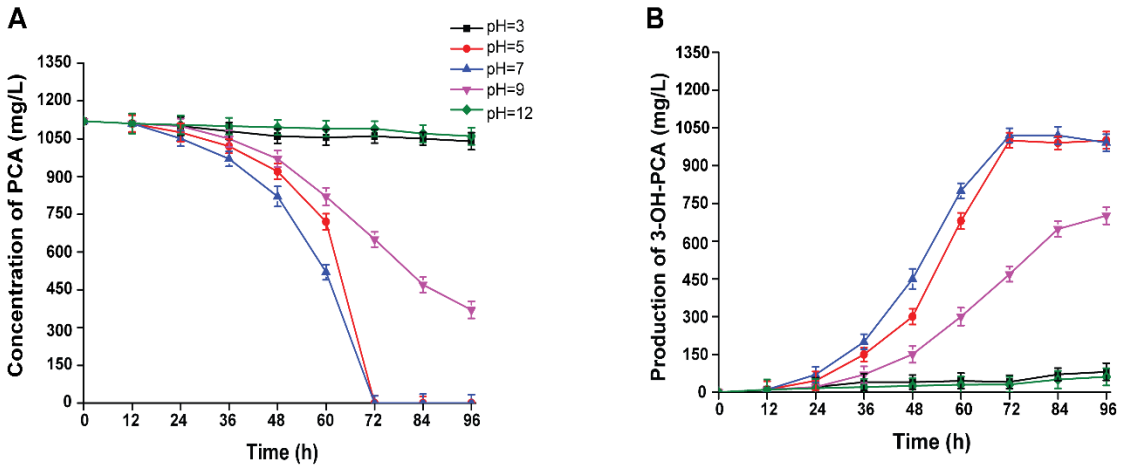

Figure 8. Biotransformation of PCA into 3-OH-PCA at various initial pHs using strain *A. sclerotiorum* in shaking flasks. (**A**) PCA (**B**) 3-OH-PCA.

3.9. Effect of Incubation Temperature on the Biotransformation of PCA

The temperature has a significant impact on the biocatalyst's stability, activity, and selectivity. Therefore, evaluated the effect of various temperatures (25°C, 30°C, 33°C, 37°C, and 42°C) on the PCA biocatalytic process. Strain *A. sclerotiorum* could completely biotrasform 1120 mg/L within 72 h under 25-30 °C. Comparing whole cell biocatalysis of PCA within 36 h and 60 h, the PCA biotransformation rate at 25 °C was the highest followed by 30 °C. The accumulated 3-OH-PCA concentration were 1040 and 1005 mg/L respectively. Although, the PCA biotransformation rate at 33 °C and 37 °C within the first 24 h was the highest among the various incubation temperatures, the biocatalysis declined significantly after 24 h. At least 84 h and 96 h are needed to biotrasform PCA, and the concentration of accumulated 3-OH-PCA was 920 and 910 mg/L respectively as shown in the Figure 9A, 9B. However, at 42°C, the enzymes were completely inhibited, and no biocatalytic activity was detected. Thus, the current study advocated the role of optimal temperature (25 °C) in effective whole-cell biotransformation of PCA.

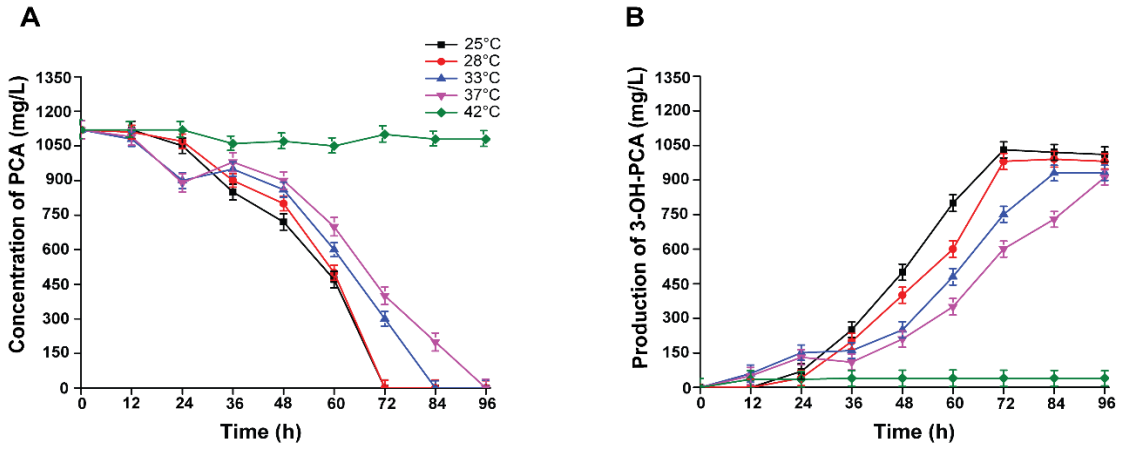

Figure 9. Biotransformation of PCA into 3-OH-PCA at various temperatures using strain *A. sclerotiorum*. (**A**) PCA (**B**) 3-OH-PCA.

3.10. Effect of Agitation Rate(rpm) on the Biotransformation of PCA

Agitation speed's effect on substrate particles' transfer rate in the biocatalysis process and play crucial role in its efficiency. So in the current study, the rotation rate(RPM) optimization for PCA biotransformation was performed at various rotatory rate (200, 100, 50,0) using strain *A. sclerotiorum*. When the agitation value was 200 rpm, the capability of PCA biotransformation was optimal and

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1120 mg/L of PCA was depleted within 72 h of incubation. As a result, 1050 mg/L of 3-OH-PCA was accumulated during the same incubation period. Moreover, the PCA biotransformation potential of the fungal strain reduced comparatively during the agitation of 100 rpm and PCA residues were sustained until the end of the experiment. The conversion efficiency of PCA was 83% and 970 mg/L 3-OH-PCA was obtained. Although, the bioconversion efficiency of PCA was not good during the agitation rate of 50 rpm and residual PCA was sustained, hence the accumulated concentration of 3-OH-PCA was 820 mg/L as shown in the Figure 10A, 10B. Apart from that, the biotransformation efficiency of PCA at 0 rpm was 19.6% and the concentration of accumulated 3-OH-PCA 220 mg/L. Briefly, the highest biocatalytic conversion of PCA was found at 200 rpm, attributable to the fact that a high rotatory rate could have altered the internal structure of the cells by increasing stress distribution on the strain *A. sclerotiorum*[49]

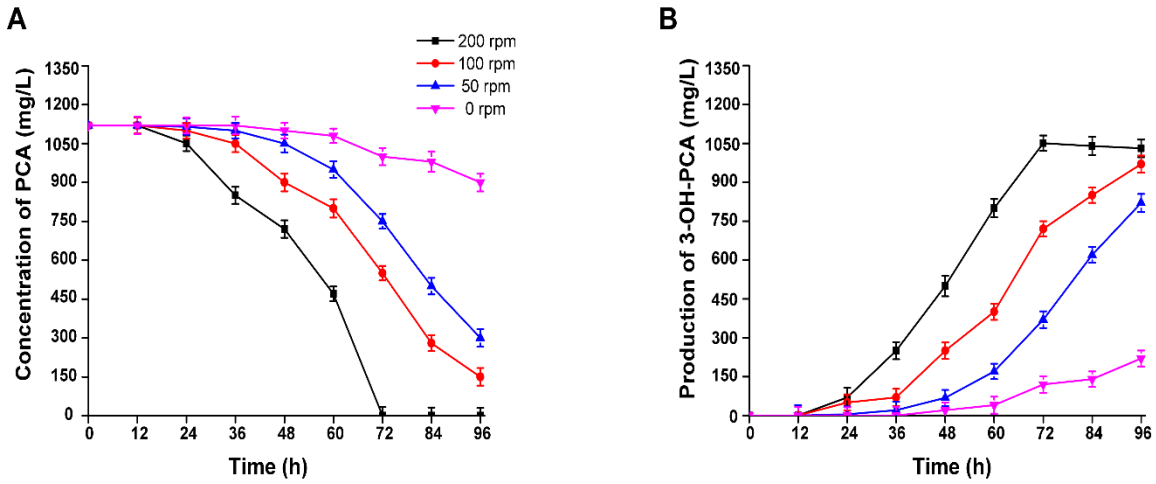

Figure 10. Biotransformation of PCA at various rotation rate ranging from 0 rpm to 200 using strain *A. sclerotiorum.* (**A**) PCA, (**B**) 3-OH-PCA.

3.11. Fungus Biomass Optimization and its Effect on the Longevity on the Biotransformation of PCA

It is apparent that, the biomass concentration will affect the longevity of whole cell biocatalysis of PCA, thus four different concentration of whole cell biomass (5,10,15,20 g/L DCW) were used and carryout biotransformation. As expected, the use of biocatalyst in higher concentration did not lead to better biotransformation results, but inversely affect the longevity of PCA biocatalysis. When the concentration of dry biomass of fungal strain was 20 g/L, the biotransformation efficiency of PCA between 12 h and 48 h was less than 15,10 g/L of biomass and took 72 h for fully depletion of PCA. Hence the concentration of accumulated 3-OH-PCA was 1000 mg/L. Moreover, when the concentration of whole cell biomass was 15 g/L, the biotransformation efficiency was the highest with the lowest longevity (60 h) of PCA biotransformation. As a result, the accumulated concentration of 3-OH-PCA was 1060 mg/L. However, when the biomass concentration was 10 g/L the longevity of the biotransformation was 72 h while its efficiency was good within 12 h and 48 h than 20 g/L of biomass. The accumulated concentration of 3-OH-PCA was 1015 mg/L. In case of 5 g/L of biomass, the efficiency of biotransformation was not good and the PCA residues were sustained until the end of the experiment as shown in the Figure 11A, 11B. Briefly, various biomass concentration 5,10,15,20 g/L showed bioconversion efficiency 89.2%, 94%, 90.6%, 62% respectively, while the concentration of accumulated 3-OH-PCA was 1000, 1060, 1015, 700 mg/L.

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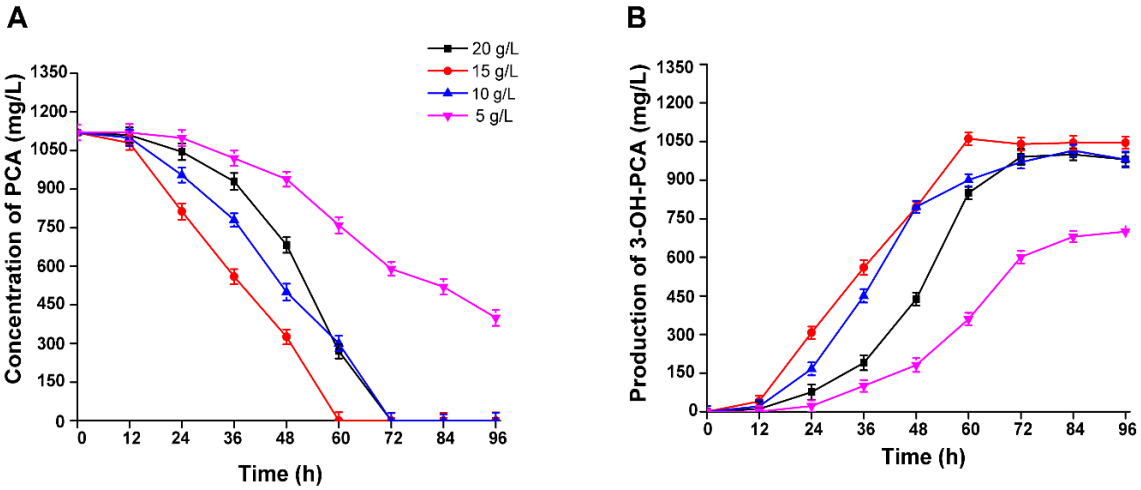

Figure 11. Biotransformation of PCA in to 3-OH-PCA at various biomass concentration using strain *A. sclerotiorum*. (**A**) PCA, (**B**) 3-OH-PCA.

3.12. Time Course of 3-OH-PCA Production in One-pot Whole Cells Biotransformation of PCA

Based on the optimized conditions mentioned above, the time course of whole cell biocatalysis of PCA for the production of 3-OH-PCA was explored as shown in the Figure 12A. Strain *A. sclerotiorum* smoothly modify PCA and the accumulation of 3-OH-PCA increased consecutively. After the incubation of 60 h, the PCA was depleted completely for the production of 3-OH-PCA. Overall, 1060 mg/L of 3-OH-PCA was produced from 1120 mg/L of PCA in 60 h, thus the conversion rate of PCA into 3-OH-PCA was 94%.

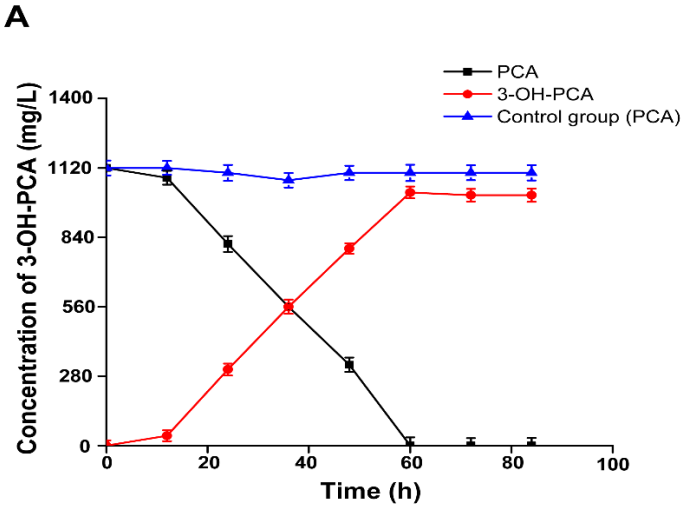

Figure 12. (**A**) Time course of biocatalytic production of 3-OH-PCA by whole cells biotransformation of PCA.

3.13. Repetition and Continuous Utilization of Fungus Pellets for Fed-batch Fermentation

Biocatalyst recycling is considered as a major element in the industrial process of whole-cell biocatalysis [50]. Therefore, the biotransformation efficiency of *A. sclerotiorum* pellets were investigated in repeated- batch mood with glucose supplement and without glucose supplement separately, where MSM medium with optimized PCA concentration (1120 mg/L) was replenished at the end of each cycle and the fungus pellet was recycled. The study was conducted over five cycles with the residence time of 60 h as shown in the Figure 13A. In all the cycles biotransformation occurred using the optimized conditions with the residence time of 60 hours. In the first cycle biocatalytic production of 3-OH-PCA was 1055 mg/L, which indicated that the biotransformation efficiency of PCA was 94%.

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Consequently, the accumulation of 3-OH-PCA decreased with each next cycle such as 980,750,500, 300 mg/L and the PCA biotransformation efficiency were 87%, 67%, 44%, 26% respectively. Nevertheless, the biotransformation efficiency of each group decreased with each subsequent cycle, but the fungal pellets retained their original shape and integrity. It might be ascribed to the fungal strain being regularly exposed to homologous concentrations of PCA in fresh flasks, causing unfavorable and inhibitory effects on the strain[51]. It is also conceivable that the cofactor's regeneration capability of the pellets reduced with each cycle attributed to nutrient deficiency, resulting in a potential catalytic reduction of the enzyme. Therefore, additional carbon sources could be used to sustain the biocatalytic capability of the fungal pellets [52]. Thus, as shown in the Figure 13B. glucose supplements (20 g/L) was added at the end of each cycle and the biocatalytic efficiency of fungal pellets were resumed and sustained until the end of the 5th cycles.

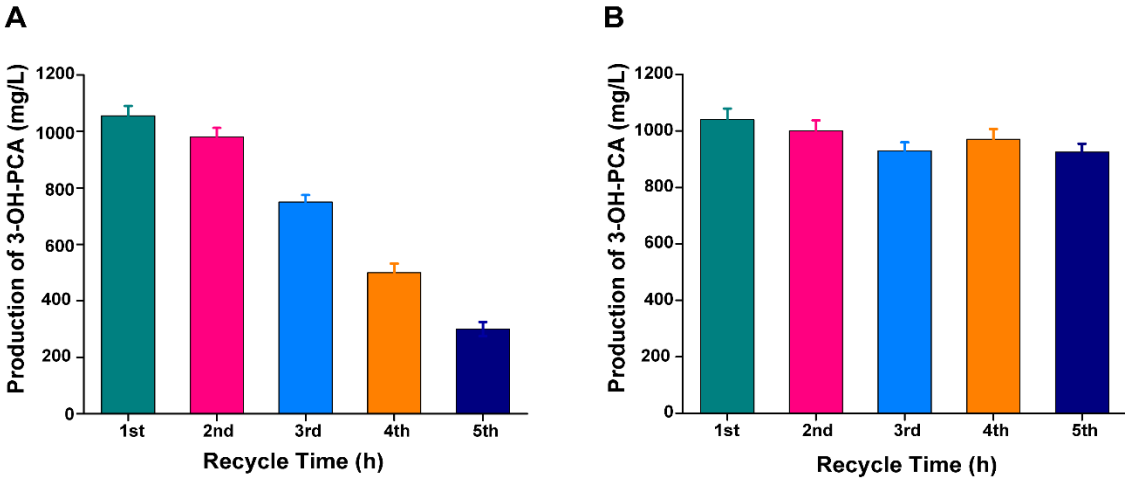

Figure 13. Biotransformation of PCA into 3-OH-PCA using Pellets of *Aspergillus sclerotiorum* in recycling mood with residence time of 60 h. (**A**) No glucose supplement addition at the end of each cycles (**B**) Glucose supplement addition at the end of each cycles.

4. Conclusion

In this study, fungus strain was isolated from the contaminated culture plate of PCA-producing bacterial strain *Pseudomonas chlororaphis* GP72. The strain was identified as *Aspergillus sclerotiorum* based on macroscopic features and complete ITS sequencing analysis. It has explored for the first time that, *A. sclerotiorum* efficiently modify PCA and produced 3-OH-PCA through whole cell biocatalysis. Gram scale (4L) biotransformation reaction was performed and following the extraction and purification process total 1 g of newly produced compound was obtained using 1.3 g of PCA. The high-resolution mass of the new compound from [M+H]+ peak was m/z 241.0539 (calculated for $C_{13}H_8N_2O_3$, 241.0539) using UPLC-MS analysis and suggesting that the compound as 3-OH-PCA. Finally, a more proficient whole cell biocatalytic system was constructed for the production of 3-OH-PCA by screening different carbon sources and optimizing physiochemical parameters. Further, we examined the fungal pellet's potential in repeated batch fermentation and observed the biotransformation efficiency reduction with each subsequent cycle with no glucose supplements, but the shape and integrity of the pellets were maintained suggesting that additional carbon sources can refuel the biocatalytic potential of the fungus pellets for the continuation of the biocatalytic process. Hence, sustainable biotransformation efficiency was observed with the addition of glucose supplement at the end of each cycle. The results of this study provide a sustainable platform for the biocatalytic production of phenazine derivatives using a new biocatalyst *A. sclerotiorum* that carried out PCA hydroxylation at carbon-3 position. The derivate metabolite 3-OH-PCA has a red condense color and might have some potential of dye in biochemical and biological industries, but further explorations are necessary.

We have conducted the first scientific study providing evidence of PCA whole-cell biocatalysis by a filamentous fungus strain *A. sclerotiorum* at an unprecedented concentration, which has not been reported previously. Furthermore, these findings will serve as a basis for future study into using this strain for the industrial-scale biocatalytic synthesis of 3-OH-PCA. The mechanism and appropriate pathway of PCA biotransformation by strain *A. sclerotiorum* are still underway. Additional research is needed to explore its commercial applicability.

Author Contributions: Conceptualization, M.J and H.H.; methodology, M.J.; validation.; formal analysis, M.J and S.Y.; investigation, M.J.; data curation, M.J.; writing—original draft preparation, M.J.; writing—review and editing, R.D, S.Y, Y.N and W.W.; project administration, H.H and X.Z.; funding acquisition, X.Z. All authors have read and agreed to the published version of the manuscript.

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