catalysts

MDPI

Article

Study on Transglucosylation Properties of Amylosucrase from *Xanthomonas campestris pv. Campestris* and Its Application in the Production of α-Arbutin

Chengyu Yang [1,2], Weiming Fan [2,3], Ruijie Zhang [1,2,4], Jiping Shi [1,2,4,*], Zorica Knežević-Jugović [5] and Baoguo Zhang [1,2,*]

1 Lab of Biorefinery, Shanghai Advanced Research Institute, Chinese Academy of Sciences, No. 99 Haike Road, Pudong, Shanghai 201210, China; yangchengyu@sari.ac.cn (C.Y.); zhangrj@sari.ac.cn (R.Z.)
2 University of Chinese Academy of Sciences, Beijing 100049, China; sxzyfwm@163.com
3 Zhejiang Zhenyuan Pharmaceutical Co., Ltd. No. 61 North Yuedong Road, Shaoxing 312000, China
4 School of Life Science and Technology, Shanghai Tech University, No. 393 Huaxia Middle Road, Pudong, Shanghai 201210, China
5 Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, Belgrade 11000, Serbia; zknez@tmf.bg.ac.rs
* Correspondence: shijp@sari.ac.cn (J.S.); zhangbg@sari.ac.cn (B.Z.); Tel.: +86-021-2035166 (J.S.); +86-021-2035166 (B.Z.)

Received: 5 December 2018; Accepted: 18 December 2018; Published: 21 December 2018

Abstract: α-Arbutin (4-hydroquinone-α-D-glucopyranoside), an effective skin-lightening agent due to its considerable inhibitory effect on human tyrosinase activity, is widely used in the pharmaceutical and cosmetic industries. Recently, α-arbutin was prepared through transglucosylation of hydroquinone using microbial glycosyltransferases as catalysts. However, the low yield and prolonged reaction time of the biotransformation process of α-arbutin production limited its industrial application. In this work, an amylosucrase (ASase) from *Xanthomonas campestris pv. campestris* str. ATCC 33913 (XcAS) was expressed efficiently in *Escherichia coli* JM109. The catalytic property of the purified XcAS for the synthesis of α-arbutin was tested. The recombinant strain was applied for highly efficient synthesis of α-arbutin using sucrose and hydroquinone as glucosyl donor and acceptor, respectively. By optimizing the biotransformation conditions and applying a fed-batch strategy, the final production yield and conversion rate of α-arbutin reached 60.9 g/L and 95.5%, respectively, which is the highest reported yield by engineered strains. Compared to the highest reported value (<1.4 g/L/h), our productivity (7.6 g/L/h) was improved more than five-fold. This work represents an efficient and rapid method for α-arbutin production with potential industrial applications.

Keywords: Amylosucrase; *Xanthomonas campestris pv. Campestris*; α-arbutin; enzymatic properties; whole-cell biocatalyst; fed-batch strategy

1. Introduction

α-Arbutin, a glycosylated hydroquinone, is a prominent natural compound used as a skin-whitening agent due to its antimelanogenesis effect without any cytotoxicity [1]. It has been reported that the inhibitory function of α-arbutin against tyrosinase is 10 times greater than that of its isomer, β-arbutin [2]. Specifically, α-arbutin only inhibits mammalian tyrosinase, while β-arbutin reduces tyrosinase activity by both melanoma and somatic cells [3]. Moreover, a study on cultured human melanoma cells and three-dimensional skin models indicates that α-arbutin does not inhibit cell viability and exhibits outstanding performance in reducing melanin synthesis [2]. α-Arbutin also

shows antioxidative, antimicrobial, and anti-inflammatory functions. Therefore, the use of α-arbutin as a safe and effective skin-whitening agent has received increasing attention in recent years.

A few strategies have been used to synthesize α-arbutin from hydroquinone (HQ). Generally, two main strategies have been employed. The first involves applying microbial strains to transform HQ substrates into α-arbutin. Native microbial strains such as *Bacillus subtilis* [4] and *Xanthomonas campestris* [5], as well as mutant strains of *Xanthomonas maltophilia* BT-112 [6–8], have been used to produce high concentrations of α-arbutin. However, the relatively low productivity (1.4 g/L/h) of α-arbutin synthesis and the use of immobilized substrate limited the large-scale application of the process. The second strategy involves HQ glycosylation via many different microbial glycosyltransferases. To date, at least seven different enzymes have been reported in α-arbutin biosynthesis, including α-glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae* and *Xanthomonas campestris* [9,10], amylosucrase (EC 2.4.1.4) from *Deinococcus geothermalis* and *Cellulomonas carboniz* [11,12], sucrose phosphorylase (EC 2.4.1.7) and dextransucrase (EC 2.4.1.5) from *Leuconostoc mesenteroides* [13,14], α-amylase (EC 3.2.1.1) from *Bacillus subtilis* X-23 [4], cyclodextrin glycosyltransferase (EC 2.4.1.19) from *Thermoanaerobacter* sp. [15], and sucrose isomerase (EC 5.4.99.11) form *Erwinia rhapontici* [16]. However, the production yield and molar conversion rate are low, which hampers their widespread application and commercialization. Compare with other microbial enzymes, amylosucrase displays prominent transglucosylation activity in α-arbutin biosynthesis with sucrose as a glucosyl donor and hydroquinone as an acceptor.

Amylosucrase (ASase), belonging to the glycoside hydrolase 13 family, is a versatile enzyme catalyzing multiple reactions [17]. With sucrose and extra glucosyl acceptors as substrates, ASase has glucosyltransferase activity and can transfer glucose molecules from sucrose to glucosyl acceptors with α-1,4 glycoside linkages only [18,19]. This enzyme utilizes the energy derived from the cleavage of glucosidic bonds in sucrose to synthesize other glycosides without involving any primer or nucleotide-activated sugars [20]. Recently, the ASase gene has been cloned from *Deinococcus geothermalis* and *Cellulomonas carboniz* for in vitro biosynthesis of α-arbutin by using sucrose as a glucosyl donor and hydroquinone as acceptor [11,12]. In these studies, the high conversion rate (90%) and low reaction time (2 h) revealed that amylosucrase has remarkably higher transglucosylation capacity, showing good potential for commercial production of α-arbutin. As compared to the other transglucosidases, amylosucrase demonstrates higher efficiency in the synthesis of α-arbutin. However, to date, its maximum production yields are still low (5.8 g/L and 0.6 g/L for ASase from *Deinococcus geothermalis* and *Cellulomonas carboniz*, respectively). Thus, it is necessary to establish a simple and cost-effective method to produce α-arbutin.

In this study, we first employed an ASase from *X. campestris pv. campestris* (XcAS) as the catalyst to synthesize α-arbutin, and the catalytic property of the purified XcAS was clarified. In addition, α-arbutin was synthesized by a whole-cell recombinant *E. coli* JM109/pet28a-xcas biocatalyst system using sucrose as glucoside donor and HQ as acceptor. Furthermore, a fed-batch strategy was employed to increase the production yield of α-arbutin. Thus, this work represents a highly efficient method for α-arbutin production with potential industrial applications.

2. Results

2.1. XcAS Heterologous Expression and Protein Purification

The XcAS encoding gene, locus XCC3359 from the *X. campestris pv. campestris* str. ATCC 33913 genome, consists of 1914 base pairs encoding 637 amino acid proteins. The target gene was synthesized and inserted into the pET28a (+) vector with 6 × histidine tagged at its carboxyl terminus, resulting in expression of the pET28a-*xcas* plasmid. Through regulated expression of the *xcas* gene under the control of a T7 promoter and *lac* repressor, it was transformed into *E. coli* JM109 [DE3]. As shown in Supplementary Figure S1A, the initial expression result of XcAS was almost manifested as inclusion bodies (IBs). To improve the production of soluble amylosucrase, the process conditions were optimized. As shown in Supplementary Figure S2, the results suggest that 0.1 mmol/L

isopropyl β-D-1-thiogalactopyranoside (IPTG) and 25 °C is the optimal inducement condition for XcAS production, and almost all of the target proteins were expressed in soluble form (Supplementary Figure S1B). The soluble fraction of the expressed protein was purified as described in Materials and Methods and analyzed by SDS-PAGE. As shown in Figure 1, the purified protein had a molecular mass of approximately 66.2 kDa.

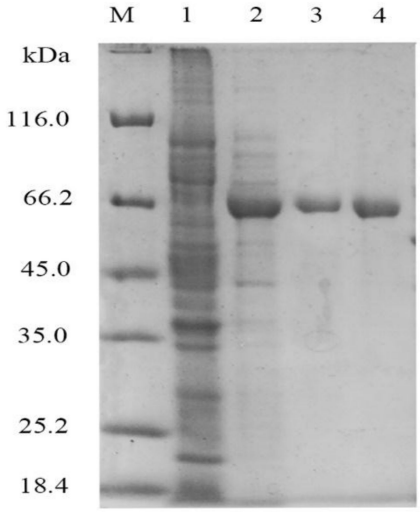

Figure 1. SDS-PAGE analysis of expressed *Xanthomonas campestris pv. campestris* (XcAS). Lane M shows the protein molecular weight marker; lane 1 shows total protein of *E. coli* JM109 cells harboring pET28a plasmid; lane 2 shows soluble supernatant of *E. coli* JM109 cells harboring the recombinant pET28a-xcas plasmid; lane 3 shows purified XcAS; and lane 4 shows purified XcAS after ultrafiltration.

2.2. Bioconversion of HQ to α-Arbutin by Purified XcAS

The transglucosylation reaction was performed using sucrose as a glucosyl donor and 10 mM hydroquinone as an acceptor in 50 mM phosphate buffer (Supplementary Figure S3A). It is known that benzoquinone, produced by the oxidation of hydroquinone, can inhibit the enzyme activity by chemically modifying and reducing its stability [9]. Thus, ascorbic acid (VC) was added to the reaction system to inhibit the oxidation of hydroquinone and improve the bioconversion rate of α-arbutin. After the reaction, the sample was analyzed by HPLC. As shown in Supplementary Figure S3B, there were two peaks at retention times (tR) of 4.5 min and 5.8 min, corresponding to α-arbutin and HQ, respectively.

2.3. Catalysis Characterization of Purified XcAS on Transglucoside Reaction

The influence of pH and temperature on the transglucoside activity of recombinant XcAS was determined by using sucrose as a glycosyl donor and hydroquinone as an acceptor. As shown in Figure 2A, the enzyme had optimal activity between pH values of 6.0 and 7.5, with specific activity of 0.9 U/mg. Moreover, in a pH stability assay (Figure 2B), XcAS transglucoside activity showed a slight reduction in pH in the range of 5.5–8.0 after being incubated in different pH at 4 °C. As shown in Figure 2C, the maximal transglucoside activity of the recombinant XcAS was at 30 °C. Furthermore, thermal stability analysis indicated that the enzyme was stable from 20 to 30 °C. However, the activity of XcAS decreased sharply when the temperature increased above 35 °C (Figure 2D). The kinetics of the XcAS-catalyzed transglucoside reaction was tested at the optimal pH and temperature using sucrose as a glucosyl donor and hydroquinone as an acceptor. As shown in Figure 3, the K_m and V_{max} values of the XcAS-catalyzed reaction were 11.29 ± 0.43 mM and 2.49 ± 0.04 U/mg, respectively.

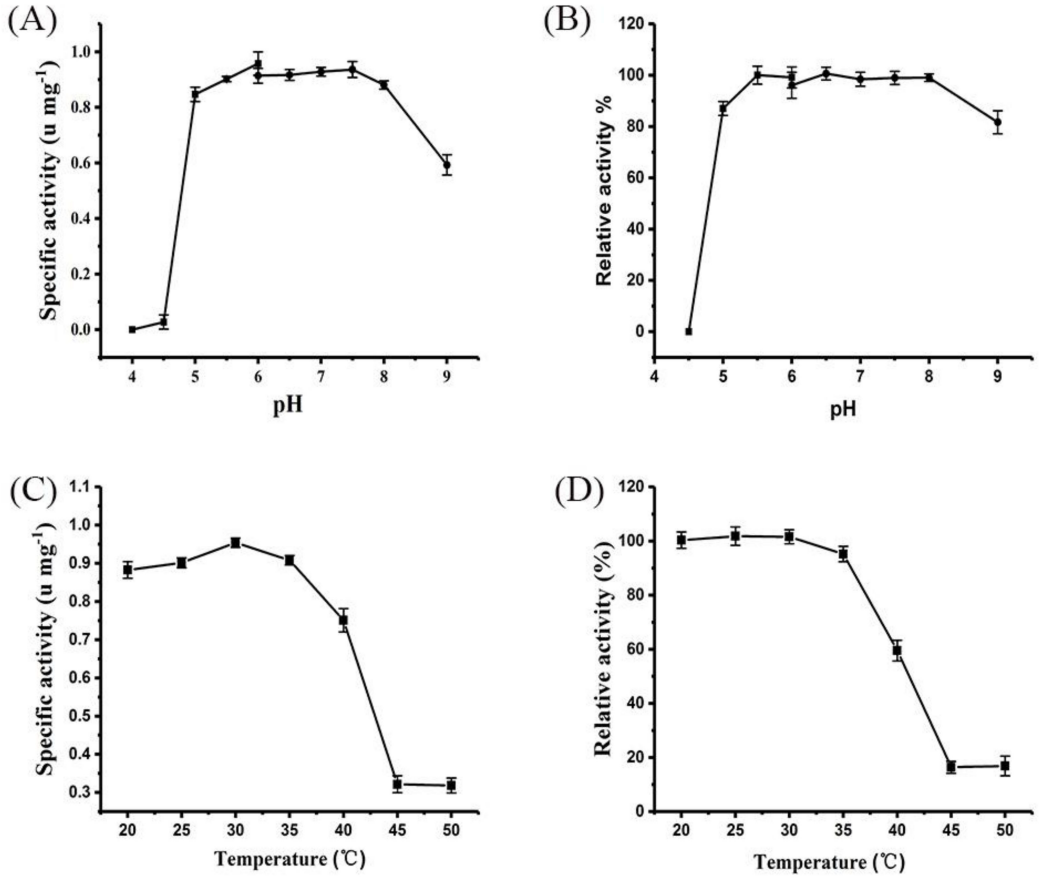

Figure 2. Effects of pH and temperature on purified XcAS transglucoside activity and stability. (**A**) Specific activity of XcAS at different external pH. Assays were performed at 30 °C in 50 mM citrate buffer (pH 4.0–6.0) and 50 mM sodium phosphate buffer (pH 6.0–9.0). (**B**) pH stability of XcAS. Purified XcAS was preincubated at pH varying from 4.5 to 9.0 for 24 h at 4 °C before enzyme activity was measured. (**C**) Specific activity of XcAS at different temperatures. Tests were performed at pH 7.0 in 50 mM sodium phosphate buffer. (**D**) Enzyme stability of XcAS at different temperatures. Assays were performed after preincubating XcAS for 1 h at different temperatures.

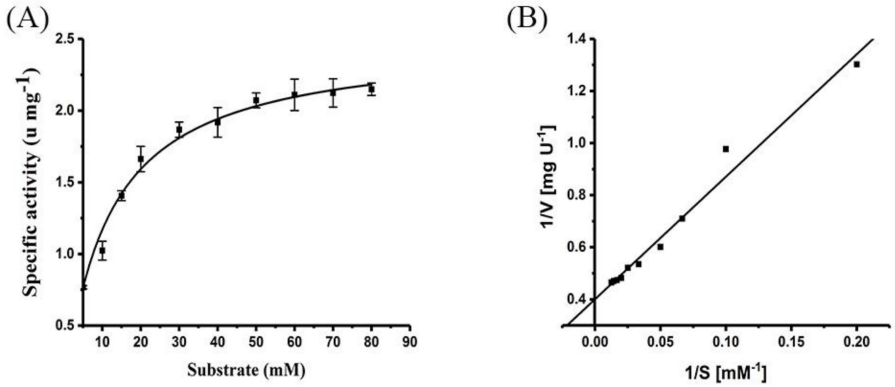

Figure 3. Determination of kinetic parameters of XcAS-catalyzed transglucoside reaction: (**A**) Michaelis–Menten plot and (**B**) double-reciprocal plot. Reactions were carried out at pH 7.0 and 30 °C by adding various amount of sucrose and hydroquinone (HQ) (ratio 10:1) to 50 mM sodium phosphate buffer.

2.4. Effects of pH and Temperature on α-Arbutin Production by Whole-Cell Biocatalysis

Bioconversion of α-arbutin from HQ by whole-cell biocatalysis is a favorable approach with many advantages [7,21]. Thus far, α-arbutin production yield is limited by the low level of enzyme activity, and research on high substrate concentration for α-arbutin production is still lacking. In the present study, *E. coli* JM109/pet28a-*xcas* with a high activity of amylosucrase, as described above, was used for the bioconversion of HQ to α-arbutin. Furthermore, the biotransformation conditions were optimized.

To optimize the efficiency of whole-cell biocatalysis on α-arbutin production, the effects of pH and temperature were investigated. As illustrated in Figure 4A, a reaction mixture containing 20 g/L wet cell weight (WCW) of the culture, 167 mM of HQ, and 1 M of sucrose in 10 mL of phosphate buffer (50 mM, pH 7.0) was incubated for 12 h under a broad range of temperatures (20 to 40 °C). The results showed that the highest conversion rate of HQ was 80.4% at 30 °C, which can be used as the optimal temperature for whole-cell biocatalysis. As the temperature increased from 30 to 40 °C, the conversion rate reduced dramatically, suggesting that XcAS activity was low and unstable at temperatures higher than 30 °C. Additionally, the effect of pH (range 4–10) was examined on whole-cell biocatalysis. All of the experiments were carried out at 30 °C. At pH 7.0, the maximum conversion rate was obtained, while the production of α-arbutin sharply decreased with a slight divergence from the optimal pH (Figure 4B). Thus, the optimum pH value of 7.0 and temperature of 30 °C were chosen for further experiments.

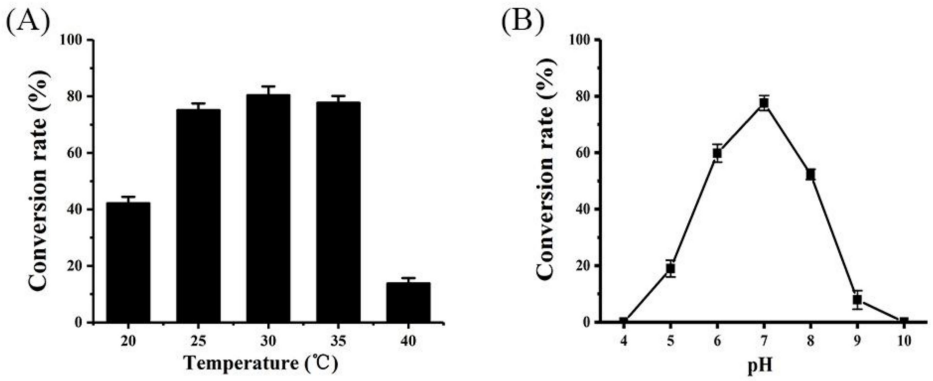

Figure 4. Effects of (**A**) temperature and (**B**) pH on α-arbutin conversion rate. All assays were performed in triplicate in three independent experiments. Standard deviations of biological replicates are represented by error bars.

2.5. Effects of Surfactant and Its Concentration on α-Arbutin Production

Surfactant additives can increase cell membrane permeability and enhance substrate transportation in whole-cell biocatalysis [22]. The effects of different surfactants (0.1% w/v) were investigated on the whole-cell *E. coli* JM109/pet28a-*xcas* biocatalyst system (Figure 5A). The additives hexadecyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) strongly inhibited the production of α-arbutin, whereas Tween-20, Tween-60, Tween-80, and TritonX-100 significantly improved its conversion rate. Of these surfactants, TritonX-100 had the greatest impact on conversion rate (90.6%), 115% higher than control, thus it was selected for further research.

To improve α-arbutin production, the effect of TritonX-100 concentration was further investigated. The results demonstrated that the conversion rate of HQ increased, attaining the highest value of 0.4% (w/v) with TritonX-100 induction (Figure 5B). However, the conversion rate decreased rapidly when a higher concentration of surfactant was added. Therefore, the optimal TritonX-100 concentration of 0.4% (w/v) was used for α-arbutin production, with a high conversion rate of 94.5%.

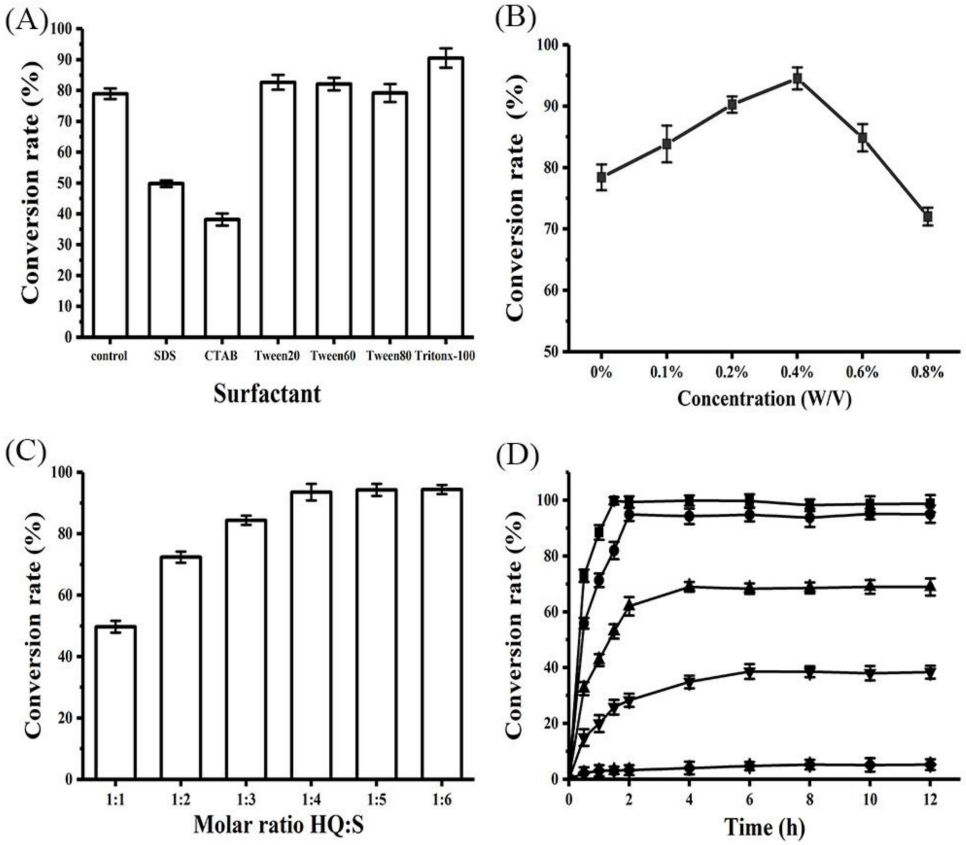

Figure 5. Effects of (**A**) surfactant, (**B**) surfactant concentration, (**C**) substrate ratio, and (**D**) substrate concentration on α-arbutin production: 125 mM (filled squares); 167 mM (filled circles); 208 mM (filled triangles); 250 mM (filled inverted triangles); 292 mM (filled diamonds). All assays were performed in triplicate in three independent experiments. Standard deviations of biological replicates are represented by error bars.

2.6. Effects of Substrate Ratio and Concentration on α-Arbutin Production

Enzymatic synthesis of α-arbutin from sucrose and HQ by *E. coli* JM109/pet28a-*xcas* is a one-step stereospecific reaction and its theoretical molar ratio is 1:1 [11]. The effect of the molar ratio of sucrose to HQ on α-arbutin production was investigated with 165 mM HQ in this study. When both substrates were in the same ratio (1:1), the conversion rate was only 49.7% (Figure 5C). By increasing the sucrose:HQ ratio to 4:1, the conversion rate increased to 93.5%. However, with further increases of the molar ratio to 5:1 and 6:1, the conversion rates remained approximately the same, indicating that the amylosucrase in *E. coli* JM109/pet28a-*xcas* was probably saturated with sucrose.

HQ can induce apoptosis in vivo through a change in cellular redox status, especially by reducing the endogenous thiol level and increasing the reactive oxygen species level [7,23]. If the concentration of HQ exceeds a certain value, the production of α-arbutin may drop sharply due to cell apoptosis. In this study, a series of HQ concentrations were tested and the time courses for these reactions were evaluated at the substrate ratio of 4:1. As shown in Figure 5D, 125 mM of HQ was completely converted within 2 h and the production of α-arbutin attained 34.1 g/L. Notably, the molar production yields of α-arbutin gradually decreased and the reaction time was extended with increasing concentration of HQ. In particular, the conversion rate decreased to 68% within 6 h after 208 mM of HQ was added. These results clearly demonstrate that the maximum HQ tolerance of *E. coli* JM109/pet28a-*xcas* was 208 mM. Furthermore, all the conversion rates retained their maximum values within 12 h.

2.7. Efficient Production of α-Arbutin through a Batch and Fed-Batch Strategy

Based on the aforementioned optimal conditions, a total yield of 33.8 g/L α-arbutin was obtained from 13.7 g/L of HQ within 2 h using the whole-cell *E. coli JM109*/pet28a-*xcas* biocatalyst system (Figure 6A). The productivity and conversion yield were 16.9 g/L/h and 99.4%, respectively. No byproduct was detected during the bioconversion process, indicating that XcAS displayed region-selective activity.

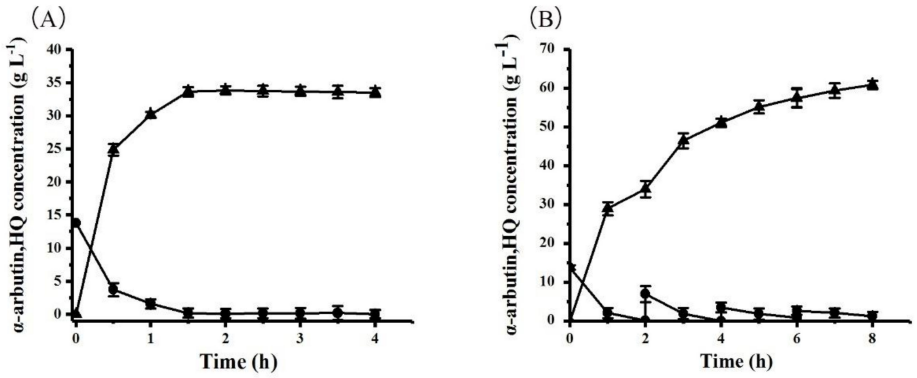

Figure 6. Time course for (**A**) batch and (**B**) fed-batch bioconversion of HQ to α-arbutin. Filled triangles represent concentration of α-arbutin; filled circles represent concentration of HQ.

A fed-batch strategy can improve the final concentration of the desired products [24]. A gradient decreasing batch was used to attain higher α-arbutin production for the sake of cell apoptosis induced by high HQ concentration. The initial concentration of HQ was 13.8 g/L (125 mM), and decreasing amounts of HQ (i.e., 62.5 mM, 31.25 mM, and 15.62 mM) and sucrose (molar ratio of HQ:sucrose was 1:4) were added to the conversion system every 2 h. After 8 h of conversion, the final α-arbutin yield reached 60.9 g/L, 180% higher than before (Figure 6B). More importantly, the final molar yield of α-arbutin reached 95.5% with a productivity of 7.6 g/L/h, which was the highest compared to other bioconversion methods (Table 1).

Table 1. Comparison of different biocatalysts or fermentative strains for α-arbutin production.

Strains and Biotransformation Model	Donor	Biotransformation Period (h)	Final Concentration (g/L)	Molar Yield (%)	Productivity (g/L/h)	Reference
Biocatalysis						
Xanthomonas campestris WU-9701	Maltose	36	11.43	93	0.32	5
B. subtilis strain X-23	Maltopentaose	0.6	1.50	24.8	0.4	13
Xanthomonas maltophilia BT-112	Sucrose	48	65.9	95.2	1.4	7
Escherichia coli JM109 (Amylosucrase)	Sucrose	8	60.9	95.5	7.6	This study
Enzymatic catalysis						
Amylosucrase (*Cellulomonas carboniz* T26)	Sucrose	2	0.608	44.7	0.31	12
Amylosucrase (*Deinococcus geothermalis*)	Sucrose	24	5.78	90	0.24	11
α-Glucosidase (*Saccharomyces cerevisiae*)	Maltose	20	1	4.6	0.05	9
Dextransucrase (*Leuconostoc mesenteroides*)	Sucrose	6	0.544	0.4	0.09	14
Fermentation						
Escherichia coli BL21 (α-glucosidase)	Maltose	36	21	76	0.58	25
Xanthomonas maltophilia BT-112	Sucrose	72	61.7	94.5	0.86	8

Biocatalysis is the transformation carried out using whole cells; enzymatic catalysis is the transformation carried out by purified enzyme; fermentation is the transformation carried out with fermentative strains.

3. Discussion

Amylosucrase is a multifunctional enzyme from the glycoside hydrolase family 13 that has powerful transglucosylation activity to generate α-1,4-linked glucose-transfer products from sucrose as glucoside donor [17]. To date, ASase from *Deinococcus geothermalis* (DG-ASase) [11,25] and *Cellulomonas carbonis* (CC-ASase) [12] has been reported to synthesize α-arbutin via the transglucosylation of inexpensive sucrose as glucosyl donor and HQ as glucosyl receptor. Compared

with other glycosyltransferases, amylosucrase does not require the addition of expensive activated sugars and exhibits predominant biotransformation ability and higher efficiency in the glycosylation of HQ (Table 1). In this study, we first employed an ASase from *X. campestris pv. campestris* (XcAS) as the catalyst to synthesize α-arbutin. The commercial expression vector pet28a+, containing an induced expression system, was used for heterologous expression of XcAS in *E. coli* JM109 (DE3). The transcriptional expression was controlled by the lac repressor, thus required IPTG as an inducer. The initial recombinant protein was mainly found in IBs (Supplementary Figure S1A) and displayed low enzymatic activity. Previous studies have reported that the heterologous protein, with a high molecular weight and complicated structure, further impedes the capacity of host cells to fold the protein intermediates, and results in the formation of IBs [26]. As a consequence, we optimized the temperature and IPTG concentrations for amylosucrase expression. The highest intracellular hydrolysis activity of 5.0 U/mg was obtained at a low temperature (25 °C) and IPTG concentration of 0.1 mM, and almost no IBs were seen in the expression results (Supplementary Figures S1B and S2). These phenomena can be explained by the reduced IB formation due to the proper folding of precursor proteins under low temperature and low inducer concentration [27,28].

The catalytic property of XcAS in the transglucoside reaction was studied by using sucrose as the glucoside donor and HQ as the receptor. The optimal pH of XcAS transglucosylation activity was in the range of 6.0–7.5 (Figure 2A) and the enzyme maintained stability between pH 5.5 and 8.0 (Figure 2B). It is assumed that the optimum working condition of this enzyme is neutral, which is similar to that of ASase from *Deinococcus geothermalis* (pH 7.0) [11], *Cellulomonas carboni* (pH 7.0) [12], and *Truepera radiovictrix* (pH 7.5) [29]. The maximal transglucosylation activity was obtained at 30 °C (Figure 2C) and dropped rapidly when the temperature was higher than 35 °C (Figure 2D). It appeared that the enzyme obtained in this study showed low thermal stability, which is a little different from CC-ASase (40 °C) and DG-ASase (45 °C). Furthermore, the apparent K_m and V_{max} values of the XcAS-catalyzed transglucoside reaction using HQ as acceptor were 11.29 ± 0.43 mM and 2.49 ± 0.04 U/mg, respectively (Figure 3). This suggests that XcAS has potential application in α-arbutin biosynthesis.

Recently, whole-cell biocatalyst has been widely used in the industrial production of glycosides, due to its efficient biotransformation process and simplified downstream purification procedure [30]. In our work, the resting cells of recombinant *E. coli* JM109/pet28a-*xcas* were prepared as a whole-cell biocatalyst in order to synthesize α-arbutin from HQ. By optimizing the biocatalysis conditions with the addition of TritonX-100 surfactant and using an HQ concentration of 125 mM, the production yield, conversion rate, and productivity of α-arbutin were found to be 33.8 g/L, 99.4%, and 16.9 g/L/h, respectively. Compared with other surfactants, TritonX-100 can obviously increase the conversion rate of α-arbutin, while SDS and CTAB have an inhibitory effect on whole-cell biocatalyst (Figure 5A). It appeared that much of this loss of amylosucrase enzymatic activity caused by ionic surfactants was due to the destruction of XcAS, while nonionic surfactants stimulated the reaction by increasing cell membrane permeability without affecting enzyme activity. In addition, the biocatalytic ability of *E. coli* JM109/pet28a-*xcas* cells was inhibited by a high HQ concentration (208 mM), which is consistent with previous studies [6,7,11,12]. A high concentration of HQ may induce cell apoptosis by changing intracellular redox status through decreasing the thiol level and increasing the reactive oxygen species level [31,32]. To prevent the toxicity of high HQ concentration while retaining α-arbutin production yield, a gradient decreasing fed-batch strategy was applied. As a result, the final production yield and molar conversion yield of α-arbutin reached 60.9 g/L and 95.5%, respectively, with an HQ concentration of 234.4 mM (Figure 6B). Furthermore, the highest productivity of α-arbutin (7.6 g/L/h) was obtained, which was five-fold higher than the previously reported value (<1.4 g/L/h).

Overall, our findings suggest that recombinant *E. coli* JM109/pet28a-*xcas* may be used as whole-cell biocatalyst system to produce α-arbutin from HQ. Furthermore, a high conversion rate in this system can simplify the downstream processing of α-arbutin purification. Hence, the proposed method may have potential applications for α-arbutin synthesis in the pharmaceutical and cosmetic industries.

4. Materials and Methods

4.1. Microorganism Plasmids and Chemicals

HQ, sucrose, and ascorbic acid (VC) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). α-Arbutin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction endonucleases, Pfu DNA polymerase, and In-Fusion Cloning Kit were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). *Escherichia coli* DH5α was used (primary host) for the construction of pet28a$^+$-xcas. *E. coli* JM109*[DE3] (recA1 supE44 endA1 hsdR17 (rk- mk+) gyrA96 thi Δ(lac-proAB) F′ [traD36 proAB laclqZΔM15] IDE3) was used as a host for pet28a$^+$-xcas to express the ASase gene.

4.2. Cloning and Recombinant Expression of ASase Gene from X. campestris pv. campestris

The full nucleotide sequence of ASase-encoding gene (GeneBank NC_003902.1, region: 40023–4004221) from *X. campestris pv. campestris* str. ATCC 33913 was commercially synthesized by Genewize Co., Ltd (Suzhou, China). The resultant plasmid pUC 57-xcas was used as a template to amplify the amylosucrase gene. Primers used in this study were as follows: As-F (5′-cgcggcagccatatgg ctagcATGATCGCTTCCTCCCCCA-3′) and As-R (5′-tggtggtggtggtgctcgagTCAACGACGCTGCAA CCAG-3′). The PCR product was inserted into pET-28a+ using In-Fusion®clone at *NheI* and *XhoI* sites, resulting in the expression vector of pET28a-*xcas*. The vector was then transformed into *E. coli* JM109*[DE3] cells for expression studies. Recombinant *E. coli* cells were grown in 50 mL Terrific Broth (TB) medium (11.8 g/L tryptone, 23.6 g/L yeast extract, 9.4 g/L dipotassium hydrogen phosphate, 2.2 g/L potassium dihydrogen phosphate, 4 mL glycerol (0.4%)) containing 0.1 mg/mL kanamycin. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD600 values of 1.0–1.2. After 24 h of induction, the cells were harvested by centrifugation at 7000 × g for 20 min at 4 °C. The resultant cell pellet was resuspended in 50 mM phosphate buffered saline (PBS) (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 0.9% NaCl, pH 7.0) and sonicated for 10 min in an ice-water bath. The lysate was centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatant was collected as the intracellular fraction.

4.3. Protein Purification

To obtain the purified XcAS, the supernatant was filtered through a 0.45 μm filter and loaded into an Ni^{2+}-chelating Sepharose Fast Flow column (Uppsala, Sweden). The column was balanced by 50 mM sodium phosphate buffer (pH 7.0) containing 500 mM NaCl and washed with the same buffer containing 50 mM imidazole after protein loading. The bound protein was eluted with 50 mM sodium phosphate buffer (pH 7.0) containing 500 mM imidazole and 500 mM NaCl. The active fractions were collected and concentrated using Amicon Ultra-15 (Millipore) with a pore size of 30 kDa. All of the purification processes were performed at 4 °C [33].

4.4. Enzymatic Activity Assay and SDS-PAGE

Determination of ASase hydrolysis activity was carried out on the basis of the hydrolysis reaction of sucrose. The fructose and glucose concentrations were determined by the 3,5-dinitrosalicylic (DNS) acid method [20]. The reaction was initiated by adding 50 μL of the enzyme solution in a reaction mixture with 100 μL of 200 mM sucrose, then incubated for 10 min at 37 °C. Subsequently, the reaction was terminated by adding 200 μL DNS solution and then incubated at 100 °C for 5 min. The resultant mixture was measured at 545 nm to determine the total reducing sugar, and fructose was used as a standard. One unit of enzymatic hydrolysis activity was defined as the amount of enzyme required to hydrolyze 1 μmol of sucrose per minute in standard assay conditions.

ASase transglucosylation activity was assayed using sucrose as glucosyl donor and HQ as acceptor. The 1 mL mixture containing 70 μg/mL XcAS, 50 mM sucrose, 1 mM VC, and 10 mM HQ in 50 mM phosphate buffer was incubated at 30 °C for 2h [12]. One unit of transglucosylation activity was defined as the amount of enzyme required to produce 1 μmol of α-arbutin per minute in the assay

conditions. Meanwhile, the soluble expression of ASase in *E. coli JM109**[DE3] cells was analyzed with SDS-PAGE [24].

$$\textbf{Specific activity } (\textbf{U/mg}) = \frac{\textbf{Total enzyme activity } (\textbf{U})}{\textbf{Purified protein } (\textbf{mg})}$$

4.5. Enzyme Properties of Purified XcAS on Transglucosylation

The optimal pH of XcAS was determined in citrate/phosphate buffer from 4.0 to 9.0 at 30 °C. To measure the pH stability, the purified XcAS was preincubated at various levels (pH 4.5–9.0) for 24 h at 4 °C before enzyme activity was measured. The optimal temperature was measured from 20 to 50 °C at pH 7.0. To determine the thermostability of the purified XcAS, the enzyme was incubated at different temperatures for 1 h at pH 7.0 buffer [34].

Kinetic assays of XcAS transglucosylation were performed under the condition of optimal temperature and pH with sucrose (25–400 mM) and HQ (5–80 mM) as substrates. The kinetic parameters, K_m and V_{max}, were determined by the Lineweaver–Burk plotting method [33]. The equation was as follows:

$$\frac{1}{\mathbf{V}} = \frac{\mathbf{Km}}{\mathbf{Vmax}} \times \frac{1}{[\mathbf{S}]} + \frac{1}{\mathbf{Vmax}}$$

where V indicates the reaction rate, K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction rate, and [S] is the substrate concentration.

4.6. Bioconversion of HQ to α-Arbutin by Whole-Cell Biocatalysis

Recombinant *E. coli JM109**[DE3]/pET28a-*xcas* strain was grown in 50 mL TB medium at different temperatures, and induced with IPTG for 12 h. The cells were harvested by centrifugation at 7000 rpm for 20 min, and washed twice with 50 mM PBS (pH 7.0). Then, the cells were resuspended with 10 mL of 50 mM PBS (pH 7.0) containing HQ (glucosyl acceptor) and sucrose (glucosyl donor). To increase production yield, ascorbic acid was added to the reaction mixture at a final concentration of 1 mM to prevent oxidation of hydroquinone [11]. Whole-cell biocatalysis was performed on a rotary shaker (200 rpm) at 30 °C, unless otherwise mentioned. The biocatalysis product was harvested by centrifugation for 10 min at 12,000 rpm, followed by HPLC analysis.

4.7. Analytical Methods

The amount of α-arbutin was determined by HPLC on an Eclipse XDB-C18 column (4.6 × 250 mm; 45 °C) connected to an SPD-10A UV detector (280 nm) and SPD-LC10 pump. The separation of α-arbutin was carried out using methanol and water (9:1 v/v) as the mobile phase at a flow rate of 0.8 mL min^{-1} with a column temperature of 45 °C [11]. The conversion rate of α-arbutin is given by:

$$\mathbf{n}\ (\%) = \frac{M_1}{M_2} \times 100\%$$

where M_1 is the mole of HQ before transformation and M_2 is the mole of α-arbutin after transformation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/9/1/5/s1, Figure S1: SDS-PAGE analysis of XcAS expression in *E. coli* JM109[DE3] (A) before and (B) after process condition optimization, Figure S2: Effects of (A) temperature and (B) IPTG concentration on *E. coli* cell growth and XcAS hydrolysis activity in *E. coli* JM109/pet28a-xcas, Figure S3: Structural determination of the transglucosylated product.

Author Contributions: Conceptualization, J.S. and B.Z.; Data curation, C.Y. and R.Z.; Formal analysis, C.Y. and B.Z.; Funding acquisition, B.Z.; Investigation, C.Y. and W.F.; Methodology, C.Y. and R.Z.; Project administration, W.F. and J.S.; Writing—original draft, C.Y.; Writing—review and editing, Z.K.-J. and B.Z.

Funding: This research was funded by the grants from the State Key Project of Research and Development Plan (grant number: 2017YFE0112700). This work was partly supported by the National Natural Science Foundation of China (grant number: 41106125).

Acknowledgments: We thank Junsong Sun (Shanghai Advanced Research Institute, Chinese Academy of Sciences) for valuable suggestions on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Seo, D.H.; Jung, J.H.; Lee, J.E.; Jeon, E.J.; Kim, W.; Park, C.S. Biotechnological production of arbutins (alpha- and beta-arbutins), skin-lightening agents, and their derivatives. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 1417–1425. [CrossRef] [PubMed]
2. Sugimoto, K.; Nishimura, T.; Nomura, K.; Sugimoto, K.; Kuriki, T. Inhibitory effects of alpha-arbutin on melanin synthesis in cultured human melanoma cells and a three-dimensional human skin model. *Biol. Pharm. Bull.* **2004**, *27*, 510–514. [CrossRef]
3. Funayama, M.; Arakawa, H.; Yamamoto, R.; Nishino, T.; Shin, T.; Murao, S. Effects of alpha- and beta-arbutin on activity of tyrosinases from mushroom and mouse melanoma. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 143–144. [CrossRef] [PubMed]
4. Nishimura, T.; Kometani, T.; Takii, H.; Terada, Y.; Okada, S. Purifiction and some properties of alpha-amylase from bacillus-subtilis X-23 that glucosylates phenolic-compounds such as hydroquinone. *J. Ferment. Bioeng.* **1994**, *78*, 31–36. [CrossRef]
5. Kurosu, J.; Sato, T.; Yoshida, K.; Tsugane, T.; Shimura, S.; Kirimura, K.; Kino, K.; Usami, S. Enzymatic synthesis of alpha-arbutin by alpha-anomer-selective-glucosylation of hydroquinone using lyophilized cells of *Xanthomonas campestris* WU-9701. *J. Biosci. Bioeng.* **2002**, *93*, 328–330. [CrossRef]
6. Liu, C.Q.; Deng, L.; Zhang, P.; Zhang, S.R.; Liu, L.; Xu, T.; Wang, F.; Tan, T.W. Screening of high alpha-arbutin producing strains and production of alpha-arbutin by fermentation. *World J. Microbiol. Biotechnol.* **2013**, *29*, 1391–1398. [CrossRef] [PubMed]
7. Liu, C.Q.; Deng, L.; Zhing, P.; Zhang, S.R.; Xu, T.; Wang, F.; Tan, T.W. Efficient production of alpha-arbutin by whole-cell biocatalysis using immobilized hydroquinone as a glucosyl acceptor. *J. Mol. Catal. B-Enzym.* **2013**, *91*, 1–7. [CrossRef]
8. Liu, C.Q.; Zhang, P.; Zhang, S.R.; Xu, T.; Wang, F.; Deng, L. Feeding strategies for the enhanced production of alpha-arbutin in the fed-batch fermentation of *Xanthomonas maltophilia* BT-112. *Bioprocess. Biosyst. Eng.* **2014**, *37*, 325–329. [CrossRef]
9. Prodanovic, R.; Milosavic, N.; Sladic, D.; Zlatovic, M.; Bozic, B.; Velickovic, T.C.; Vujcic, Z. Transglucosylation of hydroquinone catalysed by alpha-glucosidase from baker's yeast. *J. Mol. Catal. B-Enzym.* **2005**, *35*, 142–146. [CrossRef]
10. Sato, T.; Hasegawa, N.; Saito, J.; Umezawa, S.; Honda, Y.; Kino, K.; Kirimura, K. Purification, characterization, and gene identification of an alpha-glucosyl transfer enzyme, a novel type alpha-glucosidase from *Xanthomonas campestris* WU-9701. *J. Mol. Catal. B-Enzym.* **2012**, *80*, 20–27. [CrossRef]
11. Seo, D.H.; Jung, J.H.; Ha, S.J.; Cho, H.K.; Jung, D.H.; Kim, T.J.; Baek, N.I.; Yoo, S.H.; Park, C.S. High-yield enzymatic bioconversion of hydroquinone to alpha-arbutin, a powerful skin lightening agent, by amylosucrase. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1189–1197. [CrossRef] [PubMed]
12. Yu, S.H.; Wang, Y.C.; Tian, Y.Q.; Xu, W.; Bai, Y.X.; Zhang, T.; Mu, W.M. Highly efficient biosynthesis of alpha-arbutin from hydroquinone by an amylosucrase from *Cellulomonas carboniz*. *Process Biochem.* **2018**, *68*, 93–99. [CrossRef]
13. Kitao, S.; Sekine, H. Alpha-D-Glucosyl Transfer to Phenolic Compounds by Sucrose Phosphorylase from *Leuconostoc mesenteroides* and Production of alpha-Arbutin. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 38–42. [CrossRef] [PubMed]
14. Seo, E.S.; Kang, J.; Lee, J.H.; Kin, G.E.; Kim, G.J.; Kim, D. Synthesis and characterization of hydroquinone glucoside using *Leuconostoc mesenteroides* dextransucrase. *Enzym. Microb. Technol.* **2009**, *45*, 355–360. [CrossRef]
15. Mathew, S.; Adlercreutz, P. Regioselective glycosylation of hydroquinone to α-arbutin by cyclodextrin glucanotransferase from *Thermoanaerobacter* sp. *Biochem. Eng. J.* **2013**, *79*, 187–193. [CrossRef]
16. Zhou, X.; Zheng, Y.T.; Wei, X.M.; Yang, K.D.; Yang, X.K.; Wang, Y.T.; Xu, L.M.; Du, L.Q.; Huang, R.B. Sucrose Isomerase and Its Mutants from *Erwinia rhapontici* Can Synthesise alpha-Arbutin. *Protein Pept. Lett.* **2011**, *18*, 1028–1034. [CrossRef]

17. Tian, Y.; Xu, W.; Zhang, W.; Zhang, T.; Guang, C.; Mu, W. Amylosucrase as a transglucosylation tool: From molecular features to bioengineering applications. *Biotechnol. Adv.* **2018**, *36*, 1540–1552. [CrossRef]
18. De Montalk, G.P.; Remaud-Simeon, M.; Willemot, R.M.; Sarcabal, P.; Planchot, V.; Monsan, P. Amylosucrase from *Neisseria polysaccharea*: Novel catalytic properties. *FEBS Lett.* **2000**, *471*, 219–223. [CrossRef]
19. Skov, L.K.; Mirza, O.; Henriksen, A.; De Montalk, G.P.; Remaud-Simeon, M.; Sarcabal, P.; Willemot, R.M.; Monsan, P.; Gajhede, M. Amylosucrase, a glucan-synthesizing enzyme from the alpha-amylase family. *J. Biol. Chem.* **2001**, *276*, 25273–25278. [CrossRef]
20. Seo, D.-H.; Jung, J.-H.; Ha, S.-J.; Yoo, S.-H.; Kim, T.-J.; Cha, J.; Park, C.-S. Molecular Cloning of the Amylosucrase Gene from a Moderate Thermophilic Bacterium Deinococcus Geothermalis and Analysis of its Dual Enzyme Activity. *Carbohydr. Act. Enzym.* **2008**, 125–140. [CrossRef]
21. Wu, P.H.; Giridhar, R.; Wu, W.T. Surface display of transglucosidase on *Escherichia coli* by using the ice nucleation protein of *Xanthomonas campestris* and its application in glucosylation of hydroquinone. *Biotechnol. Bioeng.* **2006**, *95*, 1138–1147. [CrossRef] [PubMed]
22. King, A.T.; Davey, M.R.; Mellor, I.R.; Mulligan, B.J.; Lowe, K.C. Surfactant Effects on Yeast-Cells. *Enzym. Microb. Technol.* **1991**, *13*, 148–153. [CrossRef]
23. Khan, S.; Altaf, I.; Rasheed, M.A.; Ashraf, M.; Ishaq, S.; Bashir, R.; Munir, N. Protection of vitamin A and E against toxicity induced by hydroquinone. *Indian J. Exp. Biol.* **2017**, *55*, 383–388.
24. Shao, M.; Zhang, X.; Rao, Z.; Xu, M.; Yang, T.; Li, H.; Xu, Z.; Yang, S. Efficient testosterone production by engineered *Pichia pastoris* co-expressing human 17β-hydroxysteroid dehydrogenase type 3 and *Saccharomyces cerevisiae* glucose 6-phosphate dehydrogenase with NADPH regeneration. *Green Chem.* **2016**, *18*, 1774–1784. [CrossRef]
25. Lee, H.S.; Kim, T.S.; Parajuli, P.; Pandey, R.P.; Sohng, J.K. Sustainable Production of Dihydroxybenzene Glucosides Using Immobilized Amylosucrase from *Deinococcus geothermalis*. *J. Microbiol. Biotechnol.* **2018**, *28*, 1447–1456. [PubMed]
26. Ramshini, H.; Parrini, C.; Relini, A.; Zampagni, M.; Mannini, B.; Pesce, A.; Saboury, A.A.; Nemat-Gorgani, M.; Chiti, F. Large Proteins Have a Great Tendency to Aggregate but a Low Propensity to form Amyloid Fibrils. *PLoS ONE* **2011**, *6*, e16075. [CrossRef] [PubMed]
27. Jhamb, K.; Sahoo, D.K. Production of soluble recombinant proteins in *Escherichia coli*: Effects of process conditions and chaperone co-expression on cell growth and production of xylanase. *Bioresour. Technol.* **2012**, *123*, 135–143. [CrossRef]
28. Pinsach, J.; de Mas, C.; Lopez-Santin, J.; Striedner, G.; Bayer, K. Influence of process temperature on recombinant enzyme activity in *Escherichia coli* fed-batch Cultures. *Enzym. Microb. Technol.* **2008**, *43*, 507–512. [CrossRef]
29. Zhu, X.; Tian, Y.; Xu, W.; Bai, Y.; Zhang, T.; Mu, W. Biochemical characterization of a highly thermostable amylosucrase from *Truepera radiovictrix* DSM 17093. *Int. J. Biol. Macromol.* **2018**, *116*, 744–752. [CrossRef]
30. Chen, J.Y.; Kaleem, I.; He, D.M.; Liu, G.Y.; Li, C. Efficient production of glycyrrhetic acid 3-O-mono-beta-D-glucuronide by whole-cell biocatalysis in an ionic liquid/buffer biphasic system. *Process Biochem.* **2012**, *47*, 908–913. [CrossRef]
31. Shen, D.X.; Shi, X.; Wang, Y.; Juan-Ling, F.U.; Zhou, Z.C. Inhibitory effect of thioredoxin on cytotoxicity of hydroquinone. *Chin. J. Pharmacol. Toxicol.* **2003**, *17*, 55–60.
32. Wu, P.H.; Nair, G.R.; Chu, I.M.; Wu, W.T. High cell density cultivation of *Escherichia coli* with surface anchored transglucosidase for use as whole-cell biocatalyst for alpha-arbutin synthesis. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 95–101. [CrossRef] [PubMed]
33. Liu, Q.D.; Cheng, H.J.; Ma, X.Q.; Xu, N.; Liu, J.; Ma, Y.H. Expression, characterization and mutagenesis of a novel glutamate decarboxylase from *Bacillus megaterium*. *Biotechnol. Lett.* **2016**, *38*, 1107–1113. [CrossRef] [PubMed]
34. Liu, Q.; Ma, X.; Cheng, H.; Xu, N.; Liu, J.; Ma, Y. Co-expression of L-glutamate oxidase and catalase in Escherichia coli to produce alpha-ketoglutaric acid by whole-cell biocatalyst. *Biotechnol. Lett.* **2017**, *39*, 913–919. [CrossRef] [PubMed]