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Development of whole-cell biocatalyst for hydrolyzing high hydrophobic pyrethroid pesticide by functional display of Aminopeptidase on the surface of Saccharomyces cerevisiae

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Abstract

In this study, we developed two new whole-cell biocatalysts by immobilizing aminopeptidase (Aps) on the surface of yeast cells, using N-terminal fusion and C-terminal fusion through lectin mediated display system. After the two strains were cultured in the medium with galactose as inducer for 48 hours, the activity of expressing Aps was at a high level of 0.25 U/OD600/mL and 0.12 U/OD600/mL. The correct location of Aps was confirmed by immunofluorescence analysis and flow cytometry. Afterwards two whole cell catalysts could be reused with high stability as it retained more than 70% of initial activity after ten repeated batch reactions. Using β-cypermethrin (β-CP) as a substrate, the effectiveness of two new whole-cell catalysts in the treatment of highly hydrophobic organic pollutants was evaluated. The results showed that when the concentration of β-CP was 200 mg·L $^{-1}$, the hydrolysis rates of the two whole cell catalysts were 33.16 μmol·L $^{-1}$·day $^{-1}$ and 28.99 μmol·L $^{-1}$·day $^{-1}$, and has the ability to degrade a variety of pyrethroid pesticides. The β-CP residue in lettuce and cherry tomatoes could be removed more than 70% under the conditions of the Aga2N-Aps whole cell catalyst preparation dilution of 100 times. This is the first report on the development of surface display Aps biocatalyst, which can be used as an effective and renewable alternative for the treatment of highly hydrophobic organic pollutants.

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Abstract: In this study, we developed two new whole-cell biocatalysts by immobilizing aminopeptidase (Aps) on the surface of yeast cells, using N-terminal fusion and C-terminal fusion through lectin mediated display system. After the two strains were cultured in the medium with galactose as inducer for 48 hours, the activity of expressing Aps was at a high level of 0.25 U/OD600/mL and 0.12 U/OD600/mL. The correct location of Aps was confirmed by immunofluorescence analysis and flow cytometry. Afterwards two whole cell catalysts could be reused with high stability as it retained more than 70% of initial activity after ten repeated batch reactions. Using β-cypermethrin (β-CP) as a substrate, the effectiveness of two new whole-cell catalysts in the treatment of highly hydrophobic organic pollutants was evaluated. The results showed

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that when the concentration of β-CP was 200 mg·L^{-1}, the hydrolysis rates of the two whole cell catalysts were 33.16 μmol·L^{-1}·day^{-1} and 28.99 μmol·L^{-1}·day^{-1}, and has the ability to degrade a variety of pyrethroid pesticides. The β-CP residue in lettuce and cherry tomatoes could be removed more than 70% under the conditions of the Aga2N-Aps whole cell catalyst preparation dilution of 100 times. This is the first report on the development of surface display Aps biocatalyst, which can be used as an effective and renewable alternative for the treatment of highly hydrophobic organic pollutants.

Keywords: Biodegradation; Pyrethroids; Aminopeptidase; Yeast cell surface display; Whole-cell biocatalyst

Introduction

Pyrethroid insecticide is a kind of synthetic bionic insecticide with high efficiency and low toxicity, which is one of the most widely used and difficult to be replaced pesticides in the next few decades (Lu et al., 2021). At present, there are more than 20 kinds of commonly used pyrethroids on the market, the most commonly used pyrethroids are beta-cypermethrin, fenpropathrin, deltamethrin, cyhalothrin and fenvalerate, etc. Beta-cypermethrin (β-CP), a major synthetic pyrethroid insecticide, which is widely used to take precautions against and control pests such as fruits, vegetables, grains, etc (Tiwary and Dubey, 2016). Because of its photothermal stability and high hydrophobicity, it is easily adsorbed in soil and organic matter (Lu et al., 2021; Song et al., 2015). Furthermore, insecticides residue in agricultural products threaten human health through feeding behavior, and long-term human contact would have the risk of carcinogenesis (Zhan et al., 2020), teratogenesis and mutagenesis (Zhang et al., 2019). With the continuous harmful pollution of β-CP to the ecological environment and human health, the biodegradation of β-CP in the environment has attracted more and consideration (Luo et al., 2018; Song et al., 2015). Of the various means that are used to remediate β-CP environmental pollution, microbial degradation is considered to be one of the most promising strategies (Akbar et al., 2015).

In the current research work on the degradation of β-CP by microorganisms, most of the reported functional enzymes are intracellular enzymes, including pyrethroid-hydrolyzing esterase (EstP) from *Klebsiella sp.* strain ZD112 (Saleem et al., 2008) and*R. palustris* PSB-S esterase (Est3385) (Luo et al., 2018). Due to the high hydrophobicity and high relative molecular weight of β-CP, its low efficiency into the cell will greatly affect its decomposition by the cell (Tang et al., 2015). Therefore, how to adsorb it from the environment to the surface of microorganisms and be ingested by microorganisms is the first and key step in the detoxification and metabolism of β-cypermethrin. It has been reported that the extracellular carboxylesterase of *Bacillus licheniformis* B-1 (Zhang et al., 2021) and the extracellular aminopeptidase of*Pseudomonas aeruginosa* GF31 (A. X. Tang et al., 2017) can solve the membrane barrier problem of enzyme and substrate to a certain extent. However, the purification and recovery process of extracellular free enzyme will reduce its activity, poor stability, low repetitive utilization rate, increase production cost, and limit its large-scale production and application (Gustavsson et al., 2014).

Microbial cell-surface display (CSD) is a powerful platform to present and immobilize the protein of interest on microbial surface, it has been effectively used in various fields, including the development of live vaccines (Cheng et al., 2021), whole cell catalysis (Chordia et al., 2021; Ye et al., 2021), biosensor development (Zhao et al., 2020), environmental biosorption (Rangra et al., 2018) and so on. Among several surface display systems, the yeast surface display (YSD) system has been broadly applied as a stage for production of heterologous proteins, and a well-studied yeast surface applied is *Saccharomyces cerevisiae*(*S.cerevisiae*) (Chen et al., 2016; Yang et al., 2019). *S.cerevisiae* has been granted "Generally Recognized As Safe" (GRAS) approval by the Food and Drug Administration, suitable for industrial and food use (Chun et al., 2020). The foremost commonly utilized yeast display system were a-agglutinin and α-agglutinin mediated systems. The a-agglutinin mediated display system comprised two subunits: Aga1 and Aga2, of which Aga1 has glycosylphosphatidylinositol (GPI) anchor function (Lozančić et al., 2021; Tanaka et al., 2012). At that point Aga2 is connected with Aga1 through two disulfide bonds, and target protein can be intertwined with the N- or C-terminus of Aga2, and the ultimate target protein realizes functional expression on the yeast cell surface (Wang et al., 2015). Because the fusion site will affect the display quantity and activity of fusion protein on the engineering bacteria cell surface, steric hindrance effect between proteins must be

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considered (Kuroda and Ueda, 2013). In the yeast surface display platform constructed by selecting different fusion sites, Endolysin LysSA11 from*Staphylococcal phage* SA11 was displayed on the surface of*S.cerevisiae* by N-terminal fusion (Chun et al., 2020), and Lim has researched a new yeast display platform that involves linking two diverse heterologous proteins to Aga2, one at the C-terminus and one at the N-terminus (Lim et al., 2017).

At present, relevant studies have proved that the use of surface display technology to anchor the target protein on the outer membrane of microorganisms can solve the problem that the limitation of high hydrophobic substrate transport barrier across cell membrane (Ding et al., 2020), such as hydrolysis of organophosphorus compounds (Song et al., 2019); biodegradation of diisobutyl phthalate (DiBP) (Ding et al., 2020) and degradation of highly crystallized polyethylene terephthalate (PET) (Chen et al., 2020). This method allows the substrate to interact directly on the surface of the microbial cell, eliminating the tedious process of multi-step purification and recovery of free enzymes, and greatly reducing the cost of preparation and application of the whole-cell biocatalyst (Smith et al., 2015). Due to the unique chemical selectivity, regioselectivity and enantioselectivity of enzymes, only a few of the available functional enzymes were used for cell surface display (An et al., 2014). Hence, the improvement of novel enzymes for cell display systems an important investigate objective (Liang et al., 2019). Aminopeptidase (Aps) is an extracellular pyrethroid degrading enzyme from *Pseudomonas aeruginosa* GF31, and different from the widely reported intracellular pyrethroid degrading enzyme (carboxylesterase) (Tang et al., 2015). As a new pyrethroid degrading enzyme, Aps is a bifunctional enzyme that can hydrolyze pyrethroid and protein (Tang et al., 2017). In previous studies, the display of Aps on the cell surface and construction of whole-cell catalysts for bioremediation have not been reported yet.

In this study, we aimed to fuse the Aps of *Pseudomonas aeruginosa*GF31 to the N-terminus and C-terminus of Aga2 subunits respectively and display it on the surface of *S.cerevisiae* , constructing a novel whole-cell biocatalyst. The environmentally friendly*S.cerevisiae* engineering strain was endowed with the capacity to degrade β-CP and could be utilized for further environmental bioremediation. In addition, as a new whole cell catalyst, displayed-Aps can be used as the basis of other applied research of aminopeptidase, such as waste protein pollution treatment, milk Debittering and so on.

Methods

Strains, media and chemicals

In this study, *S.cerevisiae* EBY100 strain (Invitrogen, Carlsbad, CA) was selected as the host strain for the yeast surface display system. Transformed yeast were cultured in SD-CAA media (synthetic media, tryptophan auxotrophic, 2% D-glucose, 1 M sorbitol, pH 6.4, 25 μg/mL kanamycin). The expression of Aga2 fusion protein was induced by galactose inducible promoter in SG-CAA medium (synthetic media, tryptophan auxotrophic, 2% D-galactose, pH 6.75, 25 μg/mL kanamycin). β-Cypermethrin (98.1%), fenpropathrin (91.6%), deltamethrin (98.6%), alphacypermethrin (95.1%) and cyhalothrin (98.4%) were purchased from Guangxi Plant Protection General Station (Nanning, China). L-Leucine-p-nitroanilide (Leu-pNA) was purchased from Takara (Dalian, China). All other chemicals are purchased from commercial sources and have a purity of at least analytical grade.

Construction of displaying S.cerevisiae strain and growth curve measurement

The Aps gene (KT735188) cloned from *Pseudomonas aeruginosa* strain GF31 (Tang et al., 2017) was codon optimized for functional expression according to the codon preference of the host strain*S.cerevisiae.* The first 72 nucleotides (encoding signal peptide) and the stop codon of the Aps gene have been removed. To facilitate protein characterization and visualization, human influenza hemagglutinin (HA) was connected to the C-terminal or N-terminal of the target protein Aps. Next, the modified Aps gene sequence was fused with the N-terminus or C-terminus of the Aga2 subunit of a-agglutinin anchored protein from the yeast display system, respectively, and transformed and ligated into the pYD1 plasmid to construct the plasmids pYD1-Aga2N-Aps and pYD1-Aga2C-Aps. The fusion protein cassette was shown in **Fig. 1B** . The two vectors were transformed into*S.cerevisiae* EBY100 cells by heat-shock transformation. The engineered *S.cerevisiae*

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was cultured in SD-CAA medium at 30°C and 220 rpm for 36 h. After proliferation and culture, when the measured OD600 reaches 2.5-3.0, the engineered yeast cells were collected after centrifugation for 10 minutes under the condition of 5000×g at 4°C. After washing, they were resuspended in SG-CAA medium to control OD600 to 1.0. Then the Aps protein was induced by 2% galactose at 20°C.

The pYD1-Aga2N-Aps/pYD1-Aga2C-Aps transformants were inoculated into SD-CAA medium and cultured at 30°C until the optical density 600 (OD600) was 2.5 to 3.0. The proliferated yeast cells were centrifuged at 5000×g for 15 min and then suspended in SG-CAA medium to make its OD600 in the induction medium equal to 1.0, and cultivated at low temperature to induce the appearance of the fusion protein.*S.cerevisiae* EBY100 was used as a negative control. From 0 h to 60 h, OD600 was measured every 6 h and the growth curve was drawn. In order to explore the influence of the induction time on the enzyme activity of Aps, EBY100-pYD1-Aga2N-Aps/EBY100-pYD1-Aga2C-Aps were induced for 12, 18, 24, 30, 36, 48, 54 h for enzyme activity assay.

Expression and detection of target protein Aps

After 48 h of induction, the yeast cells were collected by centrifugation, washed with ddH2O more than three times, and resuspended in SDS-PAGE loading buffer, adding dithiothreitol (DTT) (Li et al., 2014). After the cells were boiled and centrifuged to extract proteins (12000×g, 10 min), and the supernatant was collected and used for Western blotting analysis (An et al., 2014). Soak the PVDF membrane with TBST (blocking solution) containing 5% skimmed milk powder, and seal it with a shaker at room temperature for 2 h. Dilute the corresponding primary antibody with blocking solution, soak the PVDF membrane in the primary antibody incubation solution, and incubate overnight at 4°C. TBST thoroughly washes the PVDF membrane 5-6 times to remove excess primary antibody. Dilute the corresponding HRP-labeled secondary antibody (anti-HA Tag Monoclonal antibody) (ZEN-BIOSCIENCE, China) with blocking solution 1:50000 dilution, soak the PVDF membrane in the secondary antibody (IgG goat anti-mouse antibody and HRP) incubation solution, and incubate for 2 h at 37°C on a shaker. Mix the enhancement solution in the ECL reagent with the stable peroxidase solution in a ratio of 1:1, drop the working solution on the PVDF membrane, and react for a few minutes until the fluorescence band was obvious, then was used for the visualization of the protein band.

Fluorescence microscopic assay

The strains identified as positive by PCR were inoculated into SD-CAA medium and cultured overnight, the bacterial solution was centrifuged, and the strain was replaced with SG-CAA medium to induce expression at 20°C, 200 rpm, 48 h. HA-tag (9A3) Mouse mAb (ZEN-BIOSCIENCE) was used as the primary antibody for immunostaining. Centrifuge 200μl of the bacterial solution, incubate with 1:200 concentration of Anti-Mouse IgG (H&L) (ZEN-BIOSCIENCE, China, 1:100 diluted with 1% bovine serum albumin) for 1 h in the dark, and then use 1:100 concentration of fluorescent secondary antibody (Goat anti-Mouse IgG) (H+L) Secondary Antibody, DyLight 488) incubate 30 min for 1 h. Finally, the incubated yeast cells were washed more than three times with PBS, and by the fluorescence microscope (IMAGER Z1).

Flow cytometry assay

Induced cells were collected and washed three more times with PBS buffer three times (pH 7.4). The cell density was then adjusted to approximately 10^7cell/mL and 200 μL of the adjusted cells were repelled at 12,000×g for 3 min at 4°C. After washing samples with PBSA buffer for 3-5 times, resuspend it in HA-tag (9A3) Mouse mAb (ZEN-BIOSCIENCE) in PBSA diluted at 1:200 for 1 h. At this time, it was at room temperature and kept in dark conditions. After being washed five times with PBS, the samples were resuspended in an Anti-Mouse IgG (H&L) (ZEN-BIOSCIENCE, China) and incubated on ice for 1 h. Then, the cells were washed five times and analyzed. In order to observe and determine the expression activity, the incubated yeast cells were collected and resuspended in 300μL PBSA, and analyzed with a Guava flow cytometer (Millipore).

Enzyme activity of surface-displayed Aps

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The Aps activity was measured in 50 mM Tris-HCl buffer (pH 9.0) at 60 °C, using 2 mM L-Leucine-p-nitroanilide (Leu-pNA) as the substrate. The reaction was initiated by adding *S.cerevisiae* suspension (OD600=1.0) to the preincubated substrate solution. A spectrophotometer (Beckman 600) was used to monitor the increase in absorbance at 405 nm for 20 min at intervals of 2 min to calculate the initial hydrolysis rate (Liang et al., 2019). One unit (U) of the Aps activity was defined as the amount of enzyme that produced 1 μmol of p-nitroaniline/min under standard conditions.

Effect of temperature and pH on enzyme activity and exploring the reusability

Using Leu-pNA as the substrate, the impact of temperature and pH on the activity of Aps were determined by spectrophotometry. Under standard analysis conditions, the optimal temperature for Aps activity was determined by measuring the reaction rate in the range of 10 to 80°C. The optimum pH for enzyme activity was determined at 60°C for 20 min from pH 5.0 to 10.0 in different buffers (50 mM): citrate/phosphate buffer (pH 5.0 to 8.0), Tris/HCl (pH 7.0 to 9.0), and boric acid/borax (pH 9.0 to 10.0).

The reusability of the cells used for measuring the enzyme activity was evaluated by putting the whole cells in a 50 mM Tris-HCl buffer (pH 9.0) and incubating at 60°C for 20 min. Stop the reaction with a mixture of ice and water and centrifuge at 10,000×g for 3 min. The harvested cells were resuspended in the same volume of reaction mixture and reused for up to ten subsequent reaction cycles.

*Degradation of pyrethroid pesticide by surface-**displayed Aps***

The reaction rate was determined by measuring the decrease in a flask containing 4 mL and 50-300 mg·L^{-1} β-CP. The engineering yeast cells were inoculated into the pesticide reaction system to obtain the final cell density of approximately OD600 = 1.0. The reaction took place at 60°C and was incubated on a shaker at a speed of 150 rpm, and then the reaction was stopped by adding 0.5 mL of 1 M HCl. Remaining β-CP was extricated and detected by an Ultimate 3000 high-performance liquid chromatography system (Idstein, Germany) prepared with ultraviolet detector. The specific detection method refers to the previous research report (Tang et al., 2015). Flasks were prepared as described above, and all experiments included a blank control, including cells that did not contain or were not induced. All experiments were performed at least three times.

Under the above conditions, the ability of Aps displayed by yeast to degrade other pyrethroids was also investigated.

Removal residual β-CP in vegetables using surface-displayed Aps

The stock solution of β-CP was diluted with tap water to the concentration of 500 mg/L. Then, lettuce and cherry tomatoes were immersed in this β-CP solution for 30 min to allow the β-CP to be absorbed into or attached to the vegetables. Then collect them and put them in a fume hood to dry naturally. The sample was immersed in the washing solution of 1% whole cell catalyst or tap water at 45°C for 90 minutes, and then washed with tap water once. After the washing process, the vegetable samples are placed in a cool place to dry. The control sample was not treated after β-CP coating.

First, the two vegetables were homogenized for 2 min. Then, a 20 g portion of the slurry sample was weighed to a 250 mL conical flask and extracted in 50 mL of acetonitrile for 30 min. The homogenate was filtered with a glass filter under reduced pressure and was shaken for 1 min followed by the addition of 4 g of sodium chloride. The liquid phase layer was allowed to separate and 10 mL of acetonitrile extracts were taken, evaporated dryness with a water bath at 60°C, and dissolved with 2 mL of acetonitrile. The final extract was filtered through a 0.22 μm membrane to HPLC analysis. All treatments were performed in triplicate.

Results and discussion

Construction of Aps displaying S.cerevisiae strain.

The purpose of this study research is to use a-agglutinin as an anchoring system to display Aps from *Pseudomonas aeruginosa* GF31 on *S.cerevisiae* cell surface, construct new whole cell biocatalysts and explore

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its degradation ability of pyrethroid pesticides. The subcloning of Aps and its heterologous expression in*E.coli* have been reported (Tang et al., 2017), considering that Aps from *Pseudomonas aeruginosa* GF31 is an extracellular enzyme, in comparison with the strain harboring complete enzyme encoding gene, the transformant harboring Aps gene lacking signal peptide and precursor peptides may be conducive to the expression of the enzyme. Taking into account the codon bias of *S.cerevisiae* , the codons of the Aps encoding gene were optimized. The traditional yeast surface display strategy was to express the target protein as a fusion with the C-terminus of Aga2 subunit, e.g. pCT (Tao et al., 2016) vector, or N-terminus, e.g. pTMY (Jones et al., 2011), pYD5 (Wang et al., 2005) and pCHA (Mata-Fink et al., 2013) vectors. In our study, Aps was taken as the target protein, fused to the N-terminus and C-terminus of Aga2 subunit respectively, considering that the fusion site will affect the display quantity and activity of Aps on yeast cell surface. Therefore, two kinds of surface display vectors were constructed (called pYD1-Aga2N-Aps and pYD1-Aga2C-Aps). In order to detect the fusion protein in Western blot and Flow cytometry, the epitope tag of human influenza hemagglutinin (HA) was inserted into the downstream of Aps sequence. The fusion mode between the N-terminal or C-terminal of the target protein Aps and the anchoring motif Aga2 may be the main reason affecting the enzyme activity. The expression process was regulated by the GAL1 promoter. Beneath the induction of galactose, the Aps melded with HA epitope tag and Aga2 was expressed, and the N-terminal secretion signal sequence of Aga2 actuates the conveyance of the fusion protein to the exterior of the cell. At long last, the fusion protein was automatically immobilized on the surface of yeast cells through two disulfide bonds. The disulfide bond covalently binds Aga2 and Aga1, and Aga1 was fixed on the cell wall via the GPI anchor (**Fig. 1**).

Expression of Aps in the recombinant S.cerevisiae strain

The expression pattern of fusion protein was detected by twelve alkyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that there was a band corresponding to Aga2N-Aps and Aga2C-Aps at 64 kDa and 62 kDa, respectively, which appeared on *S.cerevisiae*cells containing pYD1-Aga2N-Aps (**Fig. 2A** , Lane 3 , arrow) and pYD1-Aga2C-Aps (**Fig. 2A** , Lane 4, arrow). These results indicate that the synthesized fusion protein appeared correctly expressed in *S.cerevisiae* engineering strains, and the corresponding protein size was consistent with the calculated molecular weight.

For the recombinant plasmids, pYD1-Aga2N-Aps and pYD1-Aga2C-Aps, the HA tag was fused to the C-terminus or N-terminus of the displayed-Aps. Western blot examination with anti HA monoclonal antibody appeared that Aga2N-Aps and Aga2C-Aps fusion proteins may well be recognized in yeast cells after induction, and the molecular weight of the protein band was steady with that expected (**Fig. 2B** , Line 2, 4, arrow), which means that the Aps of N-terminal fusion and C-terminal fusion were successfully expressed in *S.cerevisiae* recombinant strains (**Fig. 2B** , Line 2, 4, arrow). Moreover, the protein bands were also detected by Western blot analysis of DTT treated protein solution (**Fig. 2B** , Line 5, 6, arrow), which indicated that the fusion proteins Aga2N-Aps and Aga2C-Aps were exactly expressed on the surface of *S.cerevisiae* cells, because DTT can break the disulfide bond, the protein displayed on the surface can be eluted. Interestingly, no matter in the results of SDS-PAGE or Western blot, similar protein bands with the fusion proteins Aga2N-Aps and Aga2C-Aps were also observed in the samples of uninduced *S.cerevisiae* engineering strains (**Fig. 2A** , Lane 1, 2; **Fig. 2B** , Lane 1, 3), but the degree of expression was weaker than that after induction, which indicated that the fusion protein had background expression even in the uninduced state (Detzel et al., 2013).

Surface localization analysis of Aps

The presence of Aga2N-Aps and Aga2C-Aps on the surface of*S.cerevisiae* cells was further confirmed by indirect immunofluorescence (**Fig. 3**). When observed under the fluorescence microscope, the cells carrying pYD1-Aga2N-Aps and pYD1-Aga2C-Aps plasmids showed green fluorescence on the cell surface after induction (**Fig. 3**), which confirmed the correct position of the protein on the cell surface. The induced cells carrying the plasmid pYD1-Aga2N-Aps showed stronger fluorescence intensity than those carrying the plasmid pYD1-Aga2C-Aps (**Fig. 3B and D**), which preliminarily indicated that the N-terminal fusion of Aga2 with the target protein Aps was more conducive to its successful expression and display on the cell

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surface.

Then, the recombinant yeast strains were investigated by flow cytometry, and the recombinant protein Aga2-Aps displayed on the surface was characterized (**Fig. 4**). The primary antibody that recognizes the HA epitope and the Goat Anti-Mouse IgG (H&L) secondary antibody were used to detect and label the fusion protein. In the flow cytometry data, the area under the peaks indicates the number of gated cells, and the fluorescence (FL) intensity reflects the number of fluorescent labels on a single cell. In this case, the relative density of Aps was represented on the surface. In the given population, the proportion of yeast cells Aga2N-Aps displaying (**Fig. 4C**) accounted for 76.87%, and the proportion of yeast cells Aga2C-Aps displaying (**Fig. 4B**) accounted for 41.70%. The proportion of yeast cells Aga2N-Aps displaying was about 35% higher than that showing Aga2C-Aps, which confirmed that the fusion target protein Aps at the N-terminal of Aga2 was indeed more conducive to the successful expression on the surface of *S.cerevisiae* than that at the C-terminal.

Characteristics of surface displayed Aps

The optimal temperature and pH of two whole-cell biocatalysts in the reaction system were investigated. As shown in **Fig. 5A** , the activity of Aps enzyme of yeast carrying plasmids pYD1-Aga2N-Aps and pYD1-Aga2C-Aps increased linearly from 10°C to 60°C and the maximal activity was detected at 60°C. When the temperature reaches 80°C, the enzyme activity of EBY100-pYD1-Aga2N-Aps still reaches 73.3% of the highest activity, it shows that the Aps displayed on the surface has good heat resistance, which is beneficial for subsequent environmental remediation. Although the maximum enzyme activity of the yeast cells expressing Aga2C-Aps fusion protein was only 53.57% of that of the yeast cells expressing Aga2N-Aps, the enzyme activity of the two engineering yeasts reached the maximum at 60°C, and maintained good enzyme activity in the range of 40 to 80°C. The optimal pH for the two entire cell biocatalysts were decided at pH 9.0 and more than 50% of enzymatic activity was kept at pH values extending from pH 7.0 to 10.0 (**Fig. 5B and C**). Under the two optimal conditions, the Aps activities of whole cells expressing Aga2N-Aps and Aga2C-Aps were 0.25 U/OD600/mL and 0.12 U/OD600/mL.

For surface expression approach, the growth inhibition of cell should be taken into consideration. To determine whether the surface display of Aga2N-Aps and Aga2C-Aps fusion protein inhibits growth of the cell, growth profile of *S.cerevisiae* strain carrying pYD1-Aga2N-Aps、pYD1-Aga2N-Aps and without vector was compared. The final cell density of the three strains was not much different after 60 h of induction (the final OD600 value only differed by 0.3), and no growth inhibition was observed for the cells expressing the Aga2-Aps fusion protein (**Fig. 5D**). During the cultivation of the recombinant EBY100 strain, the inducer galactose could induce the surface display of Aps. Some scholars pointed out that the induction time has a significant impact on the biological activity of the displayed enzyme. The optimal induction time depends on the target protein, vector and host strain used in the surface display system (Chen et al., 2020). **Fig. 5E** shows that the enzyme activity of Aps increases with time from 0-48 h after induction, and the Aps activity reaches its maximum value after 48 h induction. Then, a significant decrease in Aps activity was observed after induction for more than 48 h. The results showed that the induction time had an effect on the expression level of Aps on the surface of yeast. It was not difficult to speculate that during the growth period of the cell, the displayed-Aps accumulated continuously as the amount of cells increases, as shown in**Fig. 5D.** Hence, we chose 48 h as the induction time for producing the whole-cell biocatalyst within following studies.

The reusability of surface displayed Aps

In order to test the operational stability of two biocatalysts, which express Aga2N-Aps and Aga2C-Aps fusion proteins respectively, during repeated use, we investigated the activity of two biocatalysts in multiple reaction cycles. As shown in **Fig. 6** , the biocatalyst activity of Aps displayed on the surface of yeast well maintained ten consecutive reactions. After five consecutive reactions, the whole cell catalyst expressing Aga2N-Aps maintained more than 80% of the activity, and after ten consecutive reactions, the activity still reached more than 70% of the initial activity. Although the overall enzyme activity of the whole cell

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catalyst expressing Aga2C-Aps was lower than that of the whole cell catalyst expressing Aga2N-Aps, it still maintains more than 70% of the initial activity after ten consecutive reactions, indicating that both whole-cell biocatalysts have good properties of reusability. Generally speaking, when complex protein purification procedures (protein ultrafiltration or column chromatography, etc) cannot be carried out, it is difficult to separate a variety of soluble functional enzymes from the system, and the recovery rate is low, which will affect the reusability of free enzymes (Chen et al., 2016). While, the biocatalyst constructed on yeast host cells to express the target protein could be considered as 5-10 μm particles, coated with a functional coating, and could be separated by simple centrifugation and washed with buffer and reused.

Degradation of pyrethroid by recombinant S.cerevisiae strain

This study also explored the effect of substrate concentration on the biodegradation of whole-cell catalysts when β-CP, the most commonly used among highly hydrophobic pyrethroid pesticides, was used as the substrate. As shown in **Fig. 7A** , the whole-cell catalysts expressing Aga2N-Aps and Aga2C-Aps could degrade 200 mg·L^{-1} β-CP at a maximum rate of 33.16 μmol·L^{-1}·day^{-1} and 28.99 μmol·L^{-1}·day^{-1}. The degradation rate catalyzed increased as the substrate concentration increased from 50 to 200 mg·L^{-1}, however, the degradation rate decreased if the substrate concentration continues to increase. Although the substrate β-CP concentration was as high as 300 mg·L^{-1}, the whole-cell catalysts expressing Aga2N-Aps and Aga2C-Aps still has the degradation ability of 25.39 μmol·L^{-1}·day^{-1} and 19.65 μmol·L^{-1}·day^{-1} . Therefore, the results show that in our research, the surface display catalysts with two different fusion modes constructed based on cell surface display technology have a wide range of substrate concentration during the hydrolysis process with β-CP as the substrate. And high substrate concentration tolerance, provides a new possibility for effective restoration of water and soil contaminated by β-CP in the future.

In order to investigate the ability to degrade various pyrethroids, five commonly pyrethroids were used to evaluate the substrate specificity of displaying Aps (respectively: β-cypermethrin, fenpropathrin, deltamethrin, cyfluthrin, alphacypermethrin). As shown in **Fig. 7B** , the two whole cell catalysts had obvious degradation effects on five commonly used pyrethroid pesticide substrates. And that, the two whole cell catalysts show the best degradation ability to β-CP, followed by fenpropathrin. We also found that there was no significant difference in the enzyme activity of Aps when Aps fused to the N-terminus and C-terminus in the reaction system with pesticide as substrate compared to the reaction system with Leu-pNA as substrate. It is speculated that the mass transfer ability of substrate may play a major role in the reaction of pesticide degradation. The two whole cell catalysts can hydrolyze five different pyrethroid pesticides, indicating that the displayed Aps is a broad-spectrum pyrethroid hydrolase. Since the residual pesticide was a mixture in the environment, the broad-spectrum pyrethroid-degrading whole cell catalyst would have a wide range of application prospects in practical applications (Hu et al., 2019).

Removal residual β-CP in vegetables using surface-displayed Aps

Washing vegetables with whole cell catalyst solution was more effective than washing with tap water in reducing β-CP residues (**Figure 8**). In the process of removing high concentration pesticide residues, after washing in two whole cell catalyst solutions of Aga2N-Aps and Aga2C-Aps, the β-CP residues on cherry tomato decreased from 278.41 mg·kg^{-1} to 86.31 mg·kg^{-1} and 105.82 mg·kg^{-1} respectively, and the β-CP residues on lettuce decreased from 337.10 mg·kg^{-1} to 138.22 mg·kg^{-1} and 157.99 mg·kg^{-1}respectively (**Figure 8A**). However, only by washing with tap water, the residue removal rates were 2.91% and 7.70%, respectively. In most cases, pesticide will remain on the surface of vegetables and fruits. Usually, the pesticide residues are removed only by washing with tap water, and the high hydrophobicity of β-CP makes it difficult to be removed directly by tap water. Frying and frying are more effective in reducing residues. However, some vegetables, such as cucumber, lettuce and fresh fruit, are usually eaten raw without frying or frying. Therefore, it is very important to develop a new washing solution to remove pesticide residues from vegetables. It is found in the report that purified and concentrated extracellular enzyme solution is effective for the removal of β-CP. In this study, the two whole-cell catalysts only need a simple cell collection process to achieve the same purpose, omitting the complex and cumbersome enzyme purification process, which has more practical significance.

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Countries all over the world have set strict standards for pesticide residues in vegetables, especially edible vegetables and fruits with skin, which lead to the inability to apply pesticides before picking these vegetables and fruits, affecting the yield and quality of agricultural products. Pyrethroids are commonly used as pesticides for vegetable and fruit insecticides. Because of their high hydrophobicity, it is difficult to remove them by simple tap water washing. When the pesticide residue concentration in cherry tomato and lettuce is relatively low, tap water washing can only remove 7.39% and 10.38%. Through the further exploration of two whole cell catalysts Aga2N-Aps and Aga2C-Aps, it is found that these two whole cell catalysts not only have obvious removal effect on vegetables and fruits with high concentration of pesticide residues, but also maintain good degradation ability in vegetables and fruits with low concentration of pesticide residues. After washing cherry tomato and lettuce treated with low concentration of β-CP in two whole cell catalyst solutions, the pesticide residues on cherry tomato decreased from 4.87 $mg \cdot kg^{-1}$ to 1.36 $mg \cdot kg^{-1}$ and 1.51 $mg \cdot kg^{-1}$, and on lettuce from 5.59 $mg \cdot kg^{-1}$ to 1.84 $mg \cdot kg^{-1}$ and 1.81 $mg \cdot kg^{-1}$ respectively (**Figure 8B**). The residual concentration of β-CP in cherry tomato and leaf lettuce treated with whole cell catalyst has been lower than the Chinese pesticide residue limit standard (GB2763-2021), showing a good pesticide treatment effect. Interestingly, this phenomenon is different from that previously reported. The strains reported by Karpouzas and Walker (Karpouzas et al., 2001), showed significant differences in degradation rates when degrading high concentration pesticide substrates and low concentration pesticide substrates. In addition, the whole cell catalyst displayed on the surface of yeast has a good practical application prospect because of its good biosafety and lower price compared with purified enzyme. The yeast surface display whole cell catalyst constructed in this paper has good treatment effect on vegetables and fruits with high and low concentrations of pesticide residues. It has application potential both in agricultural production enterprises and as a household vegetable and fruit cleaning agent.

Conclusions

In this study, a-agglutinin was used as the anchor protein, and Aps was successfully displayed on the surface of *S.cerevisiae* cells by N-terminal fusion and C-terminal fusion respectively. Immunofluorescence analysis and flow cytometry analysis showed that Aps was anchored on the surface of yeast. Under the optimal reaction conditions of pH=9, 60°C, the enzyme activities of the two whole-cell catalysts reached 0.25 U/OD600/mL and 0.12 U/OD600/mL, respectively. In addition, the two whole-cell catalysts can still maintain more than 70% activity after repeated use for 10 times, and have a good reusability. It was found that the two whole-cell catalysts can also remove β-CP residues in vegetables and might be used for wash solutions in the future. Moreover, displayed-Aps is a bifunctional enzyme, which can degrade multiple pyrethroid pesticides and has the potential to remediate waste protein pollution. The broad spectrum of the substrate, good thermal stability and pH adaptation range also illustrate its high potential in restoring complex environmental scenes.

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