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Isolation of *Penicillium expansum* WH-3 for the production of L(+)-tartaric acid*#

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The L(+)-form of tartaric acid (L(+)-TA) exists extensively in nature, and is widely used in the food, chemical, textile, building, and pharmaceutical industries (Su et al., 2001). The main method for L(+)-TA production is microbial transformation by *cis*-epoxysuccinate hydrolase (CESH), which can catalyze the asymmetric hydrolysis of *cis*-epoxysuccinic acid or its salts to TA or tartrate (Bao et al., 2019). Seventeen species containing CESH have been isolated so far. However, most species for L(+)-TA production have been reported from bacteria (Xuan and Feng, 2019). The only fungus isolated from soil by our lab recently, that could be used as catalyst for the process under acidic condition, is *Aspergillus niger* WH-2 (Bao et al., 2020). In order to find strains with new characteristics, this study attempted to isolate a new CESH source from fungi and investigate its application value.

Unlike the previous isolation from soil, where the screening medium used contained a low concentration of substrate disodium *cis*-epoxysuccinate (<15 mmol/L), and other carbon sources and nitrogen sources, this study included a substrate solution of high concentration (0.2 mol/L) as the sole source of nutrients, and selected as the limiting condition to isolate strains. Disodium *cis*-epoxysuccinate (0.2 mol/L, 200 mL, pH 7.0) in a 500-mL jar without the cap was kept at room temperature for two months. The flocculant substances at the top of the solution formed the basis for fungal strain isolation by inoculating flocs onto potato dextrose agar (PDA) medium, incubation at 25 °C for 7 d, and further inoculation into potato dextrose broth (PDB) for another 5 d (Xue et al., 2018). In order to inhibit bacterial growth, ampicillin was added to the PDA medium. Finally, samples were incubated at 30 °C for 3 d with disodium *cis*-epoxysuccinate, resulting in the detection of TA content. A total of 42 colonies were isolated from six contaminated samples, which were inoculated and cultivated for a transformation test. Only the WH-3 strain was able to transform *cis*-epoxysuccinate to TA.

The identification of strain WH-3 was carried out by the observation of its morphological properties and internal transcribed spacer (ITS) sequence analysis (Wu et al., 2007). As shown in Fig. S1, the isolated strain was colored white with abundant conidiogenesis, giving the colonies a blue-green color. The colony on PDA was velutinous with an entire and narrow margin. The average colony diameter for 7-d postinoculation on PDA was about 36 mm. The conidiophores could be seen under the microscope, showing dense brush-like conidia-bearing structures with a second order of branches, bearing a cluster of phialides in turn. There were many radial stems on the branches; conidia were oblate. Its morphological properties were similar to that of *Penicillium expansum*.

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Its 579-bp long ITS sequence was submitted to the GenBank database under the accession number MN587988. Multiple alignments and the phylogenetic tree (Fig. 1) revealed that the ITS sequence was most closely related to that of *P. expansum* (NR_077154.1, 100% of similarity). Based on the above results, the WH-3 strain belongs to *P. expansum*, and was named *P. expansum* WH-3, which was submitted to the China General Microbiological Culture Collection Center, Beijing, China with the accession number CGMCC 16798.

The biotransformed product was purified and crystallized (Pan et al., 2008) to obtain colorless and scentless crystals, and was identified by nuclear magnetic resonance (NMR), mass spectrum (MS), and optical rotation analyses (Fig. S2). The ^{1}H-NMR spectrum of the biotransformation product showed that the singlets at δ 4.516 and δ 4.700 corresponded to the CH of TA and deuterium oxide, respectively. Two singlets at δ 71.717 and δ 174.391 were detected by ^{13}C-NMR, which corresponded to CHOH and COOH of TA, respectively. The MS spectrum showed a molecular weight of 149.7, indicating that the biotransformation product was TA. Its optical rotation was +12.21 ($[\alpha]_D^{25}$), which was similar to that of standard L(+)-TA, indicating that the product was L(+)-TA rather than D(–)-TA.

Fermentation conditions were optimized based on the PDB medium by the activity of fermentation supernatant and mycelial pellet formation according to Xu et al. (2018), as shown in Fig. 2. The activity

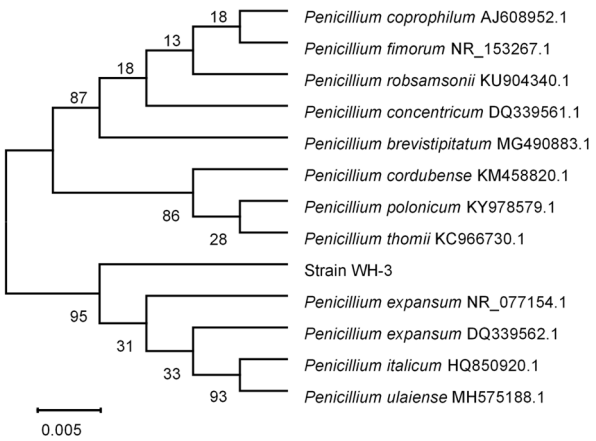

Fig. 1 Phylogenetic tree for strain WH-3 and related strains based on the internal transcribed spacer (ITS) sequence with sequence accession numbers

of mycelial pellet, fermentation supernatant, and fermentation broth was measured by activity units of U/g, U/L, and U/L, respectively, according to Bao et al. (2020). The presence of *cis*-epoxysuccinate (the substrate of CESH transformation) and L(+)-TA or D(−)-TA (the product of CESH transformation) could not improve enzyme activity (Fig. 2a), indicating that CESH is constitutively expressed. In addition, the substrate slightly inhibited the growth of colonies (whose biomass was 85% of that of the control). Therefore, no inducer was added in the subsequent optimization process. A difference in carbon source (Fig. 2b) affected the secretion and activity of CESH. The activity of fermentation supernatant and mycelial pellet was the highest when the carbon source was glucose. However, no enzyme activity was observed when lactose was used. When sucrose or maltose was applied, enzyme activity was present in the mycelial pellet, while the fermentation supernatant showed no activity. However, ethyl acetate gave the opposite result. Therefore, glucose at 10 g/L was added in the subsequent optimization process. Inorganic nitrogen source (Fig. 2c) and organic nitrogen source (Fig. 2d) only affected the level of enzyme activity, and thus the changes of activity in fermentation supernatant and mycelial pellet were synchronized with NH_4NO_3 and yeast extract, respectively, to acquire the highest level. The optimal temperature and pH were 25 °C (Fig. 2e) and pH 5.0 (Fig. 2f), respectively. Based on the above conditions, the maximum activity of mycelial pellet and fermentation supernatant was (9.1± 0.4) U/g (for 3 d) and (95.0±4.9) U/L (for 4 d), respectively, when using PDB medium with 10 g/L of glucose, 1.4 g/L of NH_4NO_3, and 20 g/L of yeast extract, and cultivating at 25 °C, pH 5.0 (Fig. 2g).

After optimization in flask, batch culturing was carried out in a 5-L fermentor (Fig. 3). The pH value decreased quickly in the first 24 h, reached its minimum level of 3.5 at 36 h, and finally increased to 4.2. The increasing trend of enzyme activity in the fermentation supernatant and mycelial pellet was obviously lagging behind biomass growth. Biomass increased quickly during the first 24 h, while the activity of mycelial pellet was almost zero. The maximum biomass, activity of mycelial pellet and fermentation supernatant were (17.1±0.8) g/L at 96 h, (10.6±0.5) U/g at 84 h, and (107.0±5.8) U/L at 72 h, respectively.

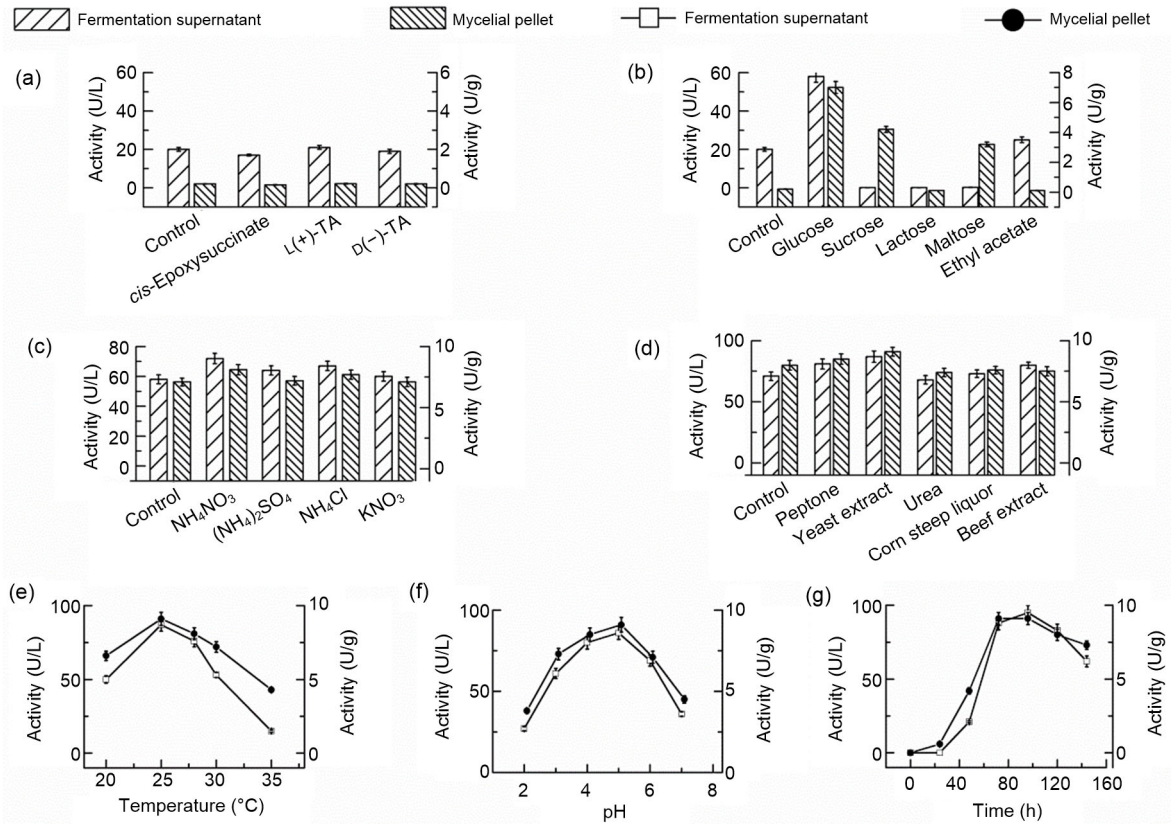

Fig. 2 Optimization of fermentation conditions for activity of mycelial pellet (U/g) and fermentation supernatant (U/L), including different inducers (a), carbon sources (b), inorganic nitrogen sources (c), organic nitrogen sources (d), temperatures (e), initial pH values (f), and fermentation time (g)

A potato dextrose broth (PDB) medium fermented at 25 °C, pH 5.0 for 3 d was used as control for (a) and (b). A PDB medium with 10 g/L of glucose was used as control for (c). A PDB medium with 10 g/L of glucose and 1.4 g/L of NH_4NO_3 was used as control for (d). TA: tartaric acid. Data were expressed as mean±standard deviation (n=3)

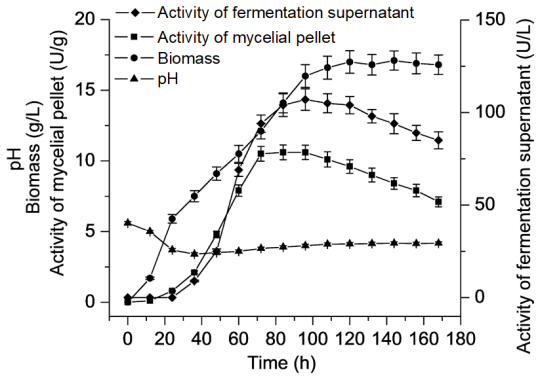

Fig. 3 Fermentation curves for pH, biomass, activity of fermentation supernatant and mycelial pellet of *Penicillium expansum* WH-3 in a 5-L fermenter

Data were expressed as mean±standard deviation (n=3)

In order to characterize CESH and biotransformation, disodium *cis*-epoxysuccinate was transformed per one liter of whole fermentation broth, mycelial pellet, and fermentation supernatant, respectively (Fig. 4a). Activity for these was (276.5±13.1), (165.3±9.4), and (108.6±5.3) U/L, respectively, which indicated that about 60% of CESH was distributed in the mycelial pellet. The conversion rate for fermentation broth and mycelial pellet reached more than 95% after 48 and 72 h, respectively, while that for fermentation supernatant was only 70% after 96 h due to its low activity and poor stability. Mycelial pellet was used for the characterization of CESH and repeated batch conversions, as it is easy to recycle and contains most of the activity.

The CESH was stable below 30 °C with an optimal temperature of 37 °C (Fig. 4b); it maintained most of the residual activity between pH 4 and 10 with an optimum of pH 8.0 (Fig. 4c). The transformation solution was analyzed by high-performance liquid chromatography (HPLC) (Fig. 4d) with 1.886 min retention time for D(−)-TA and 8.695 min for L(+)-TA, and an enantiomeric excess (EE) value for L(+)-TA of 99.6%.

The properties of CESH described in this study were compared to those from previously reported strains (Table 1). In comparison to the previous isolation from soil, *P. expansum* WH-3 was isolated from disodium *cis*-epoxysuccinate solution. However, its enzyme activity was much lower than that from previously reported bacteria and *A. niger* WH-2. Enzyme thermostability was similar to that from *A. niger* WH-2, but lower than that from bacteria. Therefore, further work such as genetic engineering and directed modification will be needed to improve its activity and stability. Although its optimal pH was similar to that from bacteria, the CESH maintained 55% of residual activity at pH 5.0 and 35% at pH 4.5, while that from bacteria lost nearly all its activity at pH<5. This indicates that CESH from *P. expansum* WH-3 could catalyze under acidic conditions showing relatively high acid stability, similarly to CESH from *A. niger* WH-2.

Disodium *cis*-epoxysuccinate, the substrate of CESH, is synthesized under acidic conditions at pH 4.0–4.5. Since CESH from bacteria lose catalytic

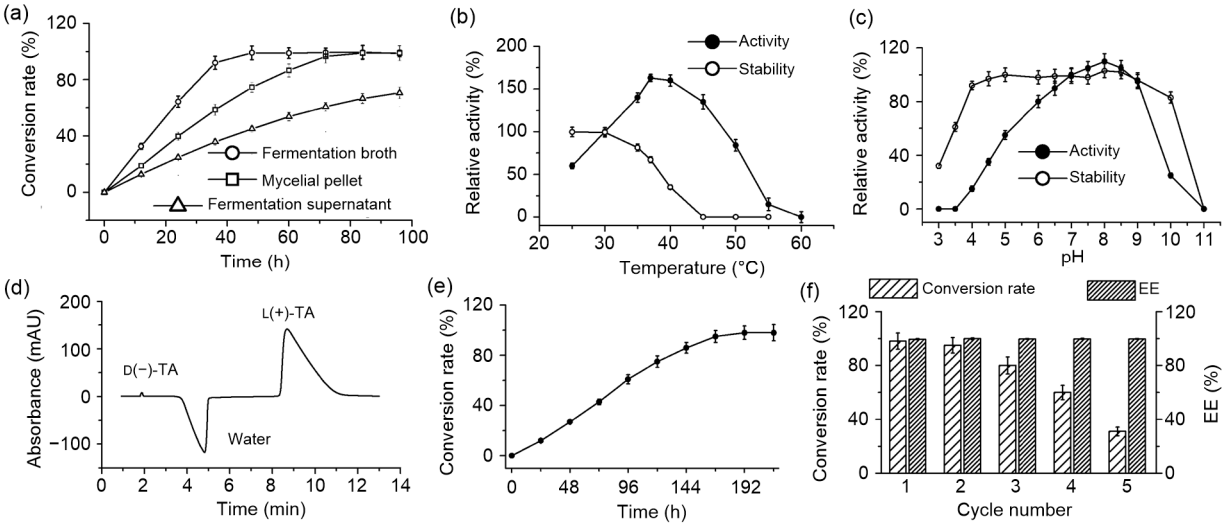

Fig. 4 Characterization and biotransformation of CESH from mycelial pellet of *Penicillium expansum* WH-3

(a) Biotransformation curves of fermentation broth, mycelial pellet, and fermentation supernatant; (b) Effects of temperature on enzyme activity and stability; (c) Effects of pH on enzyme activity and stability; (d) Enantioselectivity analysis by high-performance liquid chromatography (HPLC); (e) Biotransformation curves of mycelial pellet at pH 4.5; (f) Conversion rate and enantiomeric excess (EE) value for repeated batch conversion by mycelial pellet at pH 4.5. CESH: *cis*-epoxysuccinate hydrolase. TA: tartaric acid. Data were expressed as mean±standard deviation (n=3)

Table 1 Comparison of enzymatic properties of CESH from different species

Species	Source	Activity of cells (U/g)	Optimum temperature (°C)	Thermal stability (°C)	Optimal pH	Active pH range (>20% residual activity)	Conversion rate (%, 1 mol/L substrate)	EE value (%)	Reference
Penicillium expansum WH-3	Contaminated disodium CESH	10.6	37	<30	8.0	4.0–10.0	>98	>99.6	This study
Aspergillus niger WH-2	Soil	1278	50	<30	8.5	3.5–10.0	>95	>99.6	Bao et al., 2020
Rhodococcus opacus ML-0004	Soil	>10000	37	<35	7.5	6.0–9.0	>99	>99.5	Liu et al., 2007
Klebsiella sp. BK-58	Soil	>10000	50	<50	8.5	6.5–10.0	>99	>99.5	Cheng et al., 2014
Labrys sp. BK-8	Soil	3597	55	<55	8.5	6.0–10.0	>99	>99.5	Bao et al., 2019

CESH: *cis*-epoxysuccinate hydrolase; EE: enantiomeric excess

function under acidic conditions, the substrate solution needs to be adjusted to neutral with the administration of high volumes of sodium carbonate, increasing the load on the ion exchange column. However, CESH from *P. expansum* WH-3 retains catalytic function at pH 4.5, indicating that it can be directly transformed without adjusting the pH. This reduces the requirement for sodium carbonate greatly, thereby lowering the cost of L(+)-TA purification. Accordingly, transformation (Fig. 4e) was carried out by 20 g of mycelial pellet in total of 1 L system with 10 mmol/L of disodium *cis*-epoxysuccinate at pH 4.5 (30 °C), which resulted in a conversion rate higher than 99%. The mycelial pellet was used for transformation at pH 4.5 for 7 d to investigate its stability in repeated batch conversions (Fig. 4f). The conversion rate reached 31%, with the EE value maintaining 99.6% after five repeated batches. In order to investigate the effect of substrate concentration on CESH conversion rate, mycelial pellets from *P. expansum* WH-3 and *A. niger* WH-2 were used to transform disodium *cis*-epoxysuccinate at 10 mmol/L, 0.2 mol/L, 0.5 mol/L, and 1 mol/L. Conversion rates obtained for *P. expansum* WH-3 were 99.3%, 99.1%, 98.7%, and 98.4%, respectively, while those for *A. niger* WH-2 were 98.6%, 98.4%, 97.5%, and 95.3%, respectively. This indicates that despite the lower activity of CESH from *P. expansum* WH-3 compared to *A. niger* WH-2, higher conversion rates are achieved, especially at high substrate concentrations.

In conclusion, *P. expansum* WH-3, the second reported fungus as candidate for L(+)-TA production after *A. niger* WH-2, was isolated and identified from contaminated disodium *cis*-epoxysuccinate samples. Maximum activity of the mycelial pellet and fermentation broth supernatant, in a 5-L fermenter after optimization in a flask, was (10.6±0.5) U/g and (107.0±5.8) U/L, respectively. Transformation experiments showed that almost 60% of CESH was present in the mycelial pellet, which could catalyze at pH 4.5 with strong acidic stability, high conversion rate, and good enantioselectivity. Modification by gene cloning to improve the activity and stability of CESH from *P. expansum* WH-3 is in progress.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Contributors

The research design and manuscript were prepared by Wen-na BAO. Experiments were conducted by Yi CHEN, Hong-xiu LIAO, and Hang CHEN. The English language was polished by Shi-wang LIU. Data analysis was performed by Yong LIU. All authors have read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of data.

Compliance with ethical guidelines

Wen-na BAO, Yi CHEN, Hong-xiu LIAO, Hang CHEN, Shi-wang LIU, and Yong LIU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Morphology of *Penicillium expansum* WH-3
Fig. S2 ^{1}H-NMR (a), ^{13}C-NMR (b), and mass spectrum (c) of the biotransformed product
Materials and methods

中文概要

题　目：L(+)-酒石酸生产菌株扩展青霉 WH-3 的筛选
目　的：筛选产 L(+)-酒石酸的新真菌菌株。
创新点：首次筛选获得能同时在酸性和高底物浓度下转化生产 L(+)-酒石酸的菌株。
方　法：利用电镜、内转录间隔区（ITS）序列、核磁共振、质谱和旋光度对筛选菌株进行鉴定，并优化其发酵条件，研究其酶学性质。
结　论：扩展青霉 WH-3 的 60%活性位于菌丝体中，且该菌丝体能在酸性（pH 4.5）、高浓度底物中转化，具有高转化率和高立体特异性，为 L(+)-酒石酸的生产提供新的催化剂。
关键词：L(+)-酒石酸；扩展青霉；顺式环氧琥珀酸水解酶；筛菌