Enhancement of L-amino Acid Oxidase Production by Bacillus Subtilis HLZ-68 With Oxygen-vector and Asymmetric Degradation of DL Arginine to D-arginine

peng xu
College of Life Science and Technology, Guangxi University https://orcid.org/0000-0002-8592-2340
Changpei Pan
Nanning science and technology enterprise incubation base Co., Ltd
Gongcheng Cui
College of Life Science and Technology, Guangxi University
ChunYan Wei
College of Life Science and Technology, Guangxi University
Lijuan Wang
College of Life Science and Technology, Guangxi University
Yanting Li
College of Life Science and Technology, Guangxi University
Xiangping Li
College of Animal Science and Technology, Guangxi University
Shihai Huang (583803544@qq.com)
College of Life Science and Technology, Guangxi University

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Enhancement of L-amino acid oxidase production by *Bacillus subtilis* HLZ-68 with oxygen-vector and asymmetric degradation of DL arginine to D-arginine

Peng Xu[a,1], Changpei Pan[b,1],Gongcheng Cui[a],ChunYan Wei[a], Lijuan Wang[a],Yanting Li[a], Xiangping Li[c,*], Shihai Huang[a,*]

[a]College of Life Science and Technology, Guangxi University; [b]Nanning science and technology enterprise incubation base Co., Ltd; [c]College of Animal Science and Technology, Guangxi University

Abstract The *Bacillus subtilis* HLZ-68 can produce L-amino acid oxidase (L-AAO), and DL-arginine can be degraded asymmetrically by suspending the wet bacteria in the degradation liquid. By adding oxygen-vectors to the fermentation medium, the collected amount of wet bacteria can be increased. Taking n-dodecane, n-hexadecane, oleic acid, paraffin, and n-hexane as oxygen-vectors, the optimal oxygen-vector oleic acid was 1.2% (v/v). The weight of wet cells increased by 66.83% compared with before, and the activity of L-AAO in fermentation broth increased by 38.88% compared with before. The standard sample DL-arginine was derivatized by phenyl isothiocyanate, and then subjected to high performance liquid chromatography(HPLC), and the obtained peak area and arginine content were used as standard curves to measure the DL-arginine. The content of D-arginine and L-arginine in the initial degradation solution was 50% each, and the bacterial cells are added to the initial degradation solution of DL-arginine. After 21 hours of reaction, L-arginine was completely Degraded, remaining 47% of D-arginine.D-alanine was easily extracted from the reaction solution using cation-exchange resin,after centrifugation, decolorization, concentration and vacuum drying, and the chemical and optical purity of the extracted d-alanine was 92.68 and 97.46%, respectively.

Key points

Increase the amount of dissolved oxygen during fermentation of Bacillus subtilis by adding an oxygen-vector to obtain a large amount of wet cells and L-AAO.

De-asymmetric degradation of L-arginine in DL-arginine by L-AAO from Bacillus subtilis fermentation product, thereby obtaining D-arginine.

D-Arginine and L-Arginine are derived from phenyl isothiocyanate, and supelco chiral column (25 cm×4.6 mm, 5 μm) is used to detect D-Arginine and L-Arginine in the degradation solution. The content of lysine.

Introduction

Each an essential amino acid has its optimal intake and minimum intake(Hoffer 2016).Because D-arginine is an unnatural amino acid, its content in nature is very small, so it needs to be obtained through industrial production. D-arginine plays an important role in improving the response of glucocorticoids to normal life pressure (Griselda 2011). D-arginine plays an analgesic role during acute pain (Wolkart et al. 2004). D-arginine has protective effect on oxygen free radical induced cardiac injury (Suessenbacher et al. 2002;Martin et al. 2019). D-arginine can be used in many fields. In general, arginine can be classified into D-arginine and L-arginine according to its molecular structure of chiral (Moozeh et al. 2015) and optical rotation (Radkov et al. 2014).The existing production methods of D-arginine include microbial fermentation, bio chemical conversion, asymmetric resolution and asymmetric degradation.Asymmetric degradation is a method that directly utilizes the specificity and selectivity of enzymes in microbial cells to catalyze the oxidation of substrates. For example, L-AAO directly degrades L-amino acids in DL amino acids to produce the process of separation and purification of D-amino acids (Takahashi et al. 1997;Umemura et al. 1989). Microbial cells as a tool for the separation of l-amino acids and D-amino acids in asymmetric degradation(Singh et al. 2009).Oxygen is a significant factor affecting the growth, metabolism and morphology of microorganisms. Increasing the amount of dissolved oxygen is conducive to the collection of Bacillus subtilis.Compounds added to fermentation media that improve oxygen transfer to microorganisms are defined as oxygen-vectors(Cascaval et al. 2006).oxygen-vector is a kind of organic

solvent which has no toxicity to microbial cells, can combine with oxygen in the system, and is insoluble in water, and can transfer oxygen to the fermentation system (Xu et al. 2014). oxygen-vector addition technology is a new technology developed in 1980s. It is found that oxygen-vector can carry oxygen from gas phase to liquid phase, providing more dissolved oxygen and higher oxygen solubility than water phase, especially in aerobic fermentation system(Zhu et al. 2014).It is important that oxygen-vector can solve the high cost problem of oxygen transfer in fermentation tank. Adding oxygen-vector is economical, simple and practical.

Materials and methods

Materials

Source of bacteria

Bacillus subtilis HLZ-68 cells were obtained from Guangxi University, China.

Reagents and Solutions

Reagent A: 1.2% (V / V) PITC acetonitrile solution.

Reagent B: 14% (V / V) triethylamine (tea) acetonitrile solution.

Mobile phase A: 0.2 mol / L acetic acid sodium acetate buffer solution with pH 6.5.

Mobile phase B: acetonitrile: water: 4:1.

Methods

Selection of oxygen-vector and preparation of cell suspension

Bacillus subtilis HLZ-68 was fermented for 24 h in a fermentor with 80% filling factor, at 30 °C with an aeration of 1.0 vvm and agitation of 300 rpm.The oxygen-vector selected for fermentation and its added amount are 1.5% n-dodecane, 1.5%

n-hexadecane, 1.5% oleic acid, 1.5% paraffin, and 0.8% n-hexane.The fermentation medium was composed of (w/v) 2% dl-alanine, 0.5% yeast extract, 0.2% K_2HPO_4, 0.09% $MgSO_4 \cdot 7H_2O$, and 0.02% $CaCl_2$. Determine the effect of different oxygen-vectors on the weight of wet bacteria and the enzyme activity of L-AAO, and select the best oxygen-vector. After fermentation, the cells are collected by centrifugation（6500 g for 10 min）, washed twice with 0.9% saline and resuspended..

Determination of D-arginine and L-arginine in degradation solution

Different optical isomers of arginine were derivatized with phenyl isothiocyanate, and then the arginine isomers were separated and determined by HPLC.

Derivatization of sample: add 1 ml of diluted DL arginine into 10 ml centrifuge tube with pipette gun, add 1 ml reagent A and 1 ml reagent B into 10 ml centrifuge tube, derivatize at room temperature without light shock for 1 h, then add 3 ml n-hexane and extract for 2 min at room temperature. The lower layer solution is used as the sample.

Detection conditions: Superco chiral column (25 cm × 4.6 mm, 5 μ m); mobile phase A: mobile phase B: 5:1; total flow rate of mobile phase A and mobile phase B: 1 ml / min; manual injection 20 μ L; detection wavelength of UV detector 254 nm; column temperature of 25℃.

Determination of the ability of bacteria to degrade DL arginine

4 g DL-arginine was added into 50 / 250 ml initial degradation solution(KH_2PO_4 5 g/L、$Mg_2SO_4 \cdot 7H_2O$ 2 g/L、$CaCl_2$ 0.1 g/L、pH=7.0) containing 3 g wet cells. The degradation temperature was 30 ℃, and the rotation speed of shaking table was 180 r / min. The contents of D-arginine and L-arginine were determined every 3 hours, and each group was parallel three times. The ability of wet cells to degrade L-arginine in DL arginine was studied by HPLC.

Extraction of d-alanine from the degradation mixture

The isoelectric point of D-arginine is 10.76. The pH of the degradation solution containing amino acids is adjusted to 7, which is

lower than the isoelectric point of D-arginine. D-arginine is positively charged and can be combined with strong acid cation exchange resin. Finally, D-arginine is eluted with 2 mol / L ammonia water, and the eluent is decolorized with 10 g / L activated carbon. Then the activated carbon is centrifuged and filtered out. The decolorized eluent is at 55 The concentrated eluent was put into a 65 ℃ vacuum drying oven until D-arginine crystallized.

Determination of chemical purity and optical purity of D-arginine

The product D-arginine is dissolved in deionized water, diluted to a suitable concentration, and the content of D-arginine is measured by HPLC. The chemical purity of the product D-arginine is determined as follows(Zhang et al. 2017)

$$p_1 = \frac{wv}{m} \times 100\%$$

P_1: Chemical purity (%)

w: D-arginine concentration (mg/L)

v: Volume

m: Quality

Using deionized water as the reference solution, accurately weigh 0.1 g product D-arginine and 0.1 g pure product D-arginine, respectively, and dissolve them in 100 mL deionized water. Use a polarimeter to determine the optical rotation α, product D -The detection formula of arginine optical purity is as follows:

$$[\alpha]_{\lambda}^{T} = \frac{100a}{LC}$$

α: Optical rotation

T: Determination temperature (℃)

λ: Wavelength of light wave

L: Length of polarimeter (CM)

C: Mass of sample in 100ml solution (g)

The optical purity of the product is calculated according to the optical rotation ratio of the measured sample. The calculation formula is as follows:

$$p_2 = \frac{A_1}{A_2} \times 100\%$$

P_2: Optical purity

A_1: Specific rotation of sample

A_2: Specific rotation of pure product

Results

Selection of oxygen-vector

Table 1 Effects of different oxygen-vectors on wet cell weight and enzyme activity

oxygen-vector	Concentration %（v/v）	24h wet cell weight (g)	L-AAO enzyme activity （U/g）
Blank	0	3.02	37.45
N-hexadecane	1.5	4.3	42.91

N-dodecane	1.5	3.8	40.67
Oleic acid	1.5	5.02	46.17
Paraffin wax	1.5	3.55	38.78
N-hexane	0.8	1.64	17.35

Determination of different isomers of arginine by HPLC

It can be seen from Fig.1 that the retention time of D-arginine on elution column is 9.09 min, and that of L-arginine is 5.12 min. Therefore, it is an effective method to detect D-arginine and L-arginine in DL arginine by derivatization with phenyl isothiocyanate.

D-arginine and L-arginine standard curve

It can be seen from Fig. 2 that the peak area in HPLC is linear with the content of D-arginine, and the regression equation is y = 19226x -7632 (R^2 = 0.9998).

It can be seen from Fig. 3 that the peak area in HPLC is linear with the content of L-arginine, and the regression equation is y = 1937705x -799274 (R^2 = 0.9992).

Degradation of different isomers of arginine

It can be seen from Fig. 4 that the initial percentage content of D-arginine and L-arginine is 50%. After 21 h of reaction, the percentage content of L-arginine is 0.7%, while that of D-arginine is 47%, and that of D-arginine is 47%

At 12 h, the percentage of L-arginine decreased rapidly by 37.4% and 11.9% from 12 h to 21 h, indicating that the living bacteria had the ability of asymmetric degradation of DL arginine. Therefore, wet bacteria could consume L-arginine in DL arginine and

produce D-arginine under certain conditions.

Isolation of D-arginine and its purity

D-arginine was isolated from the reaction mixture by using a simple procedure (see “Materials and methods” section). After cation exchange and drying, resulting in a yield of 91.58%. The specific rotation of the obtained D-arginine was $[\alpha]_{\lambda}^{20} = -12.5^{\circ}$ at 20 °C. Besides, the chemical purity of the isolated and purified D-arginine was 92.68% and the optical purity was 97.46%.

Discussion

Bacillus subtilis HLZ-68 possesses L-amino acid oxidase and not D-amino acid oxidase and can carry out asymmetric oxidative degradation of L-arginine from DL-arginine. Besides, as this *Bacillus subtilis* HLZ-68 strain did not exhibit racemase activity and could not transform L-arginine into D-arginine, and just consumes L-arginine, leaving D-arginine, A similar paper has also been reported by Singh S (2009). In order to obtain a large number of wet bacteria, oxygen-vectors were added to the initial fermentation broth to increase the dissolved oxygen in the fermentation broth and solve the problem of low cell reproduction caused by insufficient oxygen in the fermentation process(Wei et al. 2012). Blaga A C (2018) increased ergosterol production by *S. cerevisiae* fed batch fermentation with n-dodecane as oxygen-vector. The oxygen-vectors commonly used in industrial production are n-hexadecane, n-dodecane, oleic acid, paraffin and n-hexane. Oleic acid was selected as the optimal oxygen-vector The weight increased by 66.83%, and the activity of L-amino acid oxidase increased by 38.88%. After cation exchange and drying, the chemical and optical purity of the obtained D-arginine was 92.68% and 97.46%.

In conclusion, L-arginine in DL arginine can be degraded asymmetrically by *Bacillus subtilis* HLZ-68 to produce D-arginine. Compared with other methods, Adding oxygen-vector solves the high cost problem of oxygen transfer in fermentation tank, which is economical, simple and practical.

Abbreviations

L-AAO,L-amino acid oxidase.

HLPC,High performance liquid chromatography.

Availability of data and materials

All data and materials are available from the corresponding authors on reasonable request.

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Author information

Peng Xu and Changpei Pan are contributed equally to this work.

Affiliations

College of Life Science and Technology, Guangxi University.

Peng Xu, Gongcheng Cui, Chunyan Wei, Lijuan Wang, Yanting Li and Shihai Huang

College of Animal Science and Technology, Guangxi University.

Xiangping Li

Nanning science and technology enterprise incubation base Co., Ltd

Changpei Pan

Contributions

Peng Xu and Changpei Pan conducted the experiments and analyzed the data. Gongcheng Cui and Chunyan Wei conceived and designed the study. Lijuan Wang and Yanting Li contributed to the experimental design and statistical analysis. Peng Xu and Changpei Pan wrote the first draft of the manuscript. Xiangping Li and Shihai Huang revised the manuscript completely. All authors read and approved the final manuscript.

Corresponding authors

Correspondence to Xiangping Li or Shihai Huang.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Additional information

Publisher's Note

References

Hoffer L J(2016) Human Protein and Amino Acid Requirements. Journal of Parenteral and Enteral Nutrition 40(4):460-474.

https://doi.org/10.1177/0148607115624084

Griselda C M(2011)D-arginine action against neurotoxicity induced by glucocorticoids in the brain. Neuroscience & Biobehavioral Reviews 35(6): 1353-1362.https://doi.org/10.1016/j.neubiorev.2011.02.009

Wolkart G, Stessel H, Brunner F(2004) In vivo administration of d-arginine: effects on blood pressure and vascular function in angiotensin II-induced hypertensive rats. Atherosclerosis1 76:219−225. https://doi.org/10.1016/j.atherosclerosis.2004.05.016

Suessenbacher A, Lass A, Mayer B(2002)Antioxidative and myocardial protective effects of L-arginine in oxygen radical-induced injury of isolated perfused rat hearts. Naunyn-Schmiedeberg's Archives of Pharmacology 365(4):269-276.https://doi.org/10.1007/s00210-001-0523-9

Martin S, Desai K(2019)The effects of oral arginine on its metabolic pathways in Sprague-Dawley rats. The British journal of nutrition 123(2).https://doi.org/10.1017/S0007114519002691

Moozeh K, So SM, Chin J(2015)Catalytic stereoinversion of L-Alanine to deuterated D-Alanine. Angew Chem 54:9381 https://doi.org/10.1002/anie.201503616

Radkov AD, Moe LA (2014) Bacterial synthesis of D-amino acids. Appl Microbiol Biotechnol 98:5363.https://doi.org/10.1007/s00253-014-5726-3

Takahashi E, Furui M, Shibatani T.(1997)D-Amion acid production from racemic amion acids by microbial asymmetric degrading Activity of Candida maltisa. Biotechnology Techniques 11(12):913-916.https://doi.org/10.1023/A:1018470502118

Umemura I, Yanagiya K, Komatsubara S(1989)D-Alanine production by Using Asymmetric Degrading Activity of Candida maltosa. Annals of the New York Academy of Sciences 613(1):659-662.https://doi.org/10.1007/BF00172182

Singh S, Gogoi B K, Bezbaruah R L(2009)Optimization of mediun and cultivation conditions for L-amino acid oxidase production by *Aspergillus fumigatus*. Canadian Journal of Microbiology 55(9):1096-1102.https://doi.org/10.1139/w09-068

Cascaval D, Galaction A L(2006)Folescu E. Comparative study on the effects of n-dodecane addition on oxygen transfer in stirred bioreactors for simulated, bacterial and yeasts broths. Biochemical Engineering Journal 33(1):56-66.https://doi.org/10.1016/j.bej.2006.05.019

Xu Z X, Bo F F, Xia J(2014)Effects of oxygen-vectors on the synthesis of epsilon-poly-lysine and the metabolic characterization of Streptomyces albulus PD-1.

Biochemical Engineering Journal (94):58-64

https://doi.org/10.1016/j.bej.2014.11.009

Zhu P, Dong S, Li S(2014)Improvement of welan gum biosynthesis and transcriptional analysis of the genes responding to enhanced oxygen transfer by oxygen vectors in *Sphingomonas sp*. Biochem. Eng. J (90): 264–271.https://doi.org/10.1016/j.bej.2014.06.011

Wei L Z, Abdul R R, Rosfarizan M(2012)Biosynthesis of high molecular weight hyaluronic acid by Streptococcus zooepidemicus using oxygen vector and optimum impeller tip speed. Journal of Bioscience and Bioengineering 114(3): 286-291.https://doi.org/10.1016/j.jbiosc.2012.04.011

Zhang Y Y, Li X P, Zhang C F(2017)Production of D-alanine from DL-alanine using immobilized cells of *Bacillus subtilis* HLZ-68. World Journal of Microbiology & Biotechnology 33(9):176-183.https://doi.org/10.1007/s11274-017-2341-3

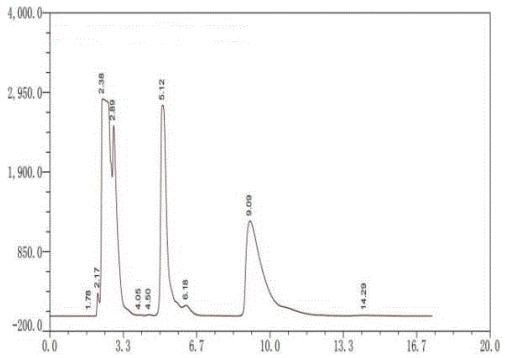

Fig.1 HPLC of different optical isomers of arginine.The standard sample DL arginine was pre -column derivatized, and the HPLC column was supelco chiral column (25 cm × 4.6 mm, 5 μ m); manual injection 20μL, UV detection wavelength 254 nm; column temperature 25 ℃; mobile phase A: mobile phase B: 5:1; total flow rate: 1 ml / min.

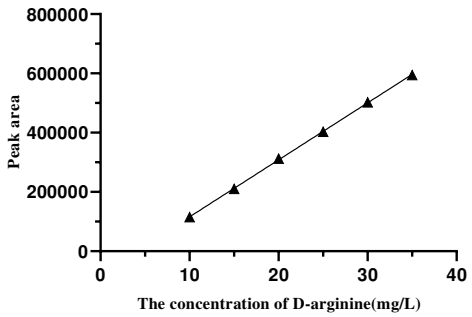

Fig. 2 Standard curve of D-arginine.Weigh a certain amount of 3.5% (w/v) D-arginine and dilute it with ultrapure water into a standard sample of a certain concentration.

The sample was injected under the above chromatographic conditions to obtain a high performance liquid chromatogram.

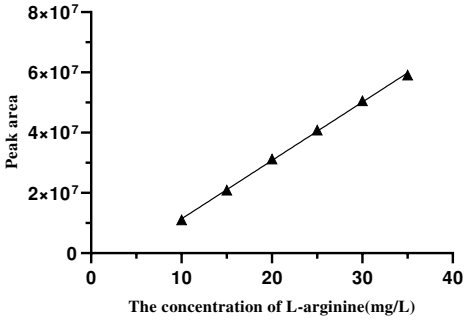

Fig. 3 Standard curve of L-arginine.Weigh a certain amount of 3.5% (w/v) L-arginine and dilute it with ultrapure water into a standard sample of a certain concentration.

The sample was injected under the above chromatographic conditions to obtain a high performance liquid chromatogram.

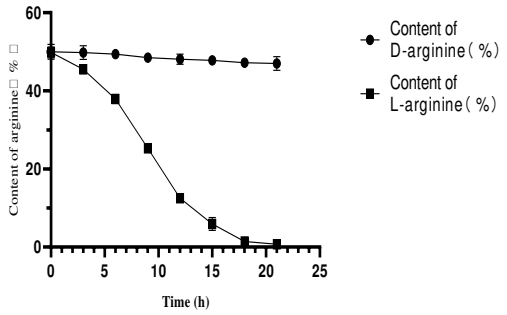

Fig.4 Dynamic changes of DL arginine degradation by *Bacillus subtilis* HLZ-68. The bacteria were added into the initial degradation solution containing DL arginine

Figures

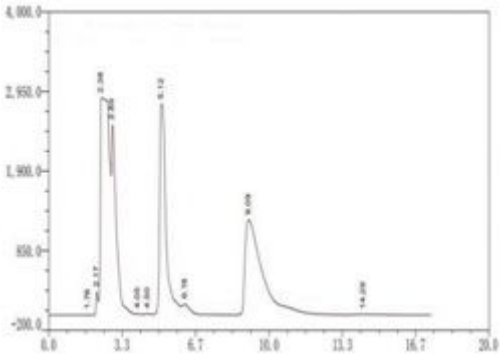

Figure 1

HPLC of different optical isomers of arginine.The standard sample DL arginine was pre -column derivatized, and the HPLC column was supelco chiral column (25 cm × 4.6 mm, 5 μ m); manual injection 20μL, UV detection wavelength 254 nm; column temperature 25 ℃; mobile phase A: mobile phase B: 5:1; total flow rate: 1 ml / min.

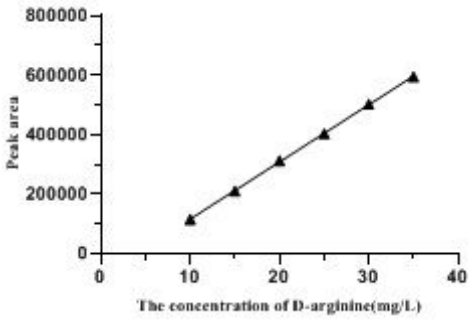

Figure 2

Standard curve of D-arginine.Weigh a certain amount of 3.5% (w/v) D-arginine and dilute it with ultrapure water into a standard sample of a certain concentration. The sample was injected under the above chromatographic conditions to obtain a high performance liquid chromatogram.

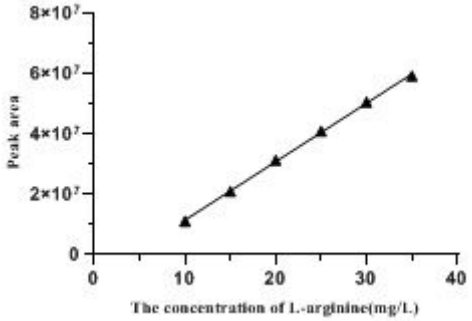

Figure 3

Standard curve of L-arginine.Weigh a certain amount of 3.5% (w/v) L-arginine and dilute it with ultrapure water into a standard sample of a certain concentration. The sample was injected under the above chromatographic conditions to obtain a high performance liquid chromatogram.

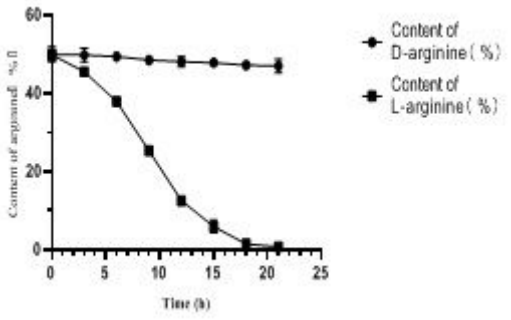

Figure 4

Dynamic changes of DL arginine degradation by Bacillus subtilis HLZ-68. The bacteria were added into the initial degradation solution containing DL arginine