MATEC Web of Conferences **22**, 05013 (2015)
DOI: 10.1051/matecconf/20152205013

Biotransformation and Production from *Hansenula Anomala* to Natural Ethyl Phenylacetate

Xun Tian, Jingwen Wang, Qianqian Li, Shimin Guan, Baoguo Cai & Shaofeng Rong*
School of Perfume and Aroma Technology, Shanghai Institute of Technology, Shanghai, China

ABSTRACT: Ethyl phenylacetate can be widely applied in many industries, such as food, medicines, cosmetics and medicinal herbs. At the moment, the production of natural ethyl phenylacetate is very limited. However, the biotransformation production of natural ethyl phenylacetate has an very extensive application prospect. This paper is written by taking the phenylacetic acid tolerance and the esterifying enzyme activity as the two indexes for screening the HA14 strain of *hansenula anomala* mutagenic which is regarded as the microorganism of ethyl phenylacetate production through biotransformation. By optimizing the production condition of phenylacetic acid and the esterification condition of ethyl phenylacetate, the production of ethyl phenylacetate accomplished through biotransformation within 72 hours can reach 864mg/L which is 171% of that of the initial bacterial strain.

Keywords: hansenula anomala, biotransformation, esterifying enzyme, ethyl phenylacetate

1 INTRODUCTION

Ethyl phenylacetate is a colorless clear liquid with rose scent. It is widely used in food industry, cosmetics industry and tobacco industry. Moreover, it can also be applied in the production of barbital hypnotic luminal [1, 2]. At present, the acquisition of ethyl phenylacetate is mainly realized by chemical synthesis preparation. The chemical synthesis method is to mix phenylacetic acid and ethanol in concentrate sulfuric acid or rare earth compound for the esterification reaction under catalyst effect. Although this esterification reaction is simple, phenylacetic acid which is the raw material in the reaction needs to be obtained through the hydrolysis of phenyl acetonitrile or phenyl acetamide. In the reaction, phenyl acetonitrile comes from the reaction of phenmethyl halide and sodium cyanide. Both the raw materials and the intermediates are violent in toxicity which will not only make it harder to separate, but will also affect the application range [3] of the product in security considerations. Natural ethyl phenylacetate can also be obtained by extracting natural essential oil or through biotransformation. The raw materials of natural essential oil are limited with high cost, and thus are unfit for industrial production and application. As the source of the reaction precursor in microorganism biotransformation is natural and abundant, the method of transforming microorganisms into natural ethyl phenylacetate has a broad application prospect [4].

The microbial transformation used in ethyl phenylacetate production is shown in Figure 1. Through the Ehrlich path inside the yeast cells, transform the L-phenylalanine into phenylacetaldehyde by transamination and decarboxylation. And then, generate phenylacetic acid by the function of $NAD(P)^+$ aldehyde dehydrogenase. The exogenouos replenished ferment ethanol will transform phenylacetic acid and ethanol into ethyl phenylacetate[5, 6] through esterification and with the effect of cell esterifying enzyme. The key parts of the biotransformation approach are the oxidation process from phenylacetaldehyde to phenylacetic acid and the esterification reaction of phenylacetic acid and ethanol. Therefore, the key of biotransformation is to find the microorganisms catalyzed by these two enzymes.

This paper applies the *hansenula anomala* including esterifying enzyme and $NAD(P)^+$aldehyde dehydrogenase genes with its mutated high-yield phenylacetic acid bacterial strains to screen for the phenylacetic acid tolerance and the esterifying enzyme activity of these bacterial strains, so as to find the high-yield bacterial strains with an industrialization prospect. Moreover, this paper aims to further improve the ethyl phenylacetate production ability of the initial bacterial strains by optimizing the biotransformation conditions of phenylacetic acid and ethyl phenylacetate.

2 MATERIALS AND METHODS

2.1 *Bacterial Strains*

The *hansenula anomala* (CICC 31006, HA) was bought from China Center of Industrial Culture Collection (CICC). The mutagenic bacterial strains of HA03、HA07、HA11、HA14 and HA17can be obtained from the initial bacterial strain HA through the mutagenic screening (nitrosoguanidine and γ-rays compound mutation) conducted in the laboratory. These five mutagenic bacterial strains contain stronger phenylacetic acid production ability of the initial bacterial strain.

*Corresponding author: rong_sf@163.com

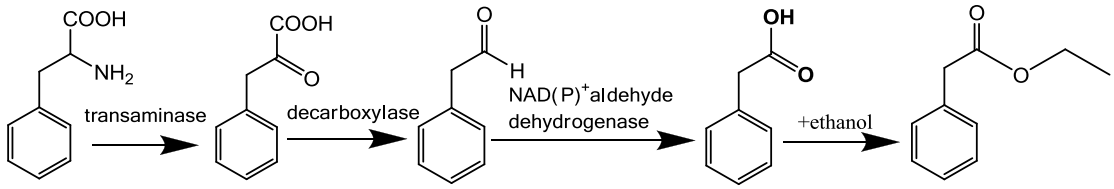

Figure 1. Ethyl Phenylacetate Production by Microbial Transformation

2.2 *Culture Media*

(1)Solid culture media: glucose 10 g/L, peptone 3 g/L, yeast extract 3 g/L, K_2HPO_4 5g/L, $MgSO_4$ 5g/L, NaCl 1 g/L, agar 20 g/L, pH7.0 and sterilization with the temperature of 121°C lasting for 20 min.

(2)Seed culture media: glucose20 g/L, peptone 3 g/L, yeast extract 3 g/L, K_2HPO_4 5g/L, $MgSO_4$ 5g/L, NaCl 1 g/L, pH7.0, sterilization with the temperature of 121°C lasting for 20 min.

(3)Biotransformation culture media: glucose 20 g/L, peptone 3 g/L, yeast extract 3 g/L, K_2HPO_4 5 g/L, $MgSO_4$ 5 g/L, NaCl 1 g/L, L-phenylalanine2 g/L, the packing volume of the culture media should be 50mL in each triangular bottle with volume of 250mL, and sterilization with the temperature of 121°C lasting for 20 min(phenylalanine should have filter sterilization alone).

2.3 *Experimental Methods*

2.3.1 *Screening Experiment for the Tolerant Bacterial Strains of Phenylacetic Acid*

Put the six *hansenula anomala* strains mentioned above into solid culture media with temperature of 28°C and keep the cultivation for 48 hours. Select single colonies and inoculate them into the test tubes containing 5mL of seeds with temperature of 28°C. Manage shaking culture at speed of 220 r/ min for 12 hours until the logarithmic period comes. Use blood counting chamber to accurately count the number of saccharomycetes. Take 1×10^7 cells/mL as the inoculum size to respectively insert 2-phenylacetic acid with different quality concentrations (0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L and 2g/L) into the culture media. Remain the temperature at 28°C and maintain the shaking culture at speed of 220 r/min for 48 hours in order to measure OD_{600}.

2.3.2 *Screening of Hansenula Anomala with High Esterifying Enzyme Activity*

Put the seed culture media of the six *hansenula anomala* strains mentioned above in the biotransformation culture media by the same cell population for cultivation (28°C, 220 r/min). Take samples once in every 12 hours. Manage centrifugation in low-temperature refrigerated centrifuge at speed of 4000 r/min with temperature of 4°C. Maintain the centrifugation for 20 minutes to complete precipitation. The resultant supernate is crude enzyme fluid. Put the crude enzyme fluid in refrigerator and maintain the temperature at 4°C. The activity of the esterifying enzyme is to be tested.

2.3.3 *Optimization of Phenylacetic Acid Production Conditions*

Inoculate the screened phenylacetic acid with strong tolerance and the bacterial strains with high esterifying enzyme activity in the culture media. Maintain the temperature at 28°C and conduct the shaking culture at speed of 220 r/min for 12 hours until the logarithmic phase comes. Change over the resultant product in the biotransformation culture media. During the experiment, $FeCl_3$ should be added into the culture media (0 g/L, 0.05 g/L, 0.1 g/L, 0.3 g/L and 0.5g/L); the temperature should be adjusted (26°C, 28°C, 30°C, 32°C and 34°C); the pH value should be varied (4, 5, 6, 7, and 8); and the rotational speed should be changed (160rpm, 180rpm, 200rpm, 220rpm and 240rpm). Study the impacts that the procedures mentioned above have on the production of phenylacetic acid.

2.3.4 *The Phenylacetic Acid and Ethanol were Catalyzed by Esterifying Enzyme in the Ethyl Phenylacetate Produced*

Cultivate the screened *hansenula anomala* in the seed culture media for 48 hours (28°C and 220r/min). Add 1g/L phenylacetic acid and 0.5g/L ethanol in a direct exogenous way. Study the optimal esterification condition of producing ethyl phenylacetate by *hansenula anomala* esterifying enzyme. Maintain the cultivation for 24 hours and takes samples to determine the content of ethyl phenylacetate.

In the optimized generation conditions of phenylacetic acid and ethyl phenylacetate mentioned above, compare the final ethyl phenylacetate production volumes and the precursor L-phenylalanine use ratios of the initial bacterial strains HA and the screened bacterial strains.

2.4 *Analytical Method*

2.4.1 *Determination of Esterifying Enzyme Activity* [7]

Take 10mL cyclohexane, 3.65 ml ethanol, 6.25 ml caproic acid (add 30g anhydrous sodium sulfate to every 500ml of all reagents) and 0.2 ml enzyme fluid. The esterification reation should be conducted in an airtight conical flask with volume of 100 ml. The reaction temperature should be set as 36°C. After 24

hours, take 0.5ml supernate in a conical flask with volume of 50 ml. Add 5 ml water with 2 drops of phenolphthalein, and use NaOH with density of 0.05 mol/L to accomplish the titration up to the terminal point. And then, determine the consumption of caproic acid. The definition of enzyme activity unit is given as follows: in the determination condition, the amount of enzyme needed in consuming 1 μmol caproic acid every minute is 1 enzyme activity unit.

2.4.2 *Determination of the Content of Phenylacetic Acid and Ethyl Phenylacetate* [8]

Take 5mL culture solution and add it into the 5mL dichloromethane. Repeat the shaking step for several times. Place the fluid still until the oil phase is separated. Repeat the above extraction process for three times. Combine the extract liquor and filter it through 0.45um Millipore filter for further test.

The chromatographic condition for the determination of phenylacetic acid content by high performance liquid chromatography: the model of the chromatographic column is XDB-C18(250 mm×4.5 mm, Agilent); the mobile phase is methyl alcohol: water =7:3; the flow rate is 1mL/min; and the sample size is 10uL. Apply the external standard method for standard phenylacetic acid to determine the standard curve of phenylacetic acid content. Measure the ethyl phenylacetate by the gas chromatographic method. The determination condition is that the model of the chromatographic column should be HP-5 (30 m × 0.32 mm × 0.25 μm, Agilent) and the temperature at the sample injection port should be 250°C. The initial temperature of the temperature programming should be 30°C. The temperature should be maintained for 5 minutes. And then, the temperature will rise to 130°C with a rate of 4°C /min. At last, the temperature will rise to 250°C with a rate of 10°C /min. The flow rate of carrier gas (nitrogen) is 1.0mL/min. Take the external standard method quantity of standard ethyl phenylacetate as the standard curve.

3 RESULTS AND DISCUSSION

3.1 *Screening of the Bacterial Strains with Phenylacetic Acid Tolerance and High Esterifying Enzyme Activity*

Put six *hansenula anomala* strains into the culture media with different phenylacetic acid concentrations. Maintain the cultivation for 48 hours and see the related OD_{600} in Figure 2. The results show that the increasing of phenylacetic acid addition can lead to enhanced growth inhibition effect on yeast. 1.0g/L phenylacetic acid can inhibit the growth of more than half of the *hansenula anomala*. After adding 1.5g/L phenylacetic acid, the growth of yeast can be almost completely inhibited. According to the results shown in the figure, it can be seen that HA07 mutagenic bacterial strain and HA14 mutagenic bacterial strain can present better phenylacetic acid tolerance. The selection of the bacterial strains with strong tolerance will not only improve the content of phenylacetic acid which is the intermediate product of the ethyl phenylacetate production process; but will also further improve the conversion of ethyl phenylacetate.

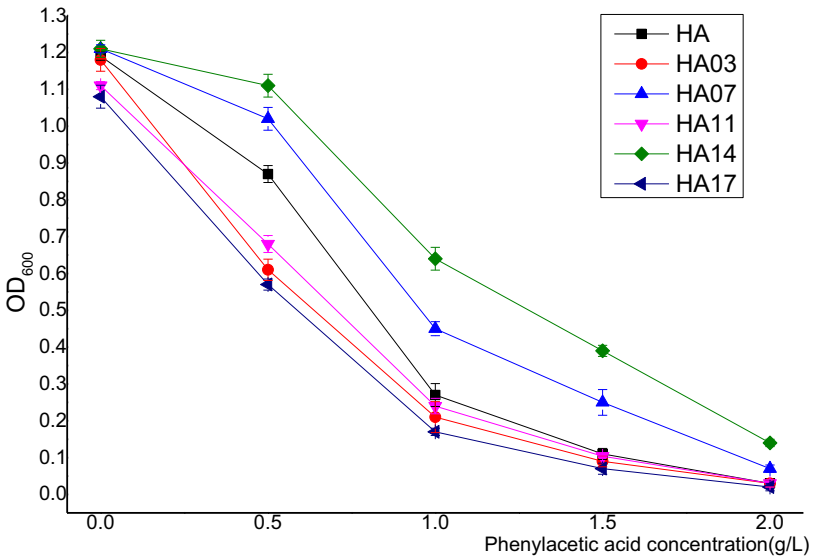

Figure 2. The 48-hour Cultivation of the six *Hansenula Anomala* Strains in the Culture Media with different Phenylacetic Acid Concentrations for the Determination of OD_{600}

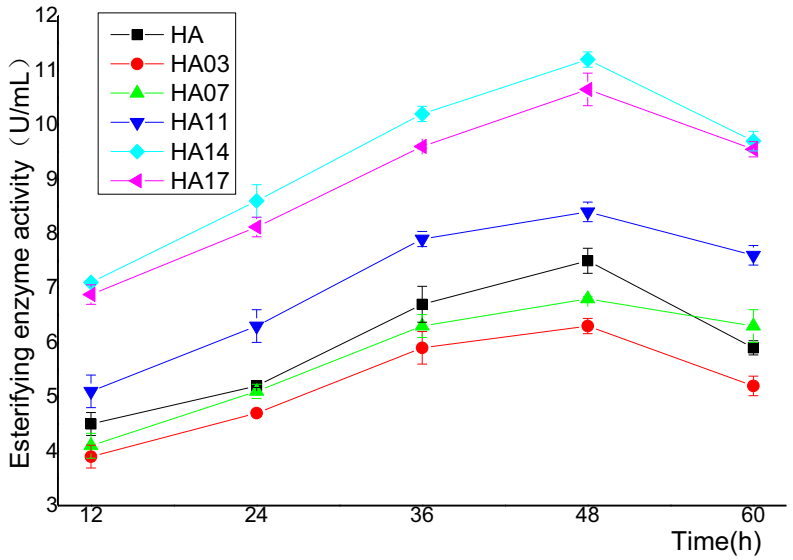

Figure 3. The Esterifying Enzyme Activity Curve of six *Hansenula Anomala* Strains

Apply the six *hansenula anomala* strains in the biotransformation culture media to determine the curve of esterifying enzyme activity. See the results in Figure 3. According to the results shown in the figure, it can be seen that the esterifying enzyme activity can reach its highest value when the six *hansenula anomala* strains have been cultivated in the biotransformation culture media for 48 hours. The highest esterifying enzyme activity of the initial bacterial strain HA is 7.5U/mL. The mutagenic strain HA 14 and HA17 of the initial bacterial strain HA can have much enhanced esterifying enzyme activity. Compared with the esterifying enzyme activity of the initial bacterial strain, the esterifying enzyme activity of HA14 and that of HA17 can be greatly improved by 49% and 42% respectively. However, the esterifying enzyme activity of the HA07 bacterial strain that has phenylacetic acid tolerance can decline by 9% compared with that of HA. The production of ethyl phenylacetate through biotransformation mainly includes the generation process off phenylacetic acid and the esterification of ethyl phenylacetate. It takes the tolerance of bacterial strain and the activity of its esterifying enzyme into comprehensive consideration. Select HA14 bacterial strain as the study bacterial strain of ethyl phenylacetate production and start the optimization of the production condition.

3.2 *Phenylacetic Acid Production through Biotransformation*

Inoculate the HA14 bacterial strains with strong phenylacetic acid tolerance and high esterifying enzyme by the same inoculum size in the biotransformation culture media. Study the optimization accomplished by the changes in pH, rotating speed, $FeCl_3$ concentration and biotransformation temperature of the biotransformation culture media. See the results in Figure 4 (A-D). The neutral meta-acid pH in the initial biotransformation culture media is the optimum condition for phenylacetic acid production. The improvement in the cultivation rotating speed of the biotransformation culture media can improve the activity of $NAD(P)^+$ aldehyde dehydrogenase, so as to further improve the production of phenylacetic acid. Temperature variation has little influence on the phenylacetic acid production through biotransformation. The addition of $FeCl_3$ can change the oxidizing condition of the culture media. The production of phenylacetic acid with 0.1g/L $FeCl_3$ can be improved by 62% compared with that of the phenylacetic acid without $FeCl_3$.

The initial culture media pH obtained from the phenylacetic acid production through biotransformation is 6. Add 0.1g/L $FeCl_3$, and control the biotransformation conditions: temperature should be 28°C; the shake cultivation at speed of 220rpm should be lasted for 48 hours; the accumulated concentration of phenylacetic acid can reach 754mg/L; and the molar conversion of L-phenylalanine can reach 0.92.

3.3 *Natural Ethyl Phenylacetate Production via Biotransformation*

After cultivating the HA14 yeast in the seed culture media for 48 hours, add 1g/L phenylacetic acid and 0.5g/L ethanol. Study the optimum temperature and the optimum pH value in the ethyl phenylacetate generation accomplished by the esterifying enzyme of the *hansenula anomala*. See Figure 5 for the results. The acidic condition when pH is 5 is the optimal condition to generate ethyl phenylacetate via the esterifying enzyme of the *hansenula anomala*. When ethyl phe-

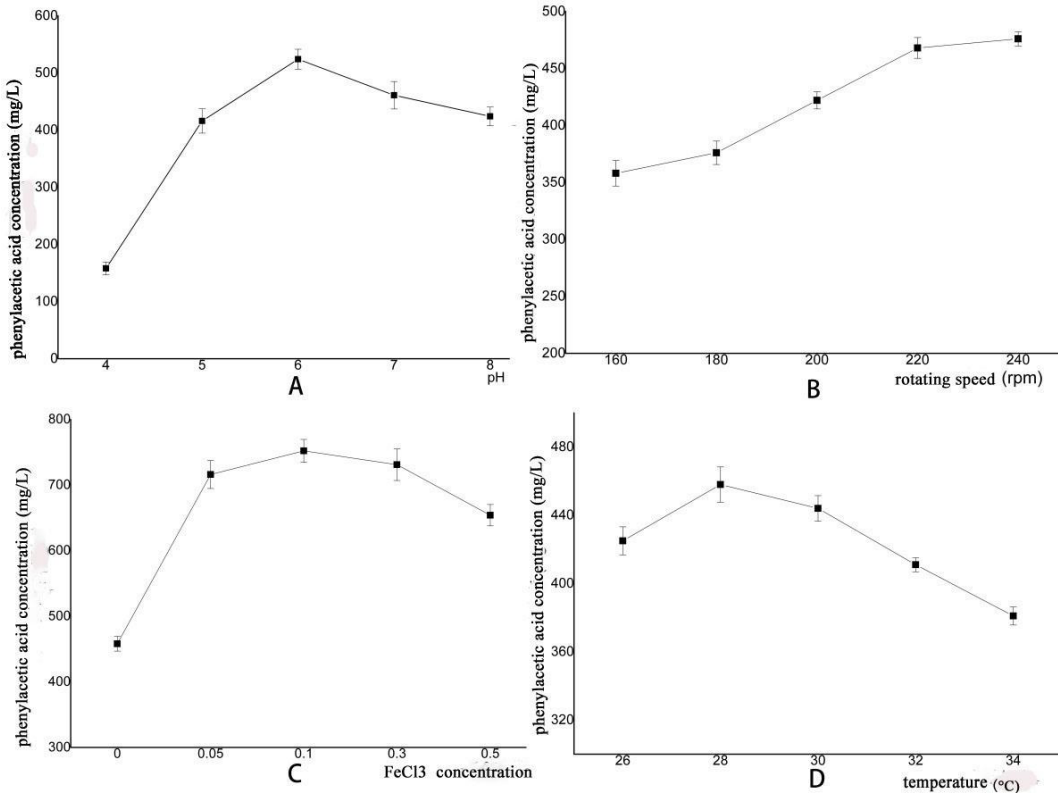

Figure 4. Conditions of Optimizing the Biotransformation for Phenylacetic Acid Production by Four Factors

Note: A, pH (3, 4, 5, 6 and 7); B, rotating speed (160rpm, 180rpm, 200rpm, 220rpm and 240rpm); C, $FeCl_3$ (0, 0.05g/L, 0.1g/L, 0.15g/L and 0.2g/L); D, temperature (26°C, 28°C, 30°C, 32°C and 34°C).

Table 1. Production of Ethyl Phenylacetate via the Biotransformation of HA and HA14 Bacterial Strains

Bacterial strain	Concentration of Phenylacetic Acid(48h, mg/L)	L-Phe Molar Conversion(mol/mol)	Concentration of Ethyl Phenylacetate(72h, mg/L)
HA	464±12	0.91	505±13
HA14	754±16	0.92	864±14

nylacetate production is realized by biotransformation, adjust the temperature from 28°C to 31°C after the phenylacetic acid production is completed, so as to improve the production of ethyl phenylacetate.

The method to produce ethyl phenylacetate via biotransformation is as follows: add 0.1g/L $FeCl_3$ in the biotransformation culture media. Keep the initial pH value as 6.0; maintain the temperature at 28℃; and retain the rate at 220rpm. After 48 hours of cultivation, add 0.5g/L ethanol and adjust the pH value to 5. Improve the biotransformation temperature until it reaches 31℃. Continue the cultivation for 24 hours. Under the biotransformation condition, compare the generation situations of ethyl phenylacetate in HA bacterial strain and HA14 bacterial strain. See Table 1 for the related results. The results show that when the cultivation lasts for 48 hours, the concentration of the mutagenic HA14 strain is improved by 63% compared with that of the initial bacterial phenylacetic acid. When the cultivation lasts for 72 hours, the ethyl phenylacetate concentration of the HA14 bacterial strain is improved by 71% compared with that of HA bacterial strain.

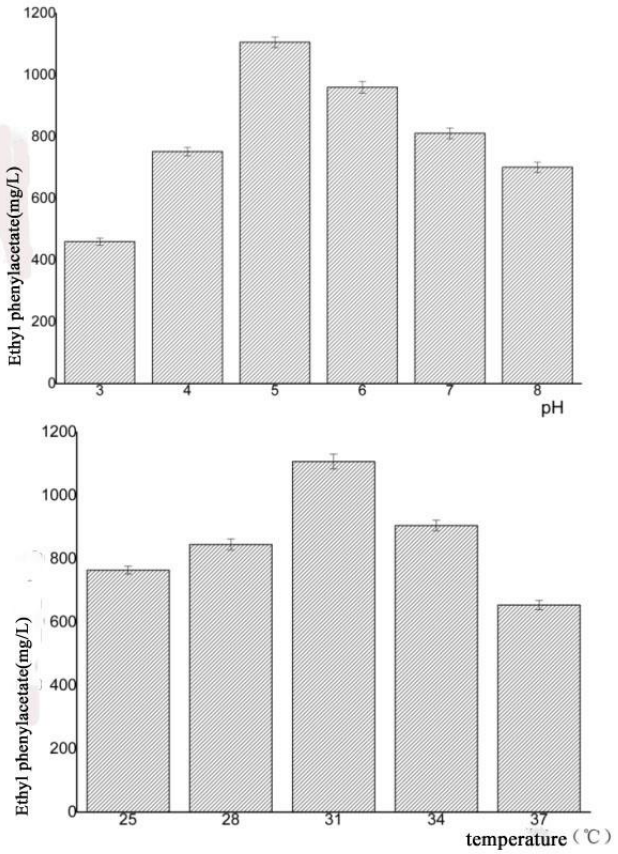

Figure 5. The Influence that pH and Temperature Have on the Esterification of *Hansenula Anomala*

4 CONCLUSIONS

The study of this paper takes natural L-phenylalanine as the precursor. It studies the phenylacetic acid production through the transminase, decarboxylase and aldehyde dehydrogenase functions. By exogenously adding ferment ethanol in the culture media, the production of natural ethyl phenylacetate can be accomplished by the biotransformation under esterifying enzyme function. The whole biotransformation process selects the *Hansenula Anomala* containing the expression of these enzymes as the microorganisms of the biotransformation. By optimizing the generation condition of phenylacetic acid and the esterification condition of esterifying enzyme, the final mutagenic bacterial strain HA14 can generate ethyl phenylacetate with volume of 840mg/L within 72 hours of cultivation. The conversion rate of L-phenylalanine and the use ratio of phenylacetic acid can reach 92% and 95% respectively.

ACKNOWLEDGEMENTS

This work was supported by Science and Technology Commission of Shanghai Municipality (Grant No. 14120503400), and National Natural Science Foundation of China (Grant No.31301475).

REFERENCES

[1] Deng B., Jiang G.B., Chen L.P. & Xu A.W. 2007. Ethyl Phenylacetate Generated through High-pressure Microwave Radiation Catalyzed Synthesis. *Fine and Specialty Chemicals*, pp: 21-23.

[2] Wu W. 2012. Study on the Ethyl Phenylacetate Synthesized by Decarboxylation Coupling and the Reaction Methodology of Its Derivatives. Hefei University of Technology.

[3] You H.Q. 2006. The Ethyl Phenylacetate Synthesized by the Phosphotungstic Heteropoly Acid Catalyzation Supported on Activated Carbon. *Journal of Sanming University*: 174-76.

[4] Hua D, Xu P. 2011. Recent Advances in Biotechnological PProduction of 2-phenylethanol. *Biotechnol Adv*; 29: 654-60.
[5] Wang X.D., Li F.L., Xu S.X., Ban S.D., Wang R.C., Shen X.C., Wu X.Y. & Qiu S.Y. 2014. The Seperation and Identification of A Produced Esterifying Enzyme Bacterial Strain and Its Enzyme Production. Brewing: 42-47.
[6] Tat L, Comuzzo P, Battistutta F. & Zironi R. 2007. Sweet-like Off-flavor in Aglianico del Vulture Wine: Ethyl Phenylacetate as the Mainly Involved Compound. J Agric Food Chem: 55:5205-12.
[7] Wang Y., Fan W.L., Xu Y., Diao Y.Q. & Lu H.Z. 2003. Research of Methods for Determining the Esterifying Enzyme in Strong-flavor Yeast Liquor. Brewing: 18-21.
[8] Li J.Q. 2011. The High Performance Liquid Chromatography Used for Detecting the Phenylacetic Acid Left in the Fermentation Broth of Penicillin. *Heilongjiang Medicine Journal*: 179-80.