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Full Length Research Paper

Research on the esterification property of esterase produced by *Monascus* sp.

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Esterification reaction by *Monascus* was produced with a mixed culture of different strains in which *Monascus* play a major role with higher activity and good specificity. The strain Q-306 was identified in the study as *Monascus* sp. with high esterification power usage and was screened from Daqu and Zhaopei in China. The esterification characteristic research showed the strain's specificity to ethyl caproate. On the basis of the single factor experiment, the optimal condition of producing esterification was as follows: temperature was 35°C, alcohol concentration was 20% (v/v), pH value was 3.5 and substrate concentration was 1%.

Key words: Esterification *Monascus,* esterifying power, catalysis characteristic.

INTRODUCTION

The esterifying enzyme belonged to the extra-cellular enzyme that consist of lipase and esterase (Wu, 2004), while it was also called lipase because of the hydrolysis or synthesis capacity and catalytic decomposition of enzymes in the ester (Du et al, 2005). It played an important role in incense making and paid extensive attention to traditional food production. The main application of esterase in liquor production is to improve the synthesized ethyl caproate, which is the main liquor fragrance ingredient. Moreover, it was used to control the traditional production process and flavoring liquor production (Araceli, 2004). Microorganisms produce esterifying enzyme, including bacteria, filamentous fungi, yeast, etc. The esterifying enzyme obtained from fungi was widely used in liquor production and food flavor strengthening at home and abroad, but very few studies had been done for the bacteria that produced the esterifying enzyme.

In foreign countries, this enzyme was used in the area of butter, cheese flavor enhancer and synthetic caproate, glyceride, etc. As it is low in energy consumption and as the fermentation process, it is easy to control with solid-state fermentation. Most importantly, fungal could grow better in a static environment and their secondary metabolites could accumulate more efficiently. So, the esterifying enzyme, obtained from fungal was widely used due to its high activity and low production costs. In the research, Q-306 was screened as the first strain, which was identified as *Monascus* sp., and its enzyme character and the optimal condition of producing esterification was studied in order for it to be used in 'Luzhou-flavor liquor' production or yellow water esterified liquid.

MATERIALS AND METHODS

The strain used in this work was obtained from Daqu and Zhaopei (country). Microorganisms were maintained on the enriched culture medium containing 10 Bx malt extract and 0.7% lactic acid, after sterilization by adding 10% ethanol. They were isolated and purified on the medium containing: 6-8 Bx sugar solution, 5% soluble starch, 3% peptone, 3% agar and 0.2% glacial acetic acid. Then, the high esterifying power of *Monascus* was screened on the liquid medium containing: 10 Bx sugar solution, 0.2% $NaNO_3$, 0.1% $MgSO_4$ and 0.25% KH_2PO_4 (natural pH).

Isolation and screening methods (Laurent and Daniel, 1996)

Enrichment culture

Erlenmeyer flasks (250 ml), containing 10 g sample, were immersed with 90 ml sterilized saline solution, which include glass breads,

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in order to break up and clarify the sample, followed by an aspiration of the supernatant (1 ml), after which its concentration was adjusted to 10^{-1} using sterilized saline solution, and then the diluents were inoculated into the enriched culture medium. Finally, the medium was incubated at 30°C for 14 days and was followed by another enriched culture medium for 14 additional days. This was repeated, until the screened target stains with red mycelium grew rapidly and strongly.

Isolation and culture

A solution of sterilized saline (9 ml) was inoculated with 1 ml enriched sample, followed by an adjustment of its concentration to 10^{-6} with sterilized saline solution. Then, 0.5 ml of the entire diluents was aspirated off and inoculated into a solid medium, after which it was incubated at 32 to 35°C for 5 to 6 days. As such, 0.2% sodium deoxycholate was added to the solution to control excessive expansion of colonies in the medium and in order to easily pick up colonies.

Purification and culture

In order to further purify the microorganisms, we selected strains which were red, purified, grown rapidly and strongly, and then inoculated them into a test-tube slant medium.

Production of esterification *Monascus* enzyme

The first order seed culture

The seed culture medium consists of wheat bran in five times distilled water. The pH was adjusted to 4.5 by boiling for 20 min, followed by filtering and addition of 2% sucrose. Then, the liquid medium was set in the test-tube and performed for sterilization at 0.1 MPa for 30 min, after which the test-tube slant was prepared. Esterification *monascus* (Q-306) was inoculated into a test-tube slant medium and was then incubated for 4 days at 35°C for formation of monascus spores and pink mycelium, which was the first order seed.

The second order seed culture

The second order seed culture medium consisted of 600 g fresh wheat bran, 100 g corn flour, 300 g waste lees and 700 ml water. Its pH was adjusted from 4.5-5.5, after which the liquid medium was set in 500 ml erlenmeyer flasks containing 150 g. The culture medium was then performed for sterilization at 0.1 MPa for 30 min, before it was finally cooled to 35°C. The aforementioned prepared first order seed, including its mycelium and medium, was inoculated into erlenmeyer flasks and incubated at 35°C. The erlenmeyer flasks were shaken for every 8 h, and the surface of the medium turned red after 4 days, which resulted to the second order seed.

The third order seed culture

The third order seed culture medium consisted of 600 g fresh wheat bran, 100 g corn flour and 300 g waste lees and 800 ml water. The pH was adjusted from 4.5-5.0, followed by boiling at the normal pressure for 60 min and then the liquid medium was set in a koji tray (with a material thickness of 5 cm). Afterwards, it was cooled to 35°C and 5% of the second order seed was inoculated for incubation at 32 to 35°C under 85 to 90% moisture. However, the medium was mixed every 8 h. As a consequence, the surface of the medium turned red after 3 days and that was the third order seed.

Preparation of enzyme solution

The seven strains were inoculated into the liquid medium and then incubated at 32 to 35°C for 7 days. In the study, we used the supernatant as the determination of ester.

Identification of microorganisms

The higher esterifying power strain, named Q-306, was identified with the molecular identification (Lakord et al., 2000).

Analytical methods

Determination of ester enzyme

Aspiration of enzyme (25 ml) was followed by an addition of 1.7 ml water, 0.3 ml caproic acid and 3 ml absolute ethanol to the medium, which was then esterified at 32 to 35°C for 7 days.

Determination of the total ester esterifying power

Saponification method was used to measure the power of the ester (Wang, 2005). The Erlenmeyer flasks (250 ml), containing 10 g sample, were shaken and immersed with 90 ml sterilized saline solution, which included glass breads, in order to break up and clarify the sample, after which the supernatant (1 ml) was aspirated off, and then its concentration was adjusted to 10^{-1} using sterilized saline solution. Consequently, the diluents were inoculated into the enriched culture medium, and were incubated further by another enriched culture medium for 14 additional days. This was repeated, until the screened target stains, with red mycelium grew rapidly and strongly.

Determination of ester yield

Saponification method (Shi, 2005) and gas chromatography (He et al., 2004) were used to characterize the ester yield.

RESULTS AND DISCUSSION

Morphological characterization

After undergoing culture for 24 h, the colonies were white, and then they grew rapidly. Three days later, the colonies grew bigger and closer and their colour changed to purple. The morphological characteristics of the seven strains were all consistent with the characteristics of *Monascus*. The mycelium was separated with horizontal, multi-core and a very complex branching, whereas the conidia of *Monascus* which were chained existed on its branches and on the top of the branches. The closed capsule shell was spherical, with handles, and there were more than 10 spherical perithecium which had ascospores inside (Figure 1).

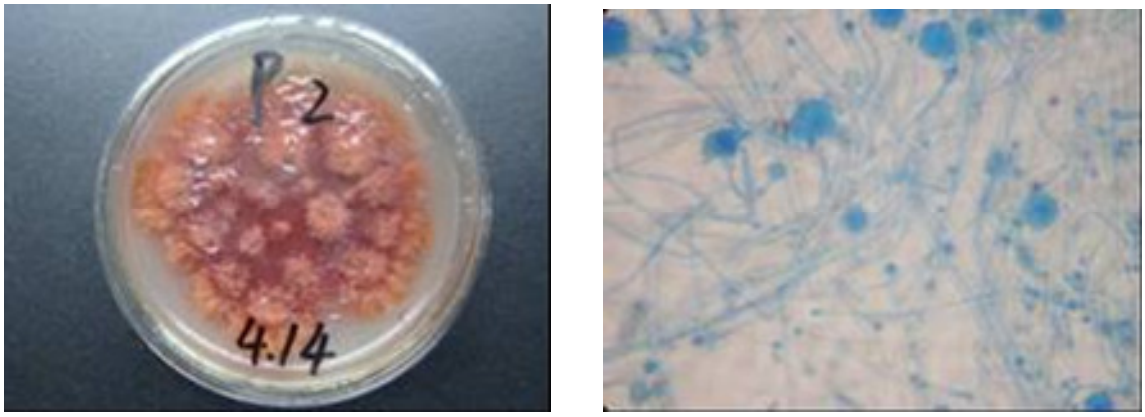

Figure 1. The Q-306 colony and micrograph pictures. A, The Q-306 colony; B, micrograph picture (400).

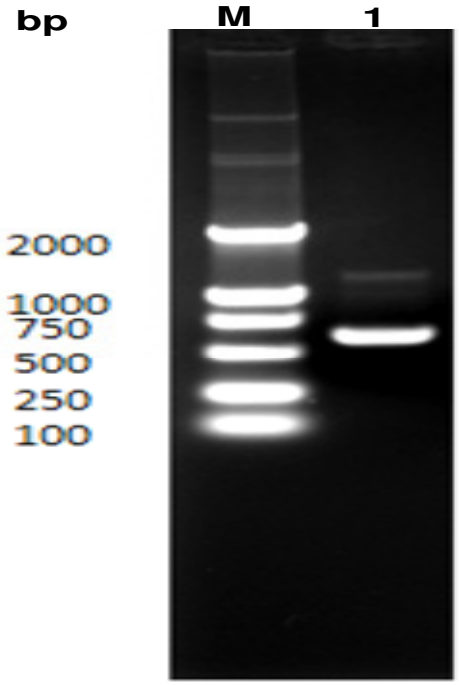

Figure 2. 26S rDNA D1/D2 region of the PCR product electrophoresis. M, Marker DI-2000; 1, 26S rDNA D1/D2 PCR product.

The 26S rDNA D1/D2 sequence analysis of Q-306

The electrophoresis of 26S rDNA D1/D2 region of the PCR product is shown in Figure 2. The sequencing result is as follows:

GGATGCCTCAGTACGGCGAGTGAAGCGGCAAGAGC
TCAAATTTGAAAGCTGGCCCCTCCGGGGTCCGCGTT
GTAATTTGCAGAGGATGCTTCGGGCTCAGCCCCCGT
CTAAGTGCCCTGGAACGGGCCGTCGGAGAGGGTGA
GAATCCCGTCTGGGACGGGGTGCCTGGGTCCATGT
GAAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGC
AGCTCTAAATGGGTGGTAAATTTCATCTAAAGCTAAA
TACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGA
TCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAC
AGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCGAT
CAGACTCGCCTGCGGGGTTCAGCCGGCATTCGTGC
CGGTGTACTTCCCCGTGGGCGGGCCAGCGTCGGTT
CGGGTGGCCGGTCAAAGGCCCCGGGAATGTGTCGC
CCTCCGGGGCGTCTTATAGCCCGGGGTGCCATGCG
GCCTACCTGGACCGAGGAACGCGCTTCGGCTCGGA
CGCTGGCGTAATGGTCGTAAGCGACCCCTCTTGAAC
AACGGACCA

When the sequencing result was submitted to NCBI-Blast online comparison, the 26SrDNA similarity of Q-306 was 97% when compared with *Monascus aurantiacus* strain CICC 5015. Therefore, Q-306 was identified as *Monascus* sp.

Measurement of the esterifying power of monascus

The caproic acid (1 ml) was aspirated off into 100 ml volumetric flask and then 20% ethanol was added, after which the medium was adjusted to 100 ml. This was followed by an addition of this solution (100 ml) into the erlenmeyer flask (250 ml) and an addition of *Daqu*, whose weight equaled 5 g dry *Daqu* [the weight of qu = 5×1/ (1 - the content of percent water) g]. They were esterified at 35°C for 100 h and then 50 ml of water was added, followed by heating and distillation. Finally, 100 ml distillated liquid was obtained to measure the content of ethyl caproate. From Table 1, we can see that the esterifying power of esterification *Monascus* enzyme was

Table 1. Esterifying power of esterification *Monascus* (mg/g).

Sample number	Esterifying power
1	62.4
2	68.5
3	65.8
Average	65.6

Table 2. Catalytic specificity to acid (mg /100 ml).

Parameter	Content
Ethyl caproate	128.4
Ethyl acetate	25.56
Ethyl butyrate	35.78
Ethyl lactate	32.38

Table 3. Selection of the esterification *Monascus* in mixed acid (mg/100 ml).

Sample number	Ethyl caproate	Ethyl acetate	Ethyl butyrate	Ethyl lactate
1	45.9	6.0	13.9	16.0
2	42.0	5.5	10.8	16.2
3	74.2	4.8	10.7	16.8

65.00 mg/g for 100 h.

Catalytic specificity of esterification *Monascus* (Zhou et al., 2006)

Catalytic specificity to acid

Four acid solutions of 100 ml were chosen, including 1% caproic acid, 1% acetic acid, 1% butyric acid and 1% lactic acid, with each solution contents having 20% ethanol and then 2 g esterification *Monascus* was added into the solutions, followed by esterification at 35℃ for 100 h. Finally, measurements for the content of ethyl caproate, ethyl acetate, ethyl butyrate and ethyl lactate were taken.

Table 2 shows that the esterification *Monascus* had high capability to the catalytic and synthesized ethyl caproate when compared with other esters. That is to say that the esterification *Monascus* had very high and specific capability to the catalytic enzymes. So, it could increase the content of esters in the production of Luzhou-flavor liquor, especially the content of ethyl caproate.

Selection of the esterification *Monascus* in the mixed acid

The esterification *Monascus* could accelerate the esterifying speed of acid and alcohol (Paiva et al., 2003), so that it could reduce the fermentation cycle of wine and increase the content of esters in the production of wine. Moreover, it could be used in the production of enhanced *Daqu*, fermented mash of grain, pressure trans-cellar, high-ester wine and yellow liquid water esterification. As there exist a large number of different scales of mixed acid in the substrate's fermented mash of grain and in the esterifying liquid, we thus designed different scales of mixed acid in this study. First, we designed Sample 1 with four equal volumes; caproic acid: lactic acid: butyric acid: acetic acid = 0.25 ml: 0.25 ml: 0.25 ml: 0.25 ml. The content of lactic acid is higher in the fermentation of wine and yellow water-esterification. In sample 2, the content of lactic acid was also higher than that of others in which the volume of scales was caproic acid: lactic acid: butyric acid: acetic acid = 0.25 ml: 0.4 ml: 0.25 ml: 0.25 ml. We could add a small amount of ethyl in the actual production of esterified liquid to increase the biological synthesis of ethyl caproate. So, we designed sample 3 with higher caproic and lactic acid and the volume of scales was caproic acid: lactic acid: butyric acid: acetic acid = 0.4 ml: 0.4 ml: 0.25 ml: 0.25 ml. At last, 20% ethanol was added to these samples and each was adjusted to 100 ml with 100 ml volumetric flask after which, 2 g esterification *Monascus* was added to the solutions followed by esterification at 35℃ for 100 h. Finally, measurement was taken for the content of esters with gas chromatography.

From Table 3, we could conclude that the esterification *Monascus* with the best catalytic selection to the lactic acid was in the mixed acid. The results of the first sample showed that the production of ethyl caproate was higher than the other two esters in the equal volume mixed acid. From the second sample, we observed that the production of ethyl lactate did not increase, but it increased the content of the butyric and mixed acid. Also, the content of the ethyl caproate did not change. From the third sample, we observed that the production of ethyl lactate did not increase, but the content of the butyric acid and the ethyl caproate of the mixed acid increased, and the content of the ethyl caproate increased as well.

Above all, we could say that the esterification *Monascus* had quite a good catalytic selection of the lactic acid in the mixed acid production of Luzhou-flavor liquor and yellow water esterified liquid, as it could increase the content of the major scent ingredient (ethyl caproate) in the mixed acid fermentation substrate during production. As a result, the esterification *Monascus* had a considerable value.

The best esterifying condition of esterification *Monascus*

Effect of temperature

Six samples of 100 ml were prepared with 2 g

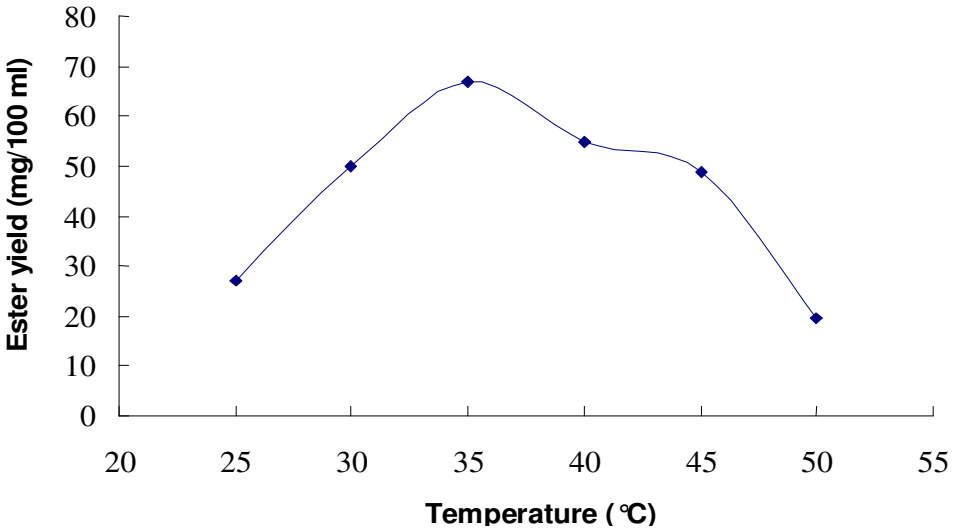

Figure 3. Effect of temperature on ester yields.

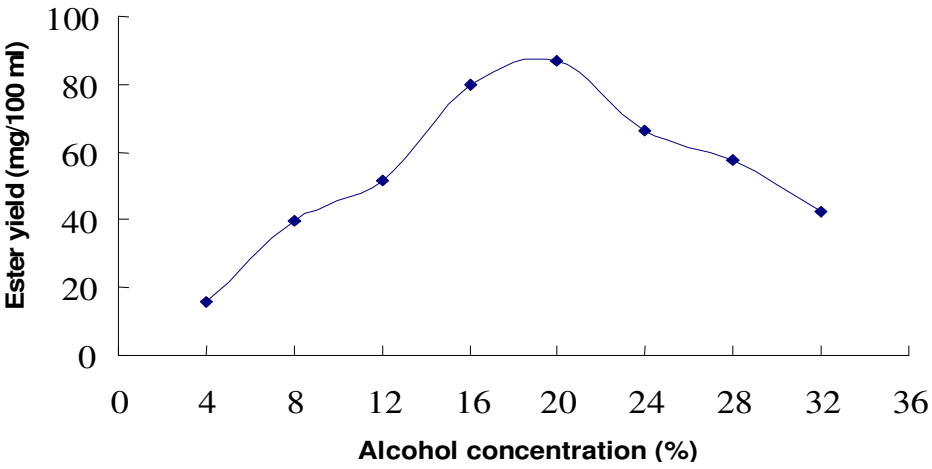

Figure 4. Effect of alcohol concentration on ester yields.

esterification *Monascus*, 0.5% caproic acid and 16% ethanol, followed by an adjustment of the pH to 3.5 after which each of the six temperature levels were esterified without oxygen (25, 30, 35, 40, 45 and 50℃) for 120 h.

As seen from Figure 3, the highest ester yield reached 66.82 mg/g at 100 h, and at 35℃. The esterifying capability of esterification *Monascus* increased, while the temperature increased between 25 and 35℃. However, when the temperature increased above 35℃, the esterifying capability of esterification *Monascus* reduced, while the temperature increased.

Effect of alcohol

Eight samples of 100 ml were prepared with 2 g esterification *Monascus*, 0.5% caproic acid and seven ethanol levels. Each (4, 8, 12, 16, 20, 24, 28 and 32%) of the levels were adjusted to a pH value of 3.5, and then esterified without oxygen at 35℃ for 120 h.

The results in Figure 4, showed that the highest ester yield reached 86.89 mg/g at 100 h, while the catalytic alcohol concentration was 20% (v/v). In addition, the esterifying capability of the esterification *Monascus* increased, while the alcohol concentration increased between 4 and 20%, but when the alcohol concentration increased above 20% (v/v), the esterifying capability of the esterification *Monascus* reduced, while the alcohol concentration increased.

Effect of pH

Seven samples of 100 ml were prepared with 2 g

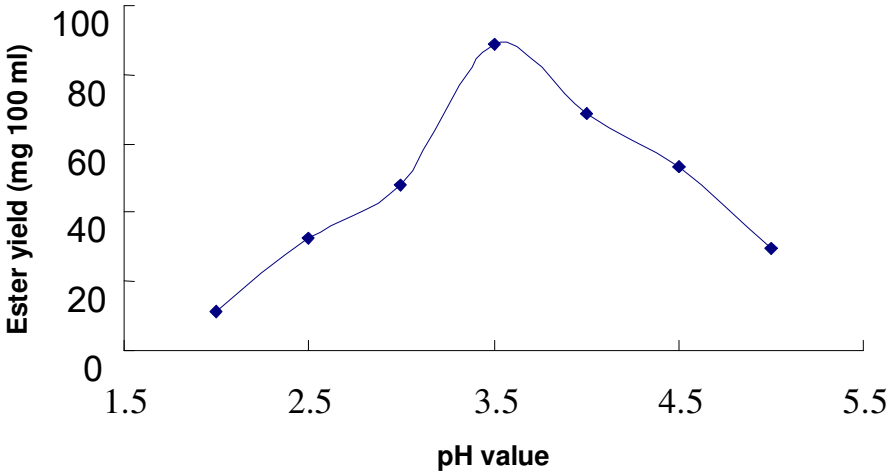

Figure 5. Effect of pH value on ester yields.

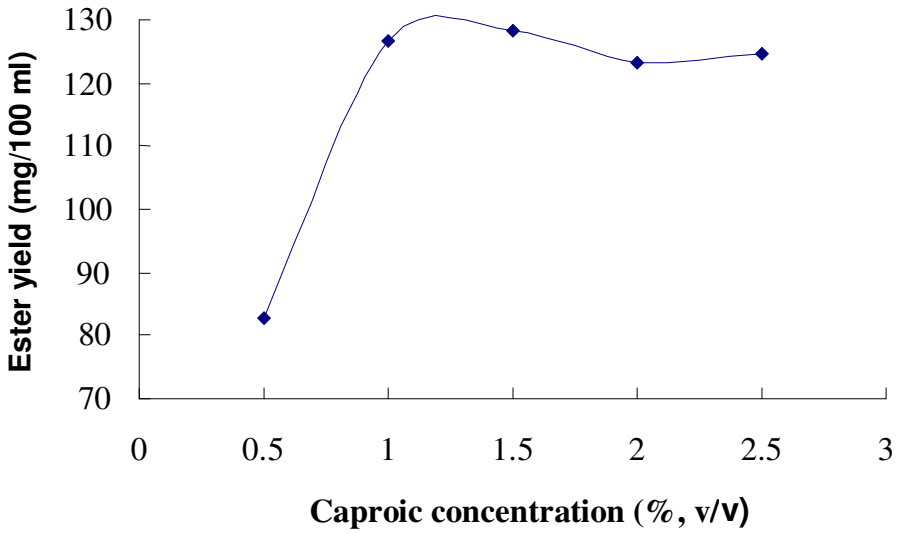

Figure 6. Effect of the substrate concentration on ester yields.

esterification *Monascus*, 0.5% caproic acid and 20% ethanol, and were then esterified without oxygen at 35℃ for 120 h at seven pH value levels (2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0). The results in Figure 5, showed that the highest ester yield reached 88.91 mg/g at 100 h, while the catalytic pH value was 3.5. Moreover, the esterifying capability of esterification *Monascus* increased, while the pH value increased between 2.0 and 3.5; but when the pH value increased above 3.5, the esterifying capability of esterification *Monascus* reduced, while the pH increased.

Influence of the substrate concentration

Six samples (100 ml) were prepared with 2g esterification *Monascus*, 20% ethanol and five substrate concentration levels (0.5, 1, 1.5, 2.0 and 2.5% (v/v)) followed by an adjustment of the pH value to 3.5, after which the samples were esterified without oxygen at 35℃ for 120 h.

The results, in Figure 6, showed that the highest ester yield reached 128.23 mg/g at100 h, while the substrate concentration was 1%. Moreover, the esterifying capability of esterification *Monascus* increased, while the concentration of caproic acid increased between 0.5 and 1%, but when the substrate concentration increased above 1%, the esterifying capability of esterification *Monascus* did not change; as the substrate concentration increased. That is to say, the esterifying capability of esterification *Monascus* would not change, when the substrate concentration only reach a certain concentration and stop there.

Conclusions

The preliminary experiments of this study showed that the esterifying power of the crude was from Q-306, which was identified as a *Monascus* sp. with quite a good catalytic selection of ethyl caproate. Above all, we could conclude that the optimal condition of production was: 35°C, 20% (v/v) alcohol concentration, 3.5 pH value and 1% substrate concentration. However, high esterification *Monascus* enzyme was produced which can be widely used in "Luzhou-flavor liquor" production or yellow water esterified liquid.

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