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Title	液体培養による紅麹菌のカルボキシペプチダーゼの生産
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Production of Carboxypeptidase by *Monascus purpureus* in Submerged Culture

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Abstract: In this study, the screening test on twenty eight strains of *Monascus* for the production of carboxypeptidasse (CPase) in submerged culture was performed, *Monascus purpureus* IFO 4478 was indicated to be the best producer of CPase. The culture medium compositions and cultivation conditions for the production of CPase by this strain during submerged culture in shake flasks were optimized. Subsequently the process was scaled up to 5 L jar fermentor. The results of carbon source test showed that glucose was the best inducer of CPase among the eleven kinds of carbon sources tested with the addition of 1% (w/v). For the nitrogen source test, soybean protein isolate (SPI) was the best inducer of CPase among the seventeen kinds of nitrogen sources tested with the addition of 2% (w/v). Moreover, the mineral salts solutions with the addition of each 0.1% (v/v) could induce the production of CPase, and when the initial pH of the culture medium was 6.0, the maximal CPase activity was obtained. In addition, the effects of shaking speed, incubation temperature and incubation time on CPase production were also investigated. The results indicated that when *M. purpureus* IFO 4478 was cultivated in a reciprocal shake incubator with a shaking speed 150 rpm and at 28℃, the maximal CPase activity was obtained after 6 days. Therefore, the optimal culture medium for the CPase production was: glucose 1.0% (w/v), SPI 2.0% (w/v), $MgSO_4 \cdot 7H_2O$ 0. 5% (w/v), KH_2PO_4 0.5 % (w/v), mineral salt solutions 0.1% (v/v) (pH 6.0), and the optimal cultivation condition was: at 150 rpm and 28℃ for 6 days.

Key words: ***Monascus purpureus,*** **carboxypeptidase, submerged culture,** ***tofuyo***

Introduction

The fungi *Monascus* species have been traditionally used in the microbial fermentation industry in East Asia (China, Japan, Indonesia et al.). In China, they are used to produce not only natural colorant but also red wine and red fermented soybean curd. In Okinawa, Japan, they are used to produce *tofuyo,* which is a vegetable protein food made from soybean curd by the action of microorganisms. *Tofuyo*-making by *Monascus* has been studied in our laboratory.[15, 17] It was found that a large number of free amino acids (e.g. glutamic acid and aspartic acid), which was considered to be important flavor and taste compounds of *tofuyo,* were produced during the maturation of *tofuyo.* This was considered to be the result of proteolytic enzymes produced by *Monascus* hydrolyzed soybean protein. However, knowledge of this process is very limited.

In order to elucidate the functions of *Monascus* enzymes in the maturation of *tofuyo,* they must be purified and characterized. In a previous study, the production, purification, and properties of an acid proteinase from *Monascus* were reported.[16] The acid proteinase is an endopeptidase that hydrolyzes proteins from their internal chain to peptides, but only to small amounts of free amino acids.[18] Thus, the action of acid proteinase is not enough to explain the occurrences of a large number of free amino acids during the maturation of *tofuyo.* By contrast, CPase is an exopeptidase that releases free amino acid residues from carboxyl termini of peptides or proteins. It is considered to serve as a key enzyme in the production of flavorful amino acids during the maturation of *tofuyo.* CPase is known to be a potential industrial applicable enzyme in food industry. Although a large number of CPase have been isolated from various species of fungi such as *Aspergillus saitoi*[4], *Aspergillus oryzae*[9, 13], *Aspergillus niger*[5, 7], *Aspergillus fumigatus*[11], *Absidia zychae*[8], *Mucor racemsus*[1], *Metarhizium anisopliae*[12], *Penicillum janthinellum*[2, 19], *Paecilonyces carneus*[14], *Pycnoporus sanguineus*[6] and yeast[3], *Monascus* CPase has not yet been

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reported.

To characterize the CPase from *Monascus,* it must be largely produced and purified. Any enzyme can be manufactured by basically two methods: submerged culture process and solid-state fermentation. Submerged fermentation is the method most familiar as it is traditionally used for the production of microbially derived enzymes. The aim of this work was primarily to screen 28 strains of *Monascus* for CPase production and strain growth in submerged culture, and subsequently to optimize the culture medium compositions and cultivation conditions of selected *Monascus* strain for the production of CPase.

Materials and Methods

1. Materials and chemicals.

Soybean protein isolate (SPI) was presented from Fuji Oil Co. Ltd., Japan. D (+)-glucose was from Kanto Chemical Co. Inc., Tokyo, Japan, Peptone was purchased form Kyokuto Pharmaceutical Industrial Co. Ltd., and a Micro BCA™ protein assay reagent kit was from Pierce, Rockford, IL USA. All chemicals used in this study were of analytical grade.

2. Microoganisms.

Twenty eight strains of *Monascus* were used for the screening test. Of them, fourteen strains were purchased from Institute for Fermentation, Osaka, Japan, and the other fourteen's were isolated from red koji made in China, Hong Kong and Taiwan. The strains were maintained on potato-glucose-agar (PGA) slants. The slants were inoculated with the strains and incubated at 30℃ for 7 days, then stored at 4℃.

3. Culture Media.

Basal liquid culture medium containing (w/v): glucose 1.0%; peptone 2.0%; $MgSO_4 \cdot 7H_2O$ 0.5%; KH_2PO_4 0.5% (pH 7.0). The basal culture medium was prepared in distilled water.

Mineral salts solutions were prepared in distilled water. A: $CaCl_2$ 15 mg/mL; B: $FeSO_4 \cdot H_2O$ 15 mg/mL; C: $CuSO_4 \cdot 5H_2O$ 7.8 μg/mL, H_3BO_3 10 μg/mL, $MnSO_4 \cdot 5H_2O$ 10 μg/mL, $ZnSO_4$ 70 μg/mL, MoO_3 10 μg/mL.

4. Inoculum.

Each time an experiment was conducted, a platinum loopful of strains maintained on PGA slants were transferred to fresh pre-autoclaved PGA slants and propagated at 30℃ for 7 days then a platinum loopful of actively growing fungal mycelia were transferred into 30 ml culture tubes containing 5 ml of previously sterilized basal culture medium and incubated in a reciprocal shake incubator at 180 rpm, 30℃ for 4 days. The cultures were used as inoculums.

5. Screening test.

A preliminary screening test was carried out with twenty eight strains of *Monascus* in the culture tubes. The cultivation of the strains was performed as that for the preparation of inoculum described above. Each culture broth was centrifuged at 10,000 *g* and 4℃ for 30 min, and the supernatants were dialyzed against 10 mM McIlvaine buffer (pH 6.0) at 4℃ overnight and then used for the detection of CPase activity. The culture tubes were run parallel in at least duplicates with the results reported as the means. The strains that exhibited higher CPase productive ability were selected for the secondary screening test.

The secondary screening test was performed with the selected six strains in shake flask. The whole volume inoculum in a culture tube was transferred into each 500 ml shake flask containing 100 mL of pre-autoclaved basal culture medium and incubated in a reciprocal shake incubator at 180 rpm and 30℃ for 7 days. Each culture broth was filtered through No. 2 filter paper (Advantec, Japan) to separate mycelium, which was washed twice with distilled water and then used for the assay of strain growth. The culture filtrates were centrifuged and dialyzed as described above, the dialysis used for CPase and protein assays. The flasks were run parallel in at least duplicates with the results reported as the means. The strain that exhibited the highest productive abilities of CPase and strain growth was selected for the purpose strain of CPase production.

6. Optimization of the culture medium composition.

A conventional method of medium optimization (variation of one factor at a time) was used to optimize the culture medium composition for the CPase production by the strain selected above and its growth in submerged culture. In the all experiments the final concentrations of carbon and nitrogen sources were adjusted to 1% and 2%, respectively, if not mentioned specially. The whole volume inoculum in a culture tube was transferred into each 500 ml shake flask containing 100 mL of pre-autoclaved experimental culture medium and incubated in a reciprocal shake incubator at 180 rpm and 30℃ for 7 days. The cultures were used for the assays of CPase activity and strain growth as described above. The basal culture medium was usually used as the control in all experiments. All experiments were conducted in duplicate shake flasks with the results reported as the means.

The effects of different carbon sources on the CPase production by the strain and its growth in shake flasks

were assayed by substituting glucose in the basal culture medium with various carbon sources. The carbon source that could best induce the CPase production and the strain growth was selected for the next experiment.

The effects of different nitrogen sources on the CPase production by the strain and its growth in shake flasks were assayed by substituting glucose in the basal culture medium with the carbon source optimized above and peptone with various nitrogen sources. The nitrogen source that could best induce the CPase production and the strain growth was selected for the next experiment.

The effect of carbon source concentration on the CPase production by the strain and its growth in shake flasks were assayed by substituting glucose and peptone in the basal culture medium with the carbon and nitrogen sources optimized above, respectively, and varying the carbon source concentration from 0.5 to 10% (w/v). The carbon source concentration, at which the CPase production and the strain growth could be induced best, was used for the next experiment.

The effect of nitrogen source concentration on the CPase production by the strain and its growth in shake flasks were assayed by substituting glucose and peptone in the basal culture medium with the carbon and nitrogen sources optimized above, respectively, adjusting the carbon concentration to that optimized above and varying the nitrogen concentration from 0.5 to 3.0% (w/v). The nitrogen source concentration, at which the CPase production and the strain growth could be best induced, was used for the next experiment.

The effects of mineral salts on the CPase production by the strain and its growth in shake flasks were assayed by substituting glucose and peptone in the basal culture medium with the carbon and nitrogen sources optimized above, and adjusting their concentrations to those optimized above, respectively, with or without the addition of mineral salts solution A, B and C at each 0.1% (v/v). According to the results whether the addition of mineral solutions could induce the CPase production or not, it was decided whether the mineral solutions were added into the culture medium or not for the next experiment.

The effects of the initial pH of the culture medium on the CPase production by the strain in shake flasks were assayed by substituting basal culture medium with that optimized above, varying the initial pH of experimental culture medium from 2.0 to 9.0. The initial pH of the culture medium, at which the maximal CPase activity was obtained, was adjusted for the next experiment.

7. Optimization of the cultivation condition.

The optimization of cultivation conditions for CPase production by the strain selected above in submerged culture was performed by varying one factor at a time. All experiments were conducted in at least duplicate shake flasks with the results reported as the means.

The effects of shake speed in the cultivation on the CPase production in shake flasks were assayed on the optimized culture medium above, varying the shake speed from 0 to 180 rpm. The shake speed, at which the maximal CPase activity was obtained, was used for the next experiment.

The effects of cultivation temperatures on the CPase production by the strain in shake flasks were assayed on the optimized culture medium at the shake speed optimized above, varying the cultivation temperature from 25 to 32 ℃. The temperature, at which the maximal CPase activity was obtained, was used for the next experiment.

The time course of CPase production by the strain in shake flasks was studied on the optimized culture medium at the shake speed and temperature optimized above for 12 days. Every duplicate flask was removed periodically (every day) from the shake incubator for the CPase assay. The cultivation time, during which the maximal CPase activity was obtained, was decided to be the optimum for the CPase production by the strain.

8. Scaling up experiment on the CPase production.

The scaling up process of CPase was performed in a stirred jar fermentor (MD-50, B. E. Marubishi, Co. Ltd. Tokyo, Japan) with an equipped with a 5 L vessel. 3 L of the optimal culture medium for the CPase production: glucose 1.0% (w/v), SPI 2.0% (w/v), $MgSO_4 \cdot 7H_2O$ 0.5% (w/v), KH_2PO_4 0.5% (w/v), mineral salts solution A, B and C each 0.1% (v/v) (pH 6.0), with the addition of 0.1% (v/v) antifoam agent Adecanol LG-126 (Asahidenka, Tokyo, Japan) were employed.

The whole volume inoculum in a culture tube was transferred into each 500 ml shake flask containing 100 mL of pre-autoclaved optimized culture medium and incubated in a reciprocal shake incubator at 150 rpm and 28℃ for 4 days. The whole volume inoculums in two shake flasks were transferred into the jar fermentor above.

The fermentation in the jar fermentor was performed at 28℃ with agitation 300 rpm and aeration 1.8 L/min for 12 days. The sampling was done periodically (24 h) with 30 ml of sample removed out from the jar fermentor. The samples were used for the assays of CPase and strain growth as described above. At least duplicate experiments were run independently with the results reported as the means.

9. CPase assay.

CPase activity was determined on the basis of the

Table 1. Various strains of *Monascus* used for the screening test.

Strains of *Monascus*	
Monascus purpureus IFO4478*	*Monascus pilosus* IFO 4525
Monascus purpureus IFO4485*	*Monascus* sp. 4820
Monascus purpureus IFO 4489	*Monascus* sp. C-1-1
Monascus purpureus IFO 6540	*Monascus* sp. C-3
Monascus purpureus NO. 3403*	*Monascus* sp. C-4*
Monascus ruber IFO 4483	*Monascus* sp. C-11
Monascus ruber IFO 4492*	*Monascus* sp. G-1
Monascus ruber IFO 4532	*Monascus* sp. H-1-1
Monascus ruber IFO 9203	*Monascus* sp. H-1-3
Monascus ruber IFO 31842	*Monascus* sp. H-2
Monascus ruber IFO 32317	*Monascus* sp. HK
Monascus ruber IFO 32318	*Monascus* sp. T1
Monascus pilosus IFO 4480*	*Monascus* sp. TSG
Monascus pilosus IFO 4520	*Monascus* sp. RC

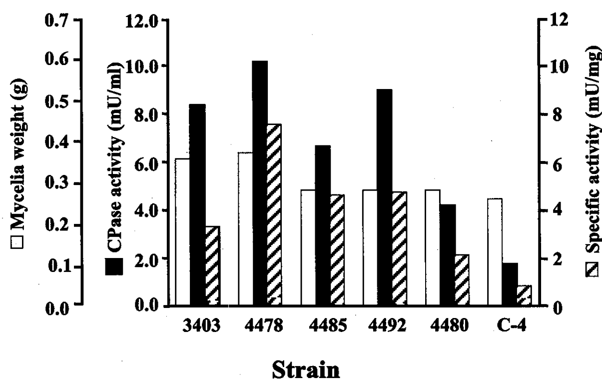

Fig. 1. Growth and carboxypeptidase production of genus *Monascus strains.*
3403, *Monascus purpureus* No. 3403;
4478, *Monascus purpureus* IFO 4478
4485, *Monascus purpureus* IFO4485;
4492, *Monascus purpureus* IFO4492
4480, *Monascus pilosus* IFO4480;
C-4, *Monascus* sp. C-4.

method of Nakadai[10]. Unless stated otherwise, enzymatic activity was assayed by adding 40 μL of enzyme sample solution to 160 μL of 5.0×10^{-4} M Z-Glu-Tyr in 0.05 M acetate buffer (pH 3.5) pre-incubated for 5min in the water bath of 37℃. The reaction mixture was incubated in a water bath of 37℃ for 30min, the reaction was stopped by adding 100 μL of ninhydrin solution, followed by heated at 100℃ for 15 min followed by cooled in ice water. Then the reaction mixture was diluted with 1 ml of mixture of 0.1 M Na_2HPO_4 and acetone (5:4) and then the optical density (O.D.) at 570 nm was measured against deionized water. One unit of enzyme activity is defined as the enzyme quantity which librated 1 μM of tyrosine from the substrate (Z-Glu-Tyr) per min under this condition. The enzyme sample inactivated at 100℃ for 10 min was served as the blank. Absorbance was compared with a tyrosine standard curve in order to calculate enzyme activity. Stock solution of Z-Glu-Tyr was prepared in 0.05 M acetate buffer (pH 3.5),

Table 2. Effect of various carbon sources on the growth of strain and the production of carboxypeptidase.

Carbon source	Mycelium weight (g/100 mL)	Total activity (mU/mL)	Specific activity (mU/mg)
Glucose	0.50	32.2	31.8
Fructose	0.72	31.1	9.9
Sucrose	0.57	20.3	6.2
Maltose	0.83	23.8	8.7
Lactose	0.49	16.9	4.9
Trehalose	0.67	24.5	9.7
Isomaltooligosaccharide	0.73	29.8	10.5
Maltotetraose	0.67	15.9	6.3
Coupling sugar	0.76	29.4	14.0
Starch	0.53	14.7	13.5
Pullulan	0.72	10.6	3.1

and when necessary dilution of enzyme sample was done in 10 mM McIlvaine buffer (pH 6.0). All pH adjustments were performed at room temperature.

10. Protein assay.

Protein concentration was determined by a Micro BCA™ protein assay reagent kit with bovine serum albumin as the standard.

11. Growth assay.

The strain growth was monitored by measuring the dry weight of mycelium. The dry weight of the mycelium of *M. purpureus* IFO 4478 was measured by the gravimetric method after dried at 105℃ for 3 h to constant weight.

Results

1. Screening test.

The result of the preliminary screening test with twenty eight strains of *Monascus* for the CPase production in submerged culture revealed that the best six producers of CPase were indicated with asterisks in Table 1.

The six strains of *Monascus* were used for the secondary screening test for the CPase production and the strain growth in submerged culture. Of them, *M. purpureus* IFO 4478 was the best producer of CPase (10.8 mU/mL) and gave the maximal growth (0.63 g/100mL) after 7 days as shown in Fig. 1. Thus *M. purpureus* IFO 4478 was selected for the purpose strain of CPase production.

2. Optimization of the culture medium composition.

1) Effect of carbon sources on CPase production.

Various carbon sources included Monosaccharides, disaccharides, oligosaccharides and polysaccharides were studied in an attempt to increase the CPase activity. The results showed that *M. purpureus* IFO 4478 could grow in

Table 3. Effect of various nitrogen sources on the production of carboxypeptidase.

Nitrogen source	Mycelium weight (g/100 mL)	Total activity (mU/mL)	Specific activity (mU/mg)
Peptone	0.50	32.2	31.8
Meat extract	0.38	19.0	8.0
Yeast extract	0.45	10.1	13.3
Urea	0.28	0	0
Casein	0.58	25.2	13.4
Polypeptone	0.55	32.6	13.1
Tryptone	0.71	53.3	17.7
Soybean protein isolate	1.62	64.2	21.1
Ammonium sulfate	0.30	1.5	6.2
Ammonium phosphate monobasic	0.35	0.7	3.6
Ammonium carbonate	0.35	0	0
Ammonium chloride	0.29	0	0
Ammonium nitrate	0.28	0	0
Sodium nitrate	0.29	0	0
Potassium nitrate	0.31	0	0

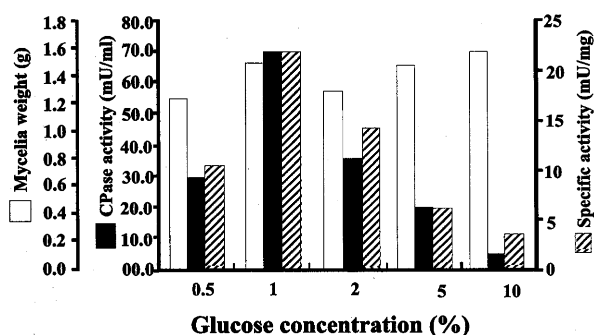

Fig. 2. Effect of glucose concentration on the production of carboxypeptidase.

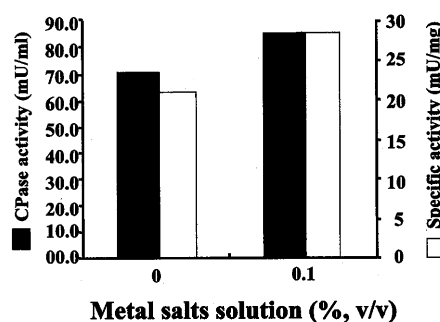

Fig. 4. Effect of mineral salts on the production of carboxypeptidase.

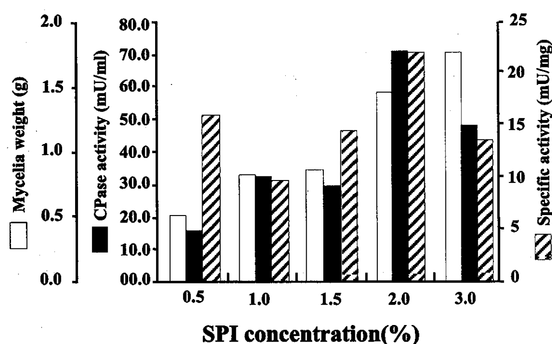

Fig. 3. Effect of SPI concentration on the production of carboxyppeptidase.

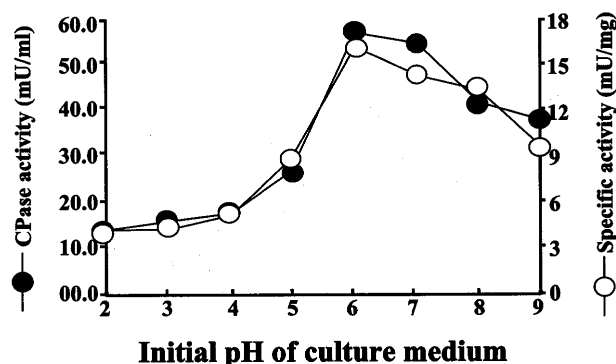

Fig. 5. Effect of initial pH of the culture medium on the production of carboxypeptidase.

all carbon sources and produce CPase well, but glucose gave the maximal CPase activity (32.2 mU/mL) followed by fructose as summarized in Table 2. Thus glucose was selected for the next experiments.

2) Effect of nitrogen sources on CPase production.

Various nitrogen sources included organic and inorganic nitrogen were studied to determine their effect on CPase production. Although all organic nitrogen sources tested except urea resulted in good strain growth and CPase production than inorganic ones, of all nitrogen sources tested, SPI resulted in the maximal growth (1.62 g/100 mL) and the highest CPase activity (64.2 mU/mL). Tryptone proved to be the second best nitrogen source for the growth of the strain and the production of CPase as summarized in Table 3.

3) Effect of carbon source concentration on CPase production.

The effect of carbon source concentration on CPase production was studied. The results indicated the maximal CPase activity was obtained when glucose concentration was 1% as shown in Fig. 2. With an increase in glucose concentration there was a decrease in CPase activity attended indicating an inhibitory effect on the CPase production.

4) Effect of nitrogen source concentration on CPase production.

The effect of nitrogen source concentration on CPase production was studied. The results revealed that the maximal CPase activity was obtained when SPI concentration

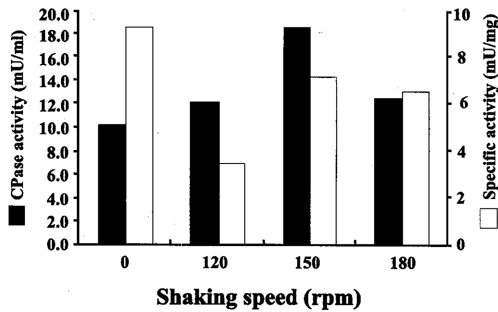

Fig. 6. Effect of shaking speed on the production of carboxypeptidase.

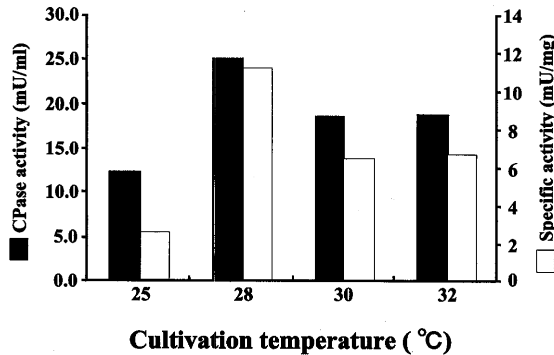

Fig. 7. Effect of cultivation temperature on the production of carboxypeptidase.

was 2% as shown in Fig. 3.

5) Effect of mineral salts on CPase production.

As already described, 1% glucose and 2% SPI resulted in the maximal CPase activity. For subsequent studies, this combination of glucose and SPI were used for testing the effect of metal salts on CPase production. The metal salts solution A, B and C was incorporated in the medium at a concentration of 0.1% each. The results revealed that the addition of metal salts resulted in the increasing of CPase activity as shown in Fig. 4.

6) Effect of initial pH of the culture medium on CPase production.

Effect of initial pH of the culture medium optimized above on CPase production was also investigated (Fig. 5). When the pH of the culture medium was 6.0, the maximal CPase activity was obtained.

3. Optimization of the cultivation condition.

1) Effect of shaking speed of the cultivation on CPase production

Effect of shaking speed of the cultivation on CPase production was determined (Fig. 6). When the cultivation was performed at 150 rpm, the maximal CPase activity was obtained.

2) Effect of temperature of the cultivation on CPase production

Effect of temperature of the cultivation on CPase production was studied (Fig. 7). When the cultivation was performed at 28℃, the maximal CPase activity was obtained.

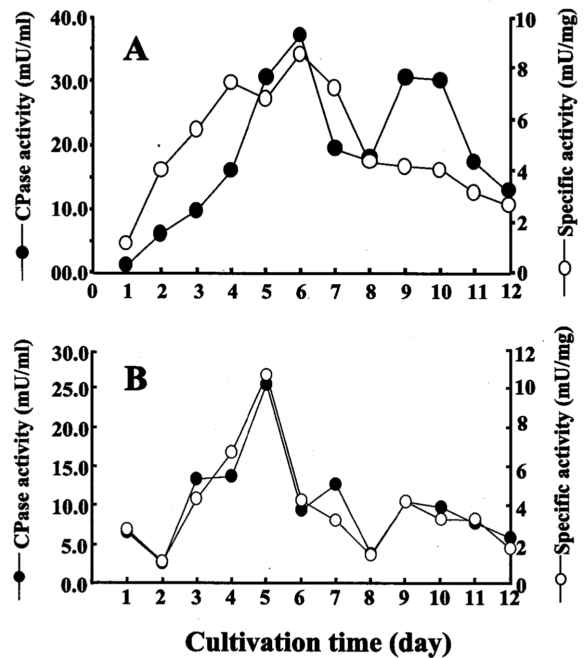

Fig. 8. The time course of carboxypeptidase production by *Monascus purpureus* IFO 4478.
A, in shake flask cultivation; B, in jar fermertor fermentation.

3) Time course of CPase production

Time courses of CPase production in shake flask cultivation (A) and in jar fermentor fermentation (B) were determined (Fig. 8). The results revealed that two CPase activity peaks were obtained after 6 and 9 days, respectively, in shake flask cultivation, while two CPase activity peaks were obtained after 5 and 9 days, respectively, in jar fermentor fermentation, and the values of the CPase activity peaks obtained in shake flask cultivation were slightly higher than those in jar fermentor fermentation.

Discussion

Monascus has been traditionally used as a source producing natural red colorent, however, the reports on enzymes from this microorganism have not been sufficiently described. The work involved screening *Monascus* for the production of CPase in submerged culture and studying several growth conditions that could increase the enzyme titers. With the aim of increasing the enzyme activity titer numerous nutritional conditions were studied first. Various carbon sources and nitrogen sources were examined individually, subsequently the combinations of the best components were used and the conditions of cultivation were investigated.

Screening test was carried out to find strains of *Monascus* that could produce a high activity of CPase. The

highest activity was found in the culture broth of *Monascus purpureus* IFO 4478. Further optimization experiments on the composition of culture medium, the best combination of the culture medium components was: glucose 1.0% (w/v), SPI 2.0% (w/v), $MgSO_4 \cdot 7H_2O$ 0.5%, KH_2PO_4 0.5%, Mineral salts solution A, B and C each 0.1% (v/v) (pH 6.0). *Mucor racemosus*[1] and *Penicillium janthinellum*[2] could also produce CPase on the medium containing glucose (pH 3.5~6.0), while the production of CPase by *Aspergillus saitoi*[5] and *Penicillium janthinellum*[19] were reported to be induced by defatted soybean. For the optimization experiments on the cultivation condition, the best combinations of the cultivation conditions was at 150 rpm and 28℃ for 6 days in shake flask, while at 300 rpm agitation, 1.8 L/min and 28℃ for 5 days in jar fermentor. Some CPases[1, 2, 5, 9, 19] were reported to be well produced at 125-300 rpm and 25-30℃ for 3-7 days.

The two CPase activity peaks were found in both shake flask cultivation and jar fermentor fermentation, this may be interpreted as either (1) the production of more than one enzyme, or (2) the existence of two different isoforms of a single enzyme produced by *M. purpureus* IFO 4478. The first peak appeared faster in jar fermentor fermentation than that in shake flask cultivation, this may be explained as that the strain could get more sufficient oxygen in jar fermentor than in shake flask. The CPase activity produced in the jar fermetor fermentation was slightly lower than that exhibited in shake flask cultivation, this may be because that antifoam agent in jar fermentor fermentation medium slightly inhibit the production of CPase. Therefore, the industrial applicability of *Monascus purpuveus* IFO 4478 will need the optimization study on the fermentation medium and the technical conditions of fermenting aimed at increasing enzyme production at the fermentation system level.

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液体培養による紅麹菌のカルボキシペプチダーゼの生産

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要 約

カルボキシペプチダーゼは，タンパク質やペプチドのカルボキシル末端からアミノ酸を順次遊離する酵素である．紅麹菌（*Monascus* 属カビ）は，中国では紅腐乳や紅酒，沖縄ではとうふようの製造に古くから使用されてきた食品微生物である．我々は，これまでにとうふようの食品科学的特性を明らかにしており，本発酵食品のアミノ酸系呈味成分の生成にカルボキシペプチダーゼが重要な役割を演じていると考えられる．本研究では，とうふよう製造および呈味形成に重要であると考えられる紅麹菌の生産するカルボキシペプチダーゼに着目し，本酵素活性の高い優良菌株のスクリーニング，本酵素生産の培地の組成と培養条件の確立を目的とした．

まず，紅麹菌28菌株を用いて，カルボキシペプチダーゼ高生産菌株の１次スクリーニングを行い，本酵素活性が高い６菌株を選択した．次に，２次スクリーニングを行い，本酵素活性及び菌体生育の最も良好な *Monascus purpureus* IFO 4478をカルボキシペプチダーゼ高産生優良菌株として選択した．続いて，本菌株を用いてカルボキシペプチダーゼの最適生産条件を調べた．その結果，本酵素生産の最適培養組成と培養条件は次のように設定した．(1)培地組成：グルコース１％（w/v），分離大豆タンパク質 ２％（w/v），$MgSO_4 \cdot 7H_2O$ 0.5%（w/v），KH_2PO_4 0.5%（w/v），ミネラル溶液 A, B and C 各0.1%（v/v）及び培地の初 pH が6.0，(2)培養条件：28℃，150 rpm，６日間振とう培養．