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SHORT COMMUNICATION

Enhanced β-carotene production by *Rhodotorula glutinis* using high hydrostatic pressure

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Abstract−High hydrostatic pressure (HHP) technology was used for improving the ability of β-carotene biosynthesis of *Rhodotorula glutinis* R68. After the treatments of five repeated cycles at 300 MPa for 15 min, the barotolerant mutant PR68 was obtained. After 72 h of culture, the biomass of mutant PR68 was 21.6 g/L, decreased by 8.5% compared to the parent strain R68, but its β-carotene production reached 19.4 mg/L, increased by 52.8% compared to the parent strain R68. The result of restriction fragment length polymorphism analysis suggested that mutant strain PR68 was likely to change in nucleic acid level, and thus enhanced β-carotene production in this strain was a result of gene mutation induced by HHP treatment. HHP technology seems a promising approach for improving industrial microbes.

Key words: β-Carotene Production, High Hydrostatic Pressure, *Rhodotorula glutinis*, Mutation

INTRODUCTION

The utility of β-carotene is well known: it is an anticancer agent, scavenger of singlet oxygen and free radicals, immune response stimulant, and coloring agent for cooked sausages, soft drinks, and baked goods [1]. Although its extraction from vegetables and chemical synthesis is standard [2], biotechnological production of carotene is of increasing interest, especially from microbial sources. *Rhodotorula glutinis* is convenient for large-scale production in fermentor due to its high growth rate and low nutritional needs. On the other hand, the quantity of β-carotene in naturally occurring strains of *R. glutinis* is low compared to other fungi [3]. The low yield of β-carotene from this yeast makes large-scale fermentation uneconomic.

High hydrostatic pressure (HHP) treatment is now a field in emergence. Elevated pressure can exert detrimental effects on microorganisms and enzymes [4], and thus the potential use of HHP technology as a novel food preservation method is currently being investigated [5,6]. Because of its effects on the gene and protein expression of microorganisms [7], a mutation induced by HHP is also possible. For example, *Escherichia coli*, a harmful microbe in the food industry, its mutant resistant to pressure has been obtained [8,9]. However, there is still little knowledge on improving applied microbes by HHP treatment. In our previous work [10,11], the HHP technology was applied to *Ganoderma lucidum Karst*, and its laccase production increased from 125 U/mL to 480 U/mL. At the same time, our experiment showed that the frequency of such mutants is higher with HHP than that after treatment with a known mutagen such as ultraviolet radiation, nitrosoguanidine. Moreover, it was also found that HHP technology could offer some advantages for improving strains such as easy handling, low cost, time saving and no polluting of the environment. Therefore, in this study we again explored the use of HHP in an attempt to raise the β-carotene production of *Rhodotorula glutinis* R68.

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MATERIALS AND METHODS

1. Reagents and Chemicals

Chromatogram-grade acetonitrile, methylene dichloride, and methanol were obtained from Kermel (Tianjin, China). Trans-β-carotene from Sigma (USA) was used as a reference compound for high performance liquid chromatography (HPLC). Four kinds of tool enzymes (HindIII, EcoRI, BamHI and HaeIII) were obtained from Huamei Biotechnology Company (Zhengzhou, China).

2. Yeast and Media

R. glutinis R68 was the stock strain. The isolate and mutants were maintained on agar medium at 4 °C and transferred every month. The fixed culture conditions used in this study were from optimum values obtained in our previous experiments.

The various culture mediums used in this experiment were as follows (per liter): Seed medium: glucose 20 g, peptone 10 g, yeast extract 10 g, and pH value of nature; Agar medium: glucose 20 g, peptone 20 g, yeast extract 10 g, agar 20 g, and pH 6.0; Selective medium: The same as agar medium but appended with diphenylamine of suitable concentration; Fermentation medium: glucose 25 g, yeast extract 3 g, $(NH_4)_2SO_4$ 5 g, tomato extract 2.5 mL, peanut oil 0.5 mL, and pH 6.0.

3. HHP Treatment and Selection of Mutant

The strains were inoculated into 30 mL of seed medium in a 250-mL Erlenmeyer flask and incubated at 28 °C on a rotary shaker at 150 rev/min; incubation time was adjusted in order to obtain initial cell counts of approximately 10^6-10^7 CFU/mL. The test samples of 10 mL were centrifuged (LD5-10, Beijing Centrifuge Factory, China) and washed twice with sterile physiological saline and suspended in Tris-HCl buffers of pH 7.30 (40 mmol/L), then transferred aseptically into sterile polyethylene pouches and heat-sealed with minimal headspace (expelling as much air as possible). The prepared test pouches and control samples were chilled in an ice bath for 2 h to prevent microbial growth.

The prepared pouches were then subjected to various combina-

tion treatments of high pressure ranging from 50 MPa to 300 MPa at 25±2 ℃ with holding time ranging from 5 min to 20 min. The HHP equipment (ZZ-900M, Inner Mongolia First Machinery Group Corporation, China) consisted of a 2-L pressure vessel with an external water jacket for temperature control, two pumps to generate the pressure, and high-pressure valves. It used castor oil as the pressure transmission fluid. The compression rate was approximately 100 MPa/min, while decompression was immediate.

To determine the viability, 0.1-mL treated samples and untreated samples after appropriate dilutions were surface plated separately on agar medium with a spiral plater and were incubated at 28 ℃ for 48 h. The viability was expressed as the CFU/mL.

To select mutants, the samples after the HHP treatment of five repeated cycles and untreated control were cultured in seed medium for 24 h at 28 ℃, centrifuged, and serially diluted with sterile physiological saline. They then were surface plated on the selective mediums containing diphenylamine and cultured for 48 h at 28 ℃. Large red or yellow colonies were isolated, and were inoculated onto inclined plane of agar medium.

These isolated colonies separately were inoculated into seed mediums of 30 mL and incubated at 28 ℃ for 20 h on rotary shakers at 150 rev/min. They were then added into fermentation mediums of 50 mL in 250-mL Erlenmeyer flasks and incubated as above for 48 h for the extractions of β-carotene.

4. Extraction of Carotene and its Analysis by HPLC

Harvested culture samples were centrifuged, washed with distilled water, and dried at 65 ℃ to a constant weight. Dry 0.1-g samples were disintegrated in 2 mL HCl (3 mol/L) for 1 h and pyrolyzed over a boiling water bath for 4 min. The pigment was extracted with acetone until all the color was removed. The extract was centrifuged and the supernatant filtered through a 0.45-μm membrane filter and subjected to HPLC analysis.

Analyses were performed on a C_{18} analytical HPLC column (Spherisorb, 4.6 mm (i.d.)×250 mm; Waters 600, USA). The mobile phase was composed of acetonitrile, methylene dichloride, and methanol (80 : 10 : 10, v/v) and had a flow rate of 0.8 ml/min. The column thermostat was set at 30 ℃. The detector was operated at 457 nm; subsequently the β-carotene reference and the samples were run as a calibration.

5. Restriction Fragment Length Polymorphism (RFLP) Analysis

The DNA of initial strain R68 and mutant PR68 was separately extracted, purified, and amplified. The primer used, S118 (5-CTG ACCAGCC-3) was chosen based on the clear band and good repetition from 130 primers (S1 to S130) purchased from Shanghai Biotechnology Company (Shanghai, China). One unit of Taq plus DNA Polymerase was then added to each tube. PCR conditions were as follows: initial denaturation at 94 ℃ for 5 min; 40 cycles of denaturing at 94 ℃ for 1 min, annealing at 35 ℃ for 1 min and extension at 72 ℃ for 2 min; and a final extension at 72 ℃ for 10 min.

The PCR products without further purification were treated with HindIII, EcoRI, BamHI and HaeIII. The enzyme reactions were controlled according to the instruction. The restriction fragments were electrophoresed with voltage 70 V for 1.5 h on 1% (w/v) multipurpose agarose stained with ethidium bromide, and then photographed.

Table 1. Survival rate of yeast treated at 300 MPa for 15 min

Cycle	Control yeasts (CFU/mL)	Treated yeasts (CFU/mL)	Survival rate (%)
1st	2.41×10^{6}	0.21×10^{2}	8.71×10^{-4}
2nd	1.36×10^{7}	3.52×10^{3}	2.59×10^{-2}
3rd	1.24×10^{7}	2.16×10^{4}	1.74×10^{-1}
4th	1.08×10^{6}	2.40×10^{3}	2.22×10^{-1}
5th	2.58×10^{7}	3.62×10^{4}	1.40×10^{-1}

Table 2. β-Carotene production by parent strain R68, mutant strain PR68

	Dry cells weight (g/L)	β-Carotene content (μg/g)	β-Carotene production (mg/L)
Strain R68	23.6±0.3	538.7±8.9	12.7±0.1
Mutant PR68	21.6±0.2	896.3±9.2	19.4±0.2
Change (%)	8.5	66.4	52.8

Table 3. Genetic quality of mutant strain PR68

Generation	Dry cells weight (g/L)	β-Carotene content (μg/g)	β-Carotene production (mg/L)
0	21.6±0.2	896.3±9.2	19.4±0.2
5	22.1±0.3	893.6±8.9	19.7±0.2
10	21.5±0.2	895.8±9.0	19.3±0.3

6. Reproducibility of Data

All experiments were done at least in triplicate. The data presented are the means of three replicate experiments.

RESULTS

1. Effect of HHP Treatment on the Growth of *R. glutinis* R68

The cell suspensions of strain R68 were subjected to different HHP treatments of combining pressure with time. In general, the yeast viability during HHP treatment decreased with the increase of processing pressure and holding time. This effect was more pronounced when cells were submitted to pressures above 100 MPa, while at 300 MPa for 20 min all yeast cells were killed (data not shown).

In order to obtain a mutant resistant to pressure, an HHP treatment at 300 MPa for 15 min was carried through by five repeated cycles after the multiplication of survival yeast. Table 1 shows that a majority of test cells had been killed after the treatment of the first cycle. The culture of survival yeast was further treated as it was found to become more resistant to pressure. After five repeated cycles, a designated mutant PR68, which survival rate increased from 8.71×10^{-4}% to 0.14%, was selected for further work.

2. Effect of HHP Treatment on the β-Carotene Production of *R. glutinis* R68

The parent strain R68, and its mutant PR68 were cultured in shake-flasks and their β-carotene productions were estimated by HPLC (Table 2). The biomass of mutant PR68 was 21.6 g/L after 72 h of culture, decreased by 8.5% compared with that of 23.6 g/L from

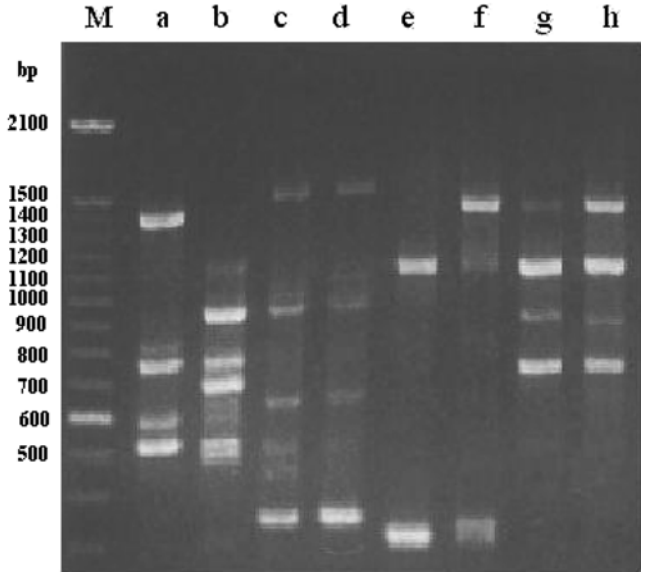

Fig. 1. Gel electrophoresis of HindIII restriction digests of DNA from R68 (a) and PR68 (b), EcoRI restriction digests of DNA from R68 (c) and PR68 (d), BamHI restriction digests of DNA from R68 (e) and PR68 (f), HaeIII restriction digests of DNA from R68 (g) and PR68 (h).

the control, but its carotene content and β-carotene production reached 896.3 μg/g cells and 19.4 mg/L, increased by 66.4% and 52.8% compared with those of 538.7 μg/g cells and 12.7 mg/L from the control, respectively.

The data from Table 3 show that mutant strain PR68 was genetically stable. This indicates that the increased carotene production was from the result of a genetic mutation HHP-induced, and the cells possessed relatively stable structures. The stability of the mutant not only means that the mutant genes in this strain were stable, but also that there was no back-mutation happening.

3. RFLP Analysis

In order to know if HHP treatment resulted in the gene mutation, the DNA of parent strain R68 and mutant strain PR68 was separately treated with the four kinds of enzymes. Fig. 1 shows the DNA segments of mutant PR68 were 3 multiples more than those of parent strain R68 when treated with HindIII. Also, the DNA segments of mutant PR68 were 1 multiple more than those of parent strain R68 when treated with BamHI. The DNA fragments of mutant PR68 cut by HindIII and BamHI were different from those of parent strain R68, and this result suggested that HHP made R68 strain changed in nucleic acid level.

DISCUSSION

1. Change of Barotolerance of Parent Strain R68

In this study, the change of barotolerance of parent strain R68 might occur in gene level. This was because the barotolerance of strain was gradually increased and passed to the next generation; thus, the mutant obtained finally should be a sort of barotolerant strain induced by HHP. Of course, another possibility was not still removed completely, that is, this barotolerant strain was from screening naturally because of the presence of potential barotolerant strains in test strains. However, the test strain used every time was from pure culture of a single colony, and thus the yeast cells in suspensions should be uniform. Therefore, there is very little possibility that the barotolerant strain obtained was from screening naturally.

Microbial growth is inhibited at high pressures, the maximum pressure allowing growth or survival depending on species and medium composition. A wide variety of high pressure-induced phenomena in living cells have been reported and reviewed [4,12], including changes in cellular morphology, biochemical reactions, genetic mechanisms, and membrane integrity. Although the mutant induced by HHP for improving microbes has not been studied in detail, several observations related to this suggest that it is a complex physiological process. Wuytack et al. [13] studied the effects of HHP on inactivation of bacteria, and found that HHP treatment can cause an accumulation of sublethal injuries ultimately leading to death. Hauben et al. [9] also considered that the high level of barotolerance observed in the *E. coli* mutants is probably the result of an accumulation of multiple mutations.

2. Pressure Resistance of Mutant Strain PR68

As a key physical parameter, the pressure has an important influence on the viability and biological activity of organisms [12]. The pressures of different magnitudes exert different effects on organisms, and different strains have different stress-response mechanism to copy with high-pressure shock. In general, slower growth usually leads to more product formation. For example [14], the biomass and special growth rate of *Saccharomyces cerevisiae* under 0.5 MPa were lower than those of control sample (0.1 MPa), but its content of glutathione increased obviously. However, based on our experimental methods (using ultra high-pressure treatment and special selective medium) and results, the pressure resistance of strain PR68 was clearly a stable characteristic and thus not the action of a physiological adaptation. Since the pressure-resistant strain exhibited a stable and significantly altered phenotype compared to the variants detected in the parent culture, it was further designated mutant.

3. Link Between High-pressure Effects and Mutation in DNA Fragments Versus Altered β-Carotene Levels

In our experiments, a selective medium containing diphenylamine was used for selecting mutants. Diphenylamine is known to affect carotenoid synthesis in a variety of organisms [15]. The diphenylamine of suitable concentration in the medium could reduce or inhibit the levels of color carotene in microorganism; however, increasing the levels of color carotene showed that mutation has occurred in the strain. Because of the change of enzymatic structure or adjusting of enzyme formation, the quantity or activity of enzyme molecules might be increased, and the inhibition effect of diphenylamine on enzymatic activity would no longer act. Thus, the enzyme reaction and metabolizing in mutant strain could still carry through. Therefore, the colonies with color on the agar plate appended with diphenylamine were able to come out. The increasing frequency of color variants results from the mutations HHP-induced and thus not from a sub-population that survived HHP, which enhanced β-carotene production in this strain was a result of gene mutation induced by HHP treatment. HHP technology seems a promising approach for improving industrial microbes. Of course, the molecular mechanism of microbial mutation HHP-induced and many factors of affecting mutagenic effect should be further investigated.

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